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Developing Protocols of Tricine-SDS-PAGE for Separation of Polypeptides in the Mass Range 1-30 kDa with Minigel Electrophoresis System

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An efficient method for the separation of proteins in the mass range 1-100 kDa with minigel electrophoresis system was developed in the present study. Tricine (N-(2-Hydroxy-1,1-bis (hydroxymethyl) ethyl) glycine)-SDS-PAGE allows a resolution for proteins smaller than 30 kDa at lower acrylamide concentration than that in other electrophoretic methods. But reliable Tricine-SDS-PAGE protocols for minigel system remain to be established. In our study, Tricine-SDS-PAGEs were conducted with different composition of gels varied with concentration of acrylamide-bisacrylamide, urea, and glycerol, to develop optimized protocols of Tricine-SDS gels for minigel system. Our results indicated that gel composition containing 10% glycerol (w/v) and 4.2M urea could provide an ideal resolution for separation of small mass proteins. Then calibration curves for different types of separating gels demonstrated effective linear mobilities, and correlation coefficients of all gels were above 0.95, and the highest is the 10%T, 3%C gel, which reached 0.97. Results from the immunoblotting experiment of a 14-kDa mouse HMGA2 polypeptide also showed that the optimized protocols of minigel had excellent compatibility with Western Blot. In conclusion, the developed protocols of Tricine-SDS-PAGE for minigel provide a new choice for efficient, reproducible and convenient separation of low molecular weight proteins.

Keywords: Low molecular weight protein/ Minigel/Tricine-SDS-PAGE/Western Blot

ABBREVIATIONS:
Tricine, N-(2-Hydroxy-1, 1-bis (hydroxymethyl) ethyl) glycine;
T, total percentage concentration of both monomers (acrylamide and bisacrylamide);
C, the percentage concentration of the crosslinker relative to the total concentration T;
AB-3, Acrylamide-bisacrylamide(AB)-3 stock solution(T=49.5%, C=3% mixture);
AB-6, Acrylamide-bisacrylamide(AB)-6 stock solution (49.5%T, 6%C mixture).

1. INTRODUCTION

The separation of low-molecular-mass proteins or polypeptides smaller than 30 kDa is a difficult task. Glycine-SDS-PAGE (also named as Leaemmli-SDS-PAGE)[1] and Tricine(N-(2-Hydroxy-1,1-bis (hydroxymethyl) ethyl) glycine)-SDS-PAGE [2-3], based on glycine-Tris and Tricine-Tris buffer systems respectively, are the two most popular SDS electrophoretic techniques for separating proteins. Together, Glycine-SDS-PAGE and Tricine-SDS-PAGE cover the protein mass range 1-500kDa. The resolution capacity of Glycine-SDS-PAGE in the large molecular mass range lies far beyond 100kDa, but proteins smaller than 20 kDa are partly or totally inseparable with this technique, unless high-acrylamide gels or gradient gels were used [4-5]. Tricine-SDS-PAGE is a preferred electrophoretic method for the separation of proteins smaller than 30 kDa, especially for proteins of 1-20 kDa, at low acrylamide concentrations. Low acrylamide concentration levels can facilitate protein blotting and staining, which could reduce the escape of small proteins because of less operation time. Compared with Glycine-SDS-PAGE, whose protocols are available for both minigel (for example, with short plate dimensions of 10.1×7.3×0.1cm) and large gel (for example, with short plate dimensions of 19×16.9×0.1cm)[6-7], Tricine-SDS-PAGE was mostly used in large gels. Although minigel equipment is convenient for SDS-PAGE[8] and there are many different application systems depending on the gel size, no effective protocols for minigel have been proposed so far. In the present study, reliable protocols of Tricine-SDS-PAGE with minigel format were developed by optimizing the composition of the gels, which will facilitate the work of separating low-molecular-mass proteins.

2. MATERIALS AND METHODS

2.1 Materials

Acrylamide, bisacrylamide, Tris base, Tricine, TEMED, ammonium persulphate, glycine, glycerol, urea, Coomassie Brilliant Blue G-250 and Protein Loading Buffer (Prod#1016) were purchased from HT-Biotech (Beijing, China). Spacer plates (Cat#1653311), short plates (Cat#1653308), comb(10well) (Cat#1653359), Mini-PROTEAN Tera Cell gel cassette, PowerPac Basic power supply and Polypeptide SDS-PAGE Standards (Cat#161-0326) were from Bio-Rad Laboratories (CA, USA). Multicolor broad range protein ladder (Cat#26634) and SuperSignal West Pico Chemiluminescent Substrate (Prod#34077) were from Thermo Scientific (Waltham, America). Mouse 3T3-L1 fibroblast cell line (Cat#CL-173) was from American Type Culture Collection (Rockefeller, America).

2.2 Solution preparation

Separation gels with different concentration of monomers (acrylamide and bisacrylamide) and percentage concentration of the crosslinker relative to the total concentration, which were afterwards indicated with T and C, respectively, were prepared[2].

The acrylamide-bisacrylamide (AB)-3 stock solution (T=49.5%, C=3% mixture) was prepared by dissolving 48 g acrylamide and 1.5 g bisacrylamide in 40ml water and then adding water up to

100ml. The acrylamide-bisacrylamide (AB)-6 stock solution (49.5%T, 6%C mixture) was prepared by mixing 46.5 g acrylamide and 3 g bisacrylamide in 40 ml water and then adding water up to 100 ml. Fixing solution, used for Coomassie staining, was prepared by adding 500ml methanol, 100ml acetic acid and 7.708g ammonium acetate to 400ml water. Staining solution was prepared by mixing 100ml acetic acid, 0.25g Coomassie dye and 900ml water. Destaining solution was 10% acetic acid solution(v/v). 80% glycerol (v/v) was prepared by diluting 80ml glycerol in 20ml water. 10%SDS solution (w/v) was obtained by dissolving 0.1g SDS in 100ml water. 10% ammonium persulphate solution (w/v) was prepared by dissolving 0.1g ammonium persulphate in 1ml water, effective for 1 week.

Table 1. Electrode and gel stock solutions for SDS-PAGE

		Anode buffer (10×)	Cathode buffer (10×)	Gel buffer[b] (3×)
Tris	(g)	121	121	36.3
Tricine	(g)	----	179	----
HCl	(ml)	10	----	4.5
SDS	(g)	----	10	0.3
pH[a]		8.9	8.25	8.45
Final Volume	(L)	1	1	0.1

a) The PH values were adjusted with concentrated hydrochloric acid; error of the PH was less than 0.25.
b) The gel buffer (3×) was filtered after adjusting PH.

Electrode and gel buffers for Tricine-SDS-PAGE were prepared as indicated in Table 1. Electrode buffer (10×) of Glycine-SDS-PAGE was prepared by dissolving 30.03g Tris, 188g glycine and 10g SDS in 1L water. Diluted it in a 1:10 ratio to water when used for electrophoresis. All solutions should be stored at room temperature, except that the acrylamide and bisacrylamide mixtures were kept at 7-10°C after filtration, to avoid crystallization at 4°C.

2.3 Gel casting

Composition for minigel was calculated referring to Tricine-SDS-PAGE protocol for large gels[3] with modification, which was optimized by selecting ideal compositions from three concentration levels of urea (6, 4.2 and 2.8M), and two mass-volume concentration levels of glycerol(10% and 13.3%). To compare the effect of two glycerol concentration levels, Tricine-SDS gels of 10% and 13.3% glycerol(w/v), both of them with 18%T, 3%C separating gel, were casted, while 18%T, 6%C separating gels with urea concentration levels of 2.8, 4.2 and 6M were also casted to optimize urea concentration in minigels.

After optimizing the concentration of glycerol and urea, five concentration levels of acrylamide-bisacrylamide (5%T, 3%C; 10%T, 3%C; 18%T, 3%C; 18%T, 6%C; 18%T, 6%C) were used to optimize the analytical electrophoresis condition. The separating gel was overlaid with several drops of water, then left for about 1 hour to polymerize adequately, finally the overlaid water was replaced by a 5%T, 3%C stacking gel.

As comparison, Glycine-SDS-PAGEs with 10%T, 3%C gel were performed, which was comprised of 2.44ml water, 1.2ml AB-3, 1.25ml Tris-HCl(1.5M, pH=8.8), 50μl 10% SDS solution(w/v), 50μl 10% ammonium persulphate solution (w/v) and 5μl TEMED. The 5%T, 3%C stacking gel was also used to cover the separating gel.

2.4 Electrophoresis

We added cathode buffer as the inner electrode buffer and anode buffer as the lateral electrode buffer. Polypeptide SDS-PAGE standards (Bio-Rad) comprised six proteins, covering the mass range of 1.4-26.6kDa. Approximately 0.5-2μg per protein band was needed for visualization after being stained by Coomassie Blue G-250[2]. The concentration of polypeptide standards was about 8μg/μl. For partial elution during the staining-destaining procedure, an amount of 4μg proteins per well was applied for all Tricine-SDS gels, including the gels used to optimize the concentration of glycerol and urea. We mixed 0.5μl protein standards with 4.5μl protein loading buffer as a loading sample. The mixture was incubated at 95℃ for 5 min and then loaded under the cathode buffer. The electrophoresis was operated at room temperature without any cooling measure except for heat conduction by the surrounding air. All running conditions were set at 60V initially and maintained at this voltage until the samples completely entered the lower separating gel. The next voltage steps were set at 100V for 1.0mm 10%T gel and 140V for 1.0mm 18%T gel. The relative electrophoretic mobility of all the proteins, exported by Quantity One software, was used to plot calibration curves.

Glycine-SDS-PAGE was performed twice, the total loading volume of the sample loaded was 5μl for each gel, and the mass of loaded polypeptides for the two gels was 4μg per well and 12μg per well, respectively.

2.5 Fixing, staining and destaining

Polypeptide bands were fixed in fixing solution for 30min (1.0-mm gels), then emerged in staining solution for 1h. An absolute background destaining of the 1.0-mm gels was performed by shaking the gels in 10% acetic acid for 2h. Destaining incubation lasted 30-60min with fresh destaining solution and several times repeated. The fixing time of less than 45 minutes and a maximum staining time of 1.5 hours were recommended. However, appropriate time was applied depending on the thickness and the concentration of the separating gel.

2.6 Western Blot

In Western Blot analysis, the Multicolor Broad Range Protein Ladder (Thermo Scientific) and samples were loaded onto SDS-PAGE, respectively. The amount of total proteins was 10μg/well, as

applied. After termination of the electrophoresis, the bands on the SDS-PAGE gel were electroblotted onto a methanol-actived PVDF membrane. Then a rabbit anti-HMGA2 antibody (1:200; Santa Cruz Biotechnology) was used to detect the 14kDa HMGA2 protein. The membrane was incubated with 1:200 dilution of anti-HMGA2 antibody in primary antibody buffer for 12 h at 4℃. The membrane was then incubated with 1:3000 dilution of goat anti-rabbit & mouse IgG-HRP universal second antibody (Abmart) in second antibody buffer at room temperature with gentle shaking. As a positive control, a 42 kDa β-actin protein was blotted with the Abmart-β-Actin antibody (1:2000; Abmart). The ECL Detection Kit (Thermo Scientific) was used for chemiluminescent substrate. Image scanner, UMAX Powerlook 2100XL-USB (UMAX Technologies), was used for scanning films. And three biological replicates were performed.

3. RESULTS

3.1 Optimizing the concentration levels of glycerol and urea

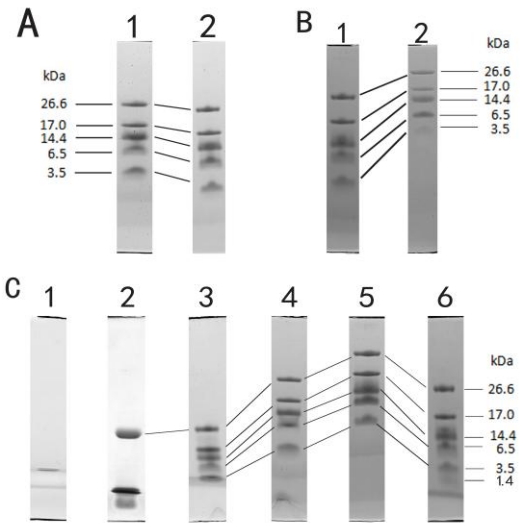

Figure 1. Separation of selected polypeptide standards on gels with different composition. (A) 18%T, 3%C Tricine-SDS gels with 13.3% glycerol (w/v) (lane 1) and 10% glycerol (w/v) (lane 2). (B) 18%T, 6%C Tricine-SDS gels with different concentration levels of urea (2.8M (lane 1) and 6M (lane 2)). (C)Lane 1, 10%T, 3%C Glycine-SDS gel (4μg polypeptides per well loaded) ; lane2, 10%T, 3%C Glycine-SDS gel (12μg polypeptides per well loaded); lane 3, 10%T, 3%C Tricine-SDS gel; lane 4, 18%T, 3%C Tricine-SDS gel; lane 5, 18%T, 6%C Tricine-SDS gel; lane 6, 18%T, 6%C Tricine-SDS gel plus 4.2M urea. The polypeptide standards were applied as 4 μg per well for all lanes of Fig.1A, Fig.1B and Fig.1C, to provide a direct comparison, except for threefold as 12 μg per well loaded in lane 2 of Fig.1C. The Quantity One software was used to crop these figures without any other manipulation.

As shown in Figure.1A, five protein bands could be sharpened on the two 18%T, 3%C separating gels with 10% and 13.3% glycerol (w/v) (lane 1 and 2 in Figure.1A), respectively, and there was no obvious difference of band patterns between the two concentration levels of glycerol, but the

less one could reduce cost. All six polypeptide bands could be detected when the concentration of urea was 4.2M as shown in lane 6 of Figure.1C, while no 1 kDa band could be detected when the concentration levels were set as 2.8M (lane 1) and 6M (lane 2) in Figure.1B. Taken together, we suggested that 10% glycerol (w/v) and 4.2M urea were optimal for Tricine-SDS-PAGE in minigel.

Optimized formula of varied concentration levels of gel was given in Table 2.

Table 2. Composition of different types of gels

		Stacking gel	Separating gels			
		5%T,3%C	10%T,3%C	18%T,3%C	18%T,6%C	18%T,6%C (4M urea) [a]
Water	(ml)	1.70	2.20	1.0	1.0	1.82
AB-3	(ml)	0.30	1.2	2.18	----	----
AB-6	(ml)	----	----	----	2.18	2.18
Gel buffer(3×)	(ml)	1	2	2	2	2
Glycerol (80%)[b]	(ml)	----	0.6	0.6	0.6	----
Urea	(g)	----	----	----	----	1.5
APS (10%)[c]	(μl)	30	30	25	25	25
TEMED	(μl)	4	3	2.5	2.5	2.5
Final Volume	(ml)	3	6	6	6	6

a) Final concentration level of urea.
b) 80% Glycerol (v/v) was prepared by diluting 80ml glycerol in 20ml water.
c) APS, Ammonium persulphate solution.

3.2 Comparison of different electrophoresis systems

In order to compare the resolution capacity of the two different electorphoresis systems, polypeptide standards were separated on Glycine-SDS gel and Tricine-SDS gel with the same acrylamide-bisacrylamide composition (10%T, 3%C), respectively[2]. However, protein bands could not be separated properly on the 10%T, 3%C Glycine-SDS gel, and all bands became visible only when the loading quantity of the standards ascended from 4 μg per well (lane 1) to 12 μg per well (lane 2), as shown in Figure. 1C. In lane 3 of Figure. 1C, five proteins could be detected, with the 26.6 kDa protein band concentrated better, which indicated that Tricine-SDS gel system was more powerful in separating the low molecular weight proteins of mass range 1-30 kDa than the Glycine-SDS gel system.

To optimize the T and C of separating gel used to do analytical electrophoresis, gels characterized by different T and C were conducted as crossover experiments of Tricine-SDS-PAGE. The results of the crossover experiments were indicated from lane 3 to lane 6 in Figure. 1C, orderly.

Within the same electrophoresis time, the decreasing migration distance from lane 3 to lane 5 indicated that the resolution of small proteins was improved as T and C ascending, which is coincident with the former research[3]. In lane 6 (18%T, 6%C plus 4.2M urea), six protein bands were sharpened, but polypeptides smaller than 14kDa were partially dispersive. Unavoidably, we could see the bromophenol blue migrated as a broad band at the bottom of each lane.

3.3 Regression analysis

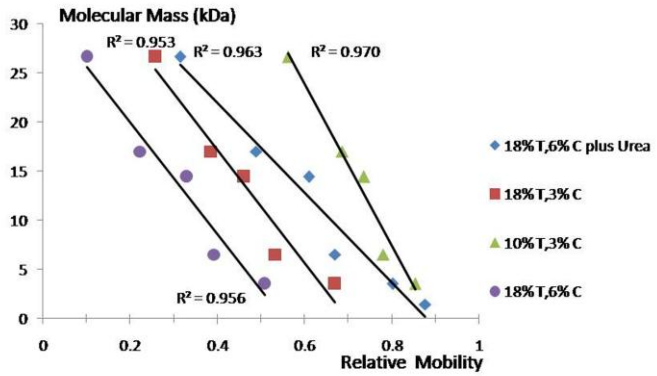

Figure 2. Standard curves for Tricine-SDS gels with different composition. 10%T, 3%C (▲), R^2=0.97; 18%T, 3%C (■), R^2=0.953; 18%T, 6%C (●), R^2=0.956; 18%T, 6%C plus 4M urea (◆), R^2=0.963. The relative mobilities of these proteins were plotted versus their molecular mass, given on a logarithmic scale.

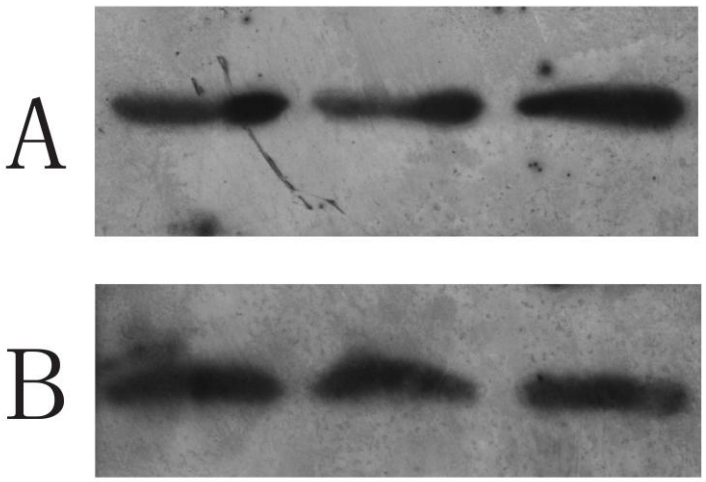

Figure 3. Polypeptide detection with immunoblotting. (A) β-actin was probed with an antibody to muscle protein (β-actin antibody, Abmart), and goat anti-rabbit&mouse IgG-HRP (Abmart) universal second antibody. (B) HMGA2 polypeptide was detected with rabbit anti-HMGA2 antibody (Santa Cruz Biotechnology) and universal second antibody, orderly. The ECL Detection Kit from Thermo Scientific was used in the experiments. The scanned films were cropped by Quantity One software, reserving the raw data.

In the present study, standard containing six polypeptides was used to optimize the T and C of gels. The mobilities of proteins, which were exported by the software Quantity One, were plotted versus their molecular masses, given on a logarithmic scale. All regression coefficients exceeded 0.95 and all calibration curves were linear, even among the bands of 1-5 kDa (Figure.2).

Furthermore, the 10% T, 3% C gel (Lane 3, Figure. 3C) showed the best linear relationship with R^2=0.97. These results also indicated that this new electrophoresis system is efficient, reproducible and convenient to separate low molecular weight proteins smaller than 30 kDa, and the developed method can play a more important role in analytical separation of low molecular proteins.

3.4 Polypeptide detection in immunoblotting

In order to test the compatibility of this mingel protocol with other technologies, Western Blot, one of the most important downstream applications of SDS-PAGE, was selected to detect a 14 kDa polypeptide, HMGA2[9].The results showed that the low molecular weight protein was successfully blotted (Figure. 3B), which indicated that our protocol for minigel shows an excellent compatibility with Western Blot.

4. DISCUSSION

Although Tricine-SDS-PAGE has been performed in minigel format for many years[10], the modified electrophoresis method described here is the only one proved to keep low molecular proteins a good linear relationship within the range from 1.4 to 26.6 kDa. For significantly different pK values of Tricine and Glycine[11], their electrophoretic mobilities relative to proteins are defined quite differently, which indicated the different resolution capacities of Tricine-SDS-PAGE and Glycine-SDS-PAGE [2].

The concentration of glycerol and urea are important in sharpening protein bands and reducing the electrophoretic mobility of small proteins[3]. In large Tricine-SDS gels, the concentrations of glycerol and urea have been optimized, but no related study has been reported in minigel, which is more convenient for SDS-PAGE[8] and compatible with many different application systems of protein analysis, so we conducted experiments to optimize the concentration levels of glycerol and urea. Glycerol in SDS gels could increase the density of solutions and reduce the time used for gel polymerizing, and also facilitates the process of stacking proteins to sharpen bands. Two mass-volume concentration levels of glycerol as 10% and 13.3% were recommended in previous studies [3, 12]. Maybe the dimensions of minigel was very limited, there was little difference on resolution between the mass-volume concentration levels of glycerol as 10% and 13.3%, and we therefore selected the less from the perspective of saving material. Urea could reduce the electrophoretic mobilities of proteins, especially for the low molecular proteins. As 6M urea was recommended in large gel[3, 12-13], and 4.2M and 2.8M were optimized for Urea-SDS-PAGE with minigel[14], the concentration gradients of urea were selected as 2.8, 4.2 and 6M. However, all six protein bands of the standard could be detected

only when the concentration was set as 4.2M, while the 1kDa protein band was lost in other two groups. However, the band patterns of 4.2M urea gel were a little dispersive, which indicated that resolution of the gel compensated for sharpness of the bands. Other reagents for the new method described in the present study were calculated based on the well established and efficient protocol of Tricine-SDS-PAGE in large gel[3].

In order to optimize the T and C of separating gel, we compared the resolving polypeptide standards on various types of gel. Acrylamide gels were usually characterized by the total percentage concentration (T) and the percentage concentration of the crosslinker (C). 10%T and 16.5% T were commonly used, while the percentage concentration of the crosslinker(C) was usually selected as 3% and 6% [2, 15]. For less space in minigel, we only increased the maximum of the total percentage concentration to 18%. The calibration curves for various types of gel showed a good linear relationship among polypeptide standard bands, and the correlation coefficients of all gels exceeded 0.95, which suggested that our method is efficient and reproducible for calculating molecular weight of small proteins. Furthermore, the correlation coefficient of 10%T, 3%C gel reached 0.97, which was preferential to analyze protein. Based on the results of the present study, the 10%T, 3%C minigel is recommended to separate small proteins in mass range 5-30 kDa. However, if the proteins below 5kDa are of major interest, using AB-6 instead of AB-3 to cast the separating gel is recommended, whose resolution could be further increased by adding 4.2M urea. Compared with Tricine-SDS-PAGE used in large gel[2], the new electrophoretic protocols are proposed for the analytical separation of low molecular proteins in the range 1-30 kDa. What's more, our protocol showed a good compatibility with the downstream application of Western Blot, which means this method would play a more important role in protein detection[16], especially for low molecular weight proteins smaller than 30 kDa.

This research provides a stable, efficient and repeatable method for Tricine-SDS-PAGE in minigel. First, this method provides a better resolution capacity in low molecular mass range. Second, the minigel electrophoresis system keeps good linear relationships among polypeptide standards. Third, the new SDS-PAGE protocol shows an excellent compatibility with Western Blot, which indicated that this method is appropriate for the downstream applications based on SDS-PAGE.

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The authors declare no competing financial interests.

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