

Protein Map Standardization of Human Saliva Using Two Dimensional Gel Electrophoresis (2-DE)

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Abstract

Over the past decade, the use of saliva as an auxiliary diagnostic tool for biomarker detection has gained considerable acceptance as a non-invasive, inexpensive alternative to conventional serum method. In proteomics, the most promising technique which can be used for detecting salivary biomarker with sufficient resolving power is two-dimensional gel electrophoresis (2-DE) that provides a unique platform for the simultaneous separation of proteins in a complex mixture. However, as a fact most of the new scientists in developing countries are still facing problems with optimization of 2-DE techniques because the performance of optimization techniques in proteomics research using 2-DE has its own limitations. Therefore, the present study was established to generate a reproducible and optimized protocol to display the 2-DE protein map of human saliva. Our results showed the standard optimization of the 2-DE protein mapping was achieved at pH 3-10 with 60 µg proteins loading. This protocol could be used by other scientists along with identification of differentially expressed proteins by mass spectrometry when 2-DE protein map of patients saliva are compared to that of normal healthy individuals. These differentially expressed proteins could be later used as specific and sensitive biomarkers for early diagnosis and prognosis of the disease in question. In conclusion, the procedure used in our study generated a highly reproducible and optimized reference 2-DE protein mapping of human saliva.

Keywords: Proteomics; 2-DE; Human saliva

Abbreviations: 2-DE: Two Dimensional Gel Electrophoresis; IEF: Isoelectric Focusing; SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; OSCC: Oral Squamous Cell Carcinoma

Introduction

Saliva is a unique and complex biofluid consisting of approximately 99% water with a mineral component consisting of electrolytes such as sodium, potassium, calcium, chloride, magnesium, bicarbonate and phosphates. The saliva component contains enzymes (α -amylase), secretory immunoglobulins (IgA, IgG and IgM) and other antimicrobial factors, mucosal glycoproteins, traces of albumin and some polypeptides and oligopeptides [1,2]. These components interact with each other resulting in various functions such as taste, digestion [3,4], protection, lubrication [5,6], cleaning, buffering capacity [3] and maintaining integrity of the tooth structure [6].

Total or whole saliva is produced by three paired major salivary glands namely parotid, submandibular, and sublingual glands along with several hundred minor salivary glands spread throughout the oral cavity together with gingival and crevicular fluid [1,2]. Over the past decade, the use of saliva as an auxiliary diagnostic tool for biomarker detection has gained considerable acceptance as a non-invasive, inexpensive alternative to conventional serum method [7,8].

The range of diseases which can be identified using salivary diagnostics are ever increasing [7-10], and salivary biomarkers have been used for detecting several diseases such as acute myocardial infarction [11,12], pancreatic cancer [13,14], oral squamous cell carcinoma (OSCC) [15,16], breast cancer [17,18], and periodontal

diseases [19,20]. The detection of various biomarkers in saliva has also been reported to help with early diagnosis of autoimmune diseases such as rheumatoid arthritis and Sjögren's syndrome [21,22].

In proteomics, the most promising technique which can be used for detecting salivary biomarker with sufficient resolving power is two-dimensional gel electrophoresis (2-DE) that provides a unique platform for the simultaneous separation of proteins in a complex mixture [23]. However, as a fact most of the new scientists in developing countries are still facing problems with optimization of 2-DE techniques because the performance of optimization techniques in proteomics research using 2-DE has its own limitations [23].

Therefore in this study, it was thought worthwhile to establish and generate a reproducible and optimized protocol to display the complete qualitative 2-DE protein map of the human saliva. This protocol could be used by other scientist along with identification of differentially expressed proteins by mass spectrometry when 2-DE protein map of

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Received July 01, 2015; **Accepted** September 28, 2015; **Published** September 30, 2015

Citation: Doustjalali SR, Yaldrum A, Al-Jashamy K, Irfan M, Zin KT, et al. (2015) Protein Map Standardization of Human Saliva Using Two Dimensional Gel Electrophoresis (2-DE). J Mol Biomark Diagn 6: 249. doi:10.4172/2155-9929.1000249

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patients saliva are compared to that of normal healthy individuals. These differentially expressed proteins could be later used as specific and sensitive biomarkers for early diagnosis and prognosis of the disease in question.

Materials and Methods

Protein extraction of human saliva

The extraction of protein from human saliva was performed as shown in the flowchart (Figure 1). Finally all samples were stored at -80°C and subjected to the similar 2-DE treatment. The research proposal was approved by the Ethics Committee of SEGi University.

2-DE gel electrophoresis optimization

The first-dimension Isoelectric Focusing (IEF) was performed by using PROTEAN IEF system (Bio-Rad Laboratories, USA). 60 µg of protein sample was supplemented with 60 µl sample buffer solution (9 M urea, 0.5% v/v Triton X-100, 2% v/v IPG buffer pH 3-10 and 60 mM DDT) and left at room temperature (20°C) for 30 minutes. The mixture was added with rehydration solution (8 M urea, 0.5% v/v Triton X-100, 0.5% v/v IPG buffer pH 3-10, 12 mM DDT and 0.002% of Orange G) to make final volume of 200 µl for 11 cm IPG Strip gel, pH 3-10 (Bio-Rad Laboratories, USA), respectively [24-27]. The IPG strips were then rehydrated with the sample mixture in the Immobiline Dry Strip Reswelling Tray. The reswelling tray and IPG strips were rehydrated at room temperature for 16 hours.

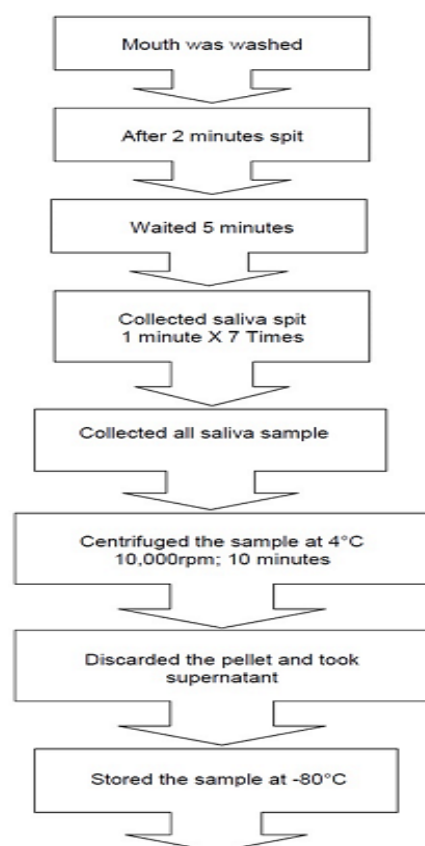


Figure 1: Flowchart of protein extraction of human saliva. The extraction of protein from human saliva was performed as shown in the flowchart. Finally all samples were stored at -80°C and subjected to the similar 2-DE treatment.

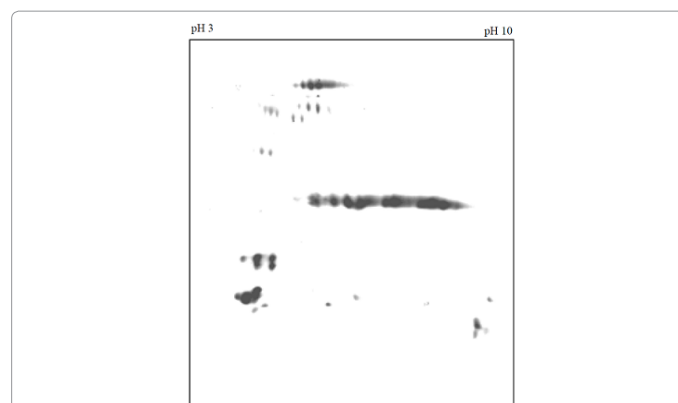


Figure 2: Typical 2-DE protein profiles of human saliva. The 2-DE analyses were optimized at pH 3-10 with 60 µg proteins loading.

Isoelectric focusing (IEF) was performed under the following conditions: 300V for 30 minutes, 3500V for remaining hours till reached 12000 V/hr. Upon completion of IEF the strips were equilibrated in equilibration buffer (6 M urea, 1.5 M Tris-HCl, pH 8.8, 30% v/v Glycerol, 2% SDS, 0.002% bromophenol blue, 0.06 M DTT) for 15 minutes, followed by the same buffer containing 240 mM iodoacetamide instead of DTT for another 15 minutes. The second dimension separation was carried out at 16°C on 12.5% SDS slab gels using 2-DE system (Bio-Rad Laboratories, USA), with the IPG strips sealed on the top of the gels with 0.5% agarose. SDS-PAGE was run for 40 mA/gel at 50V for the first 30 minutes. The voltage was subsequently increased to 600V until the bromophenol blue marker reached the bottom of the gel [24-27].

Silver staining

The 2-DE gels were developed by silver staining as previously described by Heukeshoven and Dernick [28].

Image analysis

Protein spots were analyzed and images of stained 2-DE gels were acquired with Platinum Image Master Scanner (Amersham Biosciences) and stored as TIF file. All samples were analyzed in triplicate.

Results and Discussion

Salivary biomarkers can be used for diagnosis of several diseases such as acute myocardial infarction, pancreatic cancer, oral squamous cell carcinoma (OSCC), breast cancer, periodontal diseases and autoimmune diseases such as rheumatoid arthritis and Sjögren's syndrome [7-22]. In proteomics, the most promising technique which can be used for detecting salivary biomarker with sufficient resolving power is 2-DE that provides a unique platform for the simultaneous separation of proteins in a complex mixture [23]. However, as a fact most of the new scientists in developing countries are still facing problems with optimization of 2-DE techniques because the performance of optimization techniques in proteomics research using 2-DE has its own limitations [23]. Therefore it was important to have an optimized system for developing 2-DE protein map to display the complete qualitative 2-DE protein map of the human saliva.

In our study consistency, optimization and reproducibility were achieved by standardizing the procedures for the extraction of protein from human saliva, the first and second dimension of electrophoresis, gel staining and image acquisition. Figure 2 shows the 2-DE protein maps of human saliva. The 2-DE analyses were optimized at pH 3-10 with 60 µg proteins loading. Under these conditions, proteins could

be visualized without much background noise and sacrificing gel resolution. Thus we have established a robust, reliable and optimized protocol for the 2-DE protein profile of human saliva.

This protocol could be used by other scientists along with identification of differentially expressed proteins by mass spectrometry when 2-DE protein map of patients saliva are compared to that of normal healthy individuals. These differentially expressed proteins could be later used as specific and sensitive biomarkers for early diagnosis and prognosis of the disease in question. In conclusion, the procedure used in our study generated a highly reproducible and optimized reference 2-DE protein mapping of human saliva.

Competing Interests

The authors declared they have no competing interests.

Authors' contributions

Conceived and designed the experiments: SRD. Performed the experiments: SRD NSS. Analyzed the data: SRD KAJ NSS. Wrote the paper: SRD KAJ NSS. Revised the paper: SRD AY KAJ MI KTZ NHL WML VA SYAK JN RM WAC HH AY RSYW VK CPWT NSFS HA ANAAR MM SDS NSS. All authors read and approved the final manuscript to be published.

Acknowledgements

This study was supported by Ministry of Higher Education (MOHE), Malaysia under the High Impact Research (HIR)-MOHE UM.C/625/1/HIR/MOHE/H-20001-00-E000079 and Fundamental Research Grant Scheme (FRGS) FP027-2010B.

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