Non-Gradient Blue Native Polyacrylamide Gel Electrophoresis

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Gradient blue native polyacrylamide gel electrophoresis (BN-PAGE) is a well established and widely used technique for activity analysis of high-molecular-weight proteins, protein complexes, and protein-protein interactions. Since its inception in the early 1990s, a variety of minor modifications have been made to this gradient gel analytical method. Here we provide a major modification of the method, which we call non-gradient BN-PAGE. The procedure, similar to that of non-gradient SDS-PAGE, is simple because there is no expensive gradient maker involved. The non-gradient BN-PAGE protocols presented herein provide guidelines on the analysis of mitochondrial protein complexes, in particular, dihydrolipoamide dehydrogenase (DLDH) and those in the electron transport chain. Protocols for the analysis of blood esterases or mitochondrial esterases are also presented. The non-gradient BN-PAGE method may be tailored for analysis of specific proteins according to their molecular weight regardless of whether the target proteins are hydrophobic or hydrophilic.

Keywords: blue native gel • esterase • electrophoresis • mitochondria • non-gradient

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INTRODUCTION

Blue native polyacrylamide gel electrophoresis (BN-PAGE) is a well recognized method for analysis of proteins and protein complexes (Swamy et al., 2006). While the original method was developed solely for the analysis of mitochondrial electron transport chain complexes (Schagger and von Jagow, 1991), the technique has found applications in the analysis of non-mitochondrial protein complexes such as cytosolic and nuclear proteins (Novakova et al., 2006; Deswal et al., 2010; Schlegel et al., 2010). It has also been successfully used for the analysis of proteins from other cellular organelles such as endoplasmic reticulum (Prior et al., 2016). Unlike SDS-PAGE, which denatures proteins, BN-PAGE usually retains the native protein function (Henderson et al., 2000). This is achieved by using Coomassie brilliant blue particles as the binding agent to mask a protein's inherent surface charges (Schagger, 1995). Therefore, proteins will resolve in the gel according to their size and molecular weight (Sabar et al., 2005). Additionally, the use of ϵ-amino-*N*-caproic acid in the gel buffer and in sample preparation for loading also facilitates membrane protein solubilization and resolution (Wittig and Schagger,

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2005; van den Ecker et al., 2010). Hence, the method has been widely used for analysis of membrane proteins, high-molecular-weight protein complexes, and protein-protein interactions (Bautista et al., 2000; Camacho-Carvajal et al., 2004; Krause and Seelert, 2008; Andringa et al., 2010).

Since its initial development in the early 1990s (Schagger and von Jagow, 1991), BN-PAGE has usually been performed using a gradient gel, requiring a gradient gel maker, which is not readily accessible for many laboratories. The technique has undergone numerous modifications by numerous investigators. For example, a clear BN-PAGE method without the use of Coomassie brilliant blue has been established (Wittig and Schagger, 2005; Dieguez-Casal et al., 2014). We have also modified the technique so that a blue native gel can be run non-gradiently. Therefore, in this unit, we present detailed procedures for our non-gradient gel electrophoretic method (see Basic Protocol; also see Yan et al., 2007; Thangthaeng et al., 2011; Yan et al., 2013). We demonstrate the usefulness of this non-gradient gel method by resolving mitochondrial protein complexes (I to V), dihydrolipoamide dehydrogenase (DLDH) homodimer, and serum or mitochondrial esterases followed by in-gel activity staining of each indicated complex or enzyme (Support Protocols 1 and 2). The protocols can be easily tailored based on the size or the molecular weight of a target protein that is to be analyzed. For a gradient BN-PAGE protocol, readers may refer to the method published in this manual (*UNIT 19.18*; Krause and Seelert, 2008).

BASIC PROTOCOL

NON-GRADIENT BLUE NATIVE POLYACRYLAMIDE GEL ELECTROPHORESIS FOR RESOLVING MITOCHONDRIAL PROTEINS

Numerous mitochondrial proteins exist as multimers or in protein complexes. This is especially true for those in the mitochondrial electron transport chain such as complexes I to V. Because each complex has multiple subunits with very high molecular weight, they cannot be resolved by conventional Tris/glycine native gel electrophoresis, which only works for low-molecular-weight proteins. In contrast, non-gradient BN-PAGE described in this procedure only resolves high-molecular-weight proteins, as low-molecular-weight proteins will run out of the blue native gel. Therefore, the procedure described here is very useful for resolving mitochondrial protein complexes.

Materials

Reagents for gel preparation (Table 19.29.1)
- 3× gel buffer (see recipe)
- 50% acylamide/bisacrylamide solution (see recipe)
- 10% (w/v) ammonium persulfate
- TEMED

Mitochondrial pellet (for isolation of mitochondria from rat or mouse brains, refer to Yan et al., 2007)
Mitochondria extraction buffer (see recipe)
10× sample buffer (see recipe)
Cathode buffer A (see recipe)
Cathode buffer B (see recipe)
Sonicator (Sonic Dismembrator; Fisher, cat. no. FB50110; 50 W, 110 V)

Additional reagents and equipment for polyacrylamide gel electrophoresis (*UNIT 10.1*; Gallagher, 2012), Bradford protein assay (*UNIT 3.4*; Olson and Markwell, 2007), and staining of gels (*UNIT 10.5*; Echan and Speicher, 2002)

1. Prepare gels according to the recipes given in Table 19.29.1.

   For gel assembly, pouring, and polymerization, see UNIT 10.1 (Gallagher, 2012).

Table 19.29.1 Recipes for Acrylamide Gels[a]

	Resolving	Resolving	Stacking
	8.0% (for SMP)	12% (for matrix)	4%
H_2O	5.0 ml	4.27 ml	2.9 ml
Gel buffer (3×)	3.33 ml	3.33 ml	1.67 ml
Acrylamide (50%)	1.6 ml	2.4 ml	0.4 ml
APS (10%)	50 μl	50 μl	25 μl
TEMED	7 μl	7 μl	7.5 μl
Total volume	10 ml	10 ml	5 ml

[a]For gel percentages other than the two given in the table, one can vary the volume of the 50% acrylamide solution and the volume of water. The concentrations of the remaining components remain the same. For example, if a 9% separating gel is to be made, the volume of the 50% acrylamide would be 1.8 (= 9 × 10/50) when the total volume is 10 ml, and the volume of water would be 4.8 ml. The percentage of the stacking gel is always 4%.

2. Dissolve mitochondrial pellet in mitochondria extraction buffer for a protein concentration of 1 mg/ml. Sonicate the sample (using Fisher Sonic Dismembrator or equivalent) four times at amplitude 40 for 30 sec each with a 1-min interval on ice between sonications.

   This sonication step will help solubilize mitochondrial membrane proteins.

3. Let the sample stand on ice for 10 min.

4. Centrifuge the sample 10 min at 8000 × *g*, 4°C.

   This step is required to remove any undissolved particles to clarify the sample. Otherwise, proteins may not enter the gel and will likely accumulate as a thicker protein band on top of the gel.

5. Keep the supernatant and discard the pellet.

6. Measure protein concentration in the supernatant using the Bradford assay (Bradford, 1976; described in *UNIT 3.4*, Olson and Markwell, 2007).

7. Mix the protein sample with 10× sample buffer so that the concentration of the sample buffer will be 1× and the protein concentration in the mixture will be 1 to 2 mg/ml.

8. Load the samples onto the gel.

   Usually 20 to 30 μl of the mixture from step 7 will be sufficient. Gel loading and running of gels is described in UNIT 10.1 (Gallagher, 2012).

9. Electrophorese the gel at room temperature at 200 V using cathode buffer in the upper chamber A, which contains no Serva blue G-250. The lower chamber contains anode buffer, which is used throughout the whole electrophoretic process.

10. Turn off current to the gel and change the upper chamber buffer to cathode buffer B, which contains 0.02% Serva blue G-250.

11. Continue electrophoresis at 250 V.

12. Turn off the current when the Serva blue G-250 reaches the bottom of the gel.

13. Stain the gel with Coomassie brilliant blue using previously described methods (*UNIT 10.5*; Echan and Speicher, 2002; Kang et al., 2002).

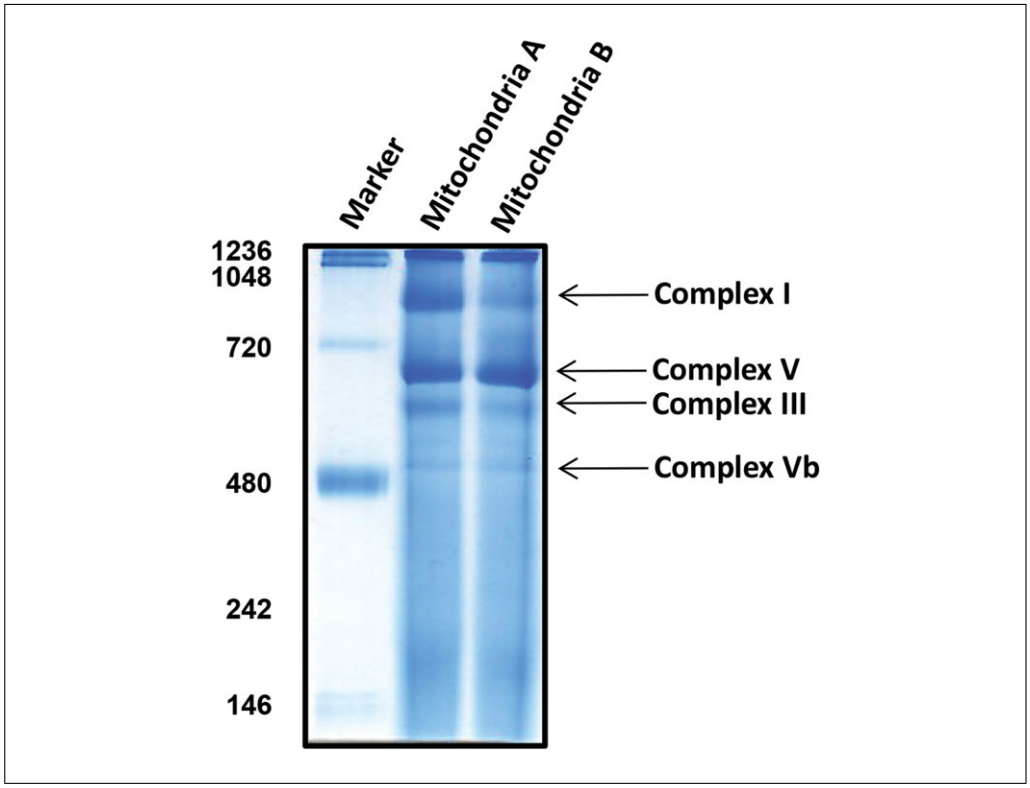

Figure 19.29.1 Representative gel image showing mitochondrial protein complexes resolved by non-gradient BN-PAGE. An 8% separating gel was used. The gel was stained by Coomassie brilliant blue after gel electrophoresis. Mitochondria isolated from rat brain were analyzed in this case, with each lane containing mitochondria prepared from independent rats. Arrows indicate mitochondrial electron transport chain complexes that could be readily visualized following Coomassie blue staining and destaining. Complexes II and IV could not be distinctly located on this gel.

14. Destain the gel in a destaining solution containing 10% methanol and 10% acetic acid.

A representative gel image showing non-gradient resolution of rat brain mitochondrial complexes is given in Figure 19.29.1. Image documentation was carried out using a digital scanner (EPSON PERFECTION 1670 in our laboratory).

SUPPORT PROTOCOL 1

PROCEDURES FOR ACTIVITY STAINING OF MITOCHONDRIAL ELECTRON TRANSPORT CHAIN COMPLEXES AND DIHYDROLIPOAMIDE DEHYDROGENASE (DLDH)

The non-gradient BN-PAGE method described in Basic Protocol 1 is designed to resolve the five mitochondrial membrane complexes I to V and dihydrolipoamide dehydrogenase (DLDH). As each complex or enzyme can only be stained by the use of its specific substrate, each complex will have to be independently stained. It should be noted that complex I and DLDH will be stained simultaneously, as the two enzymes catalyze the same reaction in the presence of NADH and nitroblue tetrazolium (NBT). If a specific complex or enzyme needs to be analyzed, the substrate and the reaction solution for that complex or enzyme will have to be used. Therefore, the staining procedure for each complex or enzyme is presented below.

NOTE: All activity staining described in this protocol can be carried out at room temperature and can be stopped by transferring the gel strips from the staining solution to a solution containing 10% acetic acid and 10% methanol. Gel strips can be kept in this solution until further imaging analysis.

Materials

Gel strip (cut from the gel prepared in Basic Protocol 1) containing mitochondrial electron transport chain complexes or dihydrolipoamide dehydrogenase (DLDH): the gel strip should not be stained with Coomassie blue or destained, as these steps will denature the enzymes on the gel strip; use the strip as it is immediately after gel electrophoresis
Nitroblue tetrazolium tablets (10 mg/tablet from Sigma)
NADH (Sigma)
50 mM potassium phosphate buffer, pH 7.0 (*APPENDIX 2E*)
5 mM Tris·Cl, pH 7.4 (*APPENDIX 2E*)
Sodium succinate
Phenazine methosulfate (Sigma, cat. no. P9625; stock solution prepared in DMSO)
50 mM sodium phosphate buffer, pH 7.2 (*APPENDIX 2E*)
3, 3′-diaminobenzidine tetrachloride (DAB)
Cytochrome *c*
Tris-glycine buffer (35 mM Tris, 270 mM glycine, pH 8.3)
$MgCl_2$
$Pb(NO_3)_2$
ATP

Activity staining of dihydrolipoamide dehydrogenase (DLDH) and Complex I

1a. Incubate the gel strip in 20 ml of 0.2 mg/ml nitroblue tetrazolium (NBT) and 0.1 mg/ml NADH in 50 mM potassium phosphate buffer, pH 7.0. Gently shake until purple deposit bands appear (as shown in Fig. 19.29.2).

NBT is yellowish in solution but becomes a purple deposit on the band that catalyzes NADH reduction of NBT. The intensity of the purple deposit on the band is positively correlated to the enzyme contained in the band.

Activity staining of Complex II

1b. Incubate the gel strip in 20 ml of Tris·Cl (pH 7.4) containing 50 mM sodium succinate, 215 mM phenazine methosulfate, and 20 mg nitroblue tetrazolium (NBT). Gently shake until a blue band appears near the bottom of the gel.

Sodium succinate serves as complex II substrate, while phenazine methosulfate is an artificial electron acceptor that can relay electrons to NBT, which then forms a purple deposit on the gel band that contains complex II.

A representative complex II activity staining is shown in Fig. 19.29.3A.

Activity staining of Complexes III and IV

1c. Incubate the gel strips in 50 ml sodium phosphate buffer (pH 7.4) containing 20 mg DAB and 50 mg cytochrome *c*. Shake gently until reddish bands appear.

The lower band is that of complex IV, while the upper band is that of complex III (Fig. 19.29.3B). DAB can be oxidized by hydrogen peroxide and forms a dark brown color on the gel band which contains either complex III or complex IV. Hydrogen peroxide can be generated by these complexes in the presence of cytochrome c.

For unknown reasons, we have found that complex IV can also be stained in the above solution in the absence of cytochrome c. However, the development of a visible complex IV staining band usually takes more than 6 hr.

Complex III activity staining can also be achieved by the method described by Smet et al. (2011), whereby a kit from Pierce called 1-step TMB blotting is used.

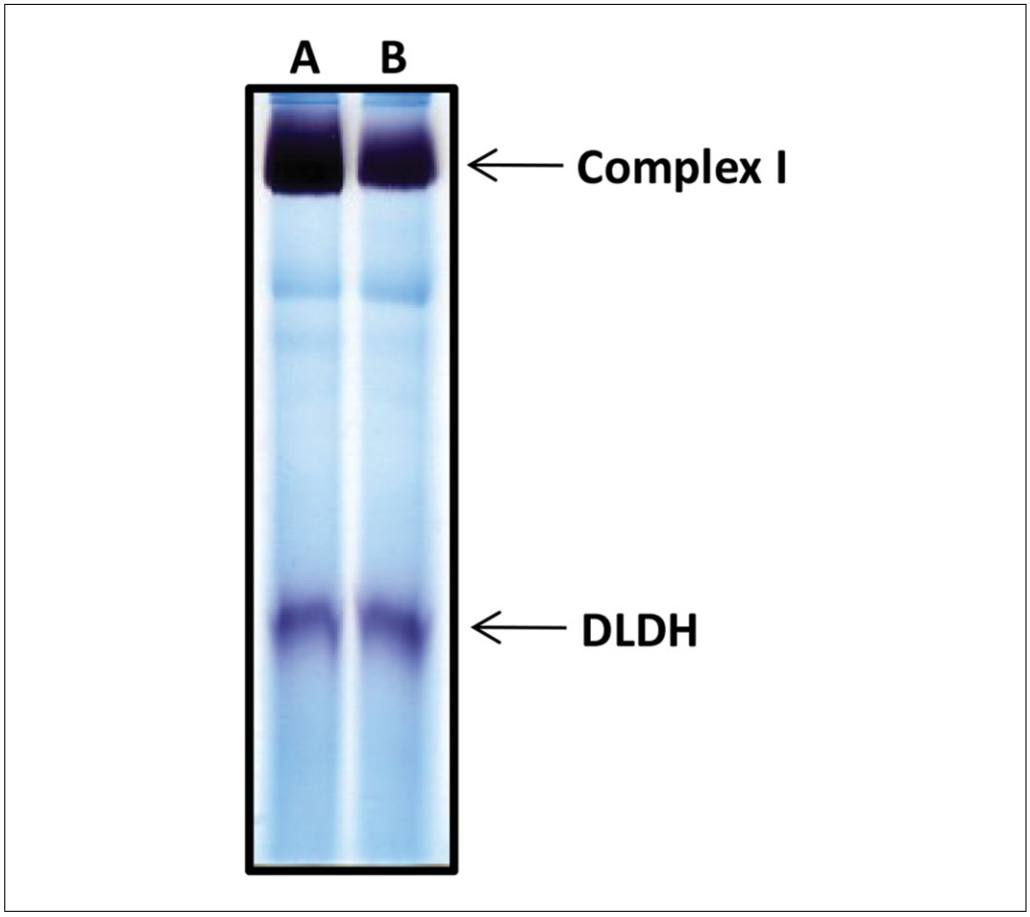

Figure 19.29.2 In-gel activity staining of mitochondrial complex I and dihydrolipoamide dehydrogenase (DLDH). Both enzymes can be detected simultaneously on one gel. The gel strip was incubated in a solution containing NADH (0.2 mg/ml) and nitroblue tetrazolium (NBT; 0.1 mg/ml). Activity staining was stopped by transferring the gel strip to a solution containing 10% methanol and 10% acetic acid. Each lane represents one brain mitochondrial preparation from one rat.

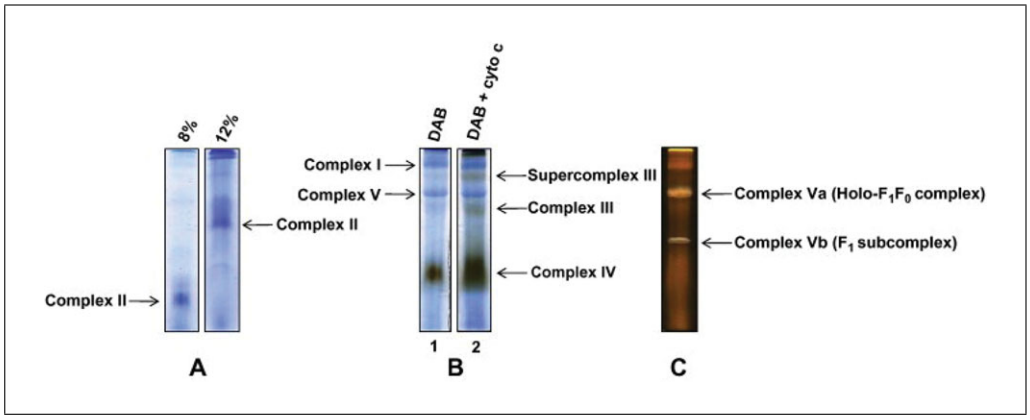

Figure 19.29.3 In-gel activity staining of mitochondrial complexes II to V. (**A**) Complex II activity staining on both 8% and 12% gels. (**B**) Complexes III and IV activity staining with DAB in the absence (left panel) or presence (right panel) of cytochrome *c*. There was also a supercomplex above the complex V band that could be stained by complex III substrate, which was named supercomplex III (Yan and Forster, 2009). (**C**) Complex V activity staining showing two activity bands representing complex Va and complex Vb, respectively. This figure was reprinted with permission from Yan and Forster (2009).

Activity staining of Complex V

1d. Incubate the gel strip in 50 ml Tris-glycine buffer containing 14 mM $MgCl_2$, 0.2% $Pb(NO_3)_2$, and 8 mM ATP. Gently shake until visible white color bands develop.

Complex V staining is based on the reaction between lead nitrate and phosphate that is released from ATP by complex V. Lead phosphate then deposits on complex V.

For a given gel strip, there are usually two bands of complex V that can be stained. The one having a high molecular weight is that of complex Va (so called holo-F_1F_0 complex), while the one having a low molecular weight belongs to that of complex Vb (F_1 subunit) (Fig. 19.29.3C).

PROCEDURES FOR ESTERASE ACTIVITY STAINING

SUPPORT PROTOCOL 2

The purpose of this protocol is to detect cellular or tissue esterases resolved by non-gradient BN-PAGE, described in Basic Protocol 1. Following gel electrophoresis, the gel band that contains the esterases to be visualized and analyzed is stained by incubating in a solution containing naphthyl acetate and Fast blue BB. Naphthyl acetate serves as the substrate of the esterases to be detected, while Fast blue BB is a diazonium salt that can form a colored, insoluble azo dye upon reaction with naphthol on the band that catalyzes formation of naphthol from naphthyl acetate. As the substrate used in this staining is naphthyl acetate, only those esterases that catalyze the release of acetate from naphthyl acetate will be stained. Nonetheless, the protocol can be adapted for analysis of a specific esterase if the substrate of such an esterase is known and available.

Materials

Gel strips: prepared following gel electrophoresis (Basic Protocol) by cutting each lane out of the slab gel
50 mM Tris·Cl, pH 7.4 (*APPENDIX 2E*)
α- or β-naphthyl acetate (Sigma, cat. no. N8505 or N6875, respectively)
Ethanol
Fast blue BB (Sigma-Aldrich, cat. no. 44670)
Acetic acid
Methanol

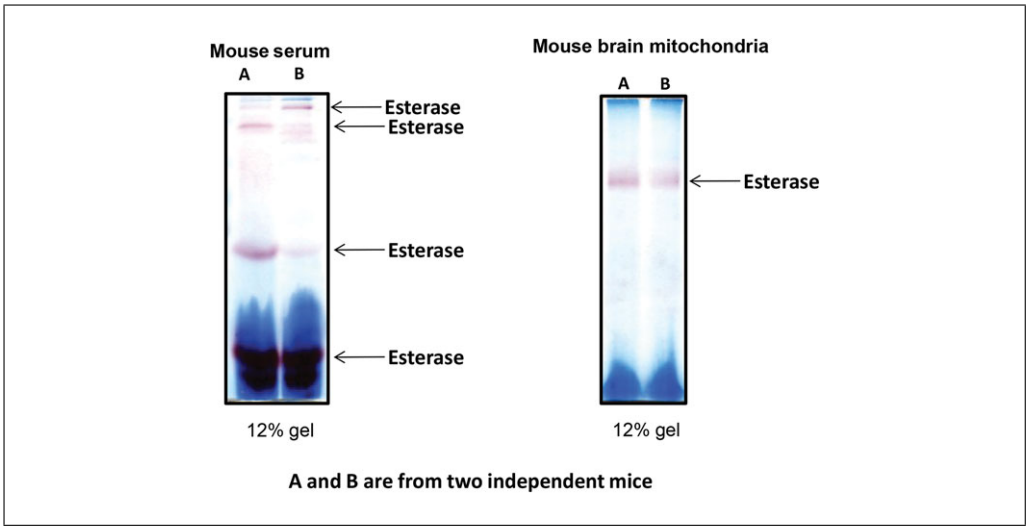

Figure 19.29.4 In-gel activity staining of esterases resolved by non-gradient BN-PAGE. Shown are esterases that were detected in mouse serum (left panel) and in mouse brain mitochondria (right panel) using the protocol described in this unit. It should be noted that the identities of these esterases remain unknown.

NOTE: This esterase activity staining method can be used to analyze esterase activities from a variety of sources such as serum, mitochondria, and cytosol. Regardless of the source of the enzymes to be analyzed, the final protein concentration in the loading sample should usually be 1 to 2 mg/ml. Gel running conditions are the same as described in the Basic Protocol for mitochondrial samples.

1. Incubate the gel strips in 100 ml of 50 mM Tris·Cl, pH 7.4, containing 50 mg naphthyl acetate (prepared in 1 ml ethanol) and 50 mg Fast blue BB (prepared in 50 mM Tris·Cl, pH 7.4. Shake gently until colored bands develop.
2. Stop the staining by transferring the gel strips to a solution containing 10% acetic acid and 10% methanol.

   Gels can be kept in this solution until further imaging analysis.

   A representative in-gel esterase activity staining is shown in Figure 19.29.4.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent for the preparation of all reagents. For common stock solutions, see APPENDIX 2E.

Acrylamide:bisacrylamide solution, 50% (acrylamide:bisacrylamide = 100:1 w/w)

Dissolve 100 g acrylamide and 1 g bisacrylamide in 200 ml water. This will give a 50% solution. Store at room temperature for up to 1 year. At 4°C or a lower temperature, acrylamide tends to precipitate, which will dilute the concentration of each chemical.

Anode buffer

Prepare 50 mM Bis-Tris, pH 7.0 (lower chamber). Store up to 6 months at 4°C.

Cathode buffer A

50 mM Tricine
15 mM Bis-Tris pH 7.0
Store up to 6 months at 4°C

There is no need to adjust the pH; it will be 7.0 using the given concentrations of the two chemicals. For upper chamber, without Serva blue G-250 (see Basic Protocol, step 9).

Cathode buffer B

50 mM Tricine
15 mM Bis-Tris pH 7.0
Store up to 6 months at 4°C

There is no need to adjust the pH; it will be 7.0 using the given concentrations of the two chemicals. For upper chamber, containing Serva blue G-250 (see Basic Protocol, step 10).

Gel buffer, 3×

1.5 M ε-amino-*N*-caproic acid (Sigma-Aldrich, cat. no. 1381)
150 mM Bis-Tris, pH 7.0
Store up to 6 months at 4°C
For 500 ml, weigh out 98.4 g ε-amino-*N*-caproic acid and 15.69 g Bis-Tris; adjust pH to 7.0 with concentrated HCl.

Final concentrations in gel: 500 mM ε-amino-N-caproic acid and 50 mM Bis-Tris, pH 7.0).

Mitochondria extraction buffer

0.75 M ε-amino-*N*-caproic acid (Sigma-Aldrich, cat. no. 1381)

5 mM Bis-Tris, pH 7.0
1% *n*-dodecyl-β-D-maltoside (DDM; Sigma, cat. no. D4641; add from 10% stock stored up to 6 months at 4°C)
Store up to 6 months at 4°C

This buffer can be diluted 2× from the concentrated gel buffer and supplemented with 1% DDM.

Sample buffer, 10×

3% (w/v) Serva blue G-250 (Serva Electrophoresis GmbH, cat. no. 35030)
0.5 M ε-amino-*N*-caproic acid (Sigma-Aldrich, cat. no. 1381)
Store in aliquots up to 1 year at –20°C

No pH adjustment is needed.

COMMENTARY

Background Information

Cellular proteins seldom exist individually. Rather, most proteins interact with other proteins and exist in a protein complex. This is particularly true for mitochondria, in which pyruvate dehydrogenase complex (Williams, 1992) and the major electron transport chain components (complexes I to V) exist as protein complexes. The development of gradient BN-PAGE in the early 1990s by Schagger and von Jagow (1991) has greatly facilitated detection and quantitation of mitochondrial protein complexes (Jung et al., 2000). The method has also found application in the analysis of non-mitochondrial proteins (Heinemeyer et al., 2004), and has been widely used for investigating ill-assembled protein complexes and abnormal protein-protein interactions in disease (Schagger, 1995; Van Coster et al., 2001; Ramos-Miguel et al., 2015). While the original gradient-gel technique has been modified by numerous investigators (Beriault et al., 2005; Singh et al., 2005; Dresler et al., 2011), none of the modifications have changed the gradient nature of this technique. As gradient gel analysis requires a gradient maker, which is expensive and not readily accessible, we have modified the method so that a non-gradient gel method can be employed to achieve what has been achieved by the gradient gel technique in analysis of mitochondrial proteins and tissue esterases (Yan et al., 2007; Yan and Forster, 2009; Thangthaeng et al., 2011; Yan et al., 2013; Wu et al., 2015). This unit is therefore a comprehensive summary of our non-gradient BN-PAGE method that may find use in analysis of protein-protein interactions and protein complexes.

Critical Parameters and Troubleshooting

For non-gradient BN-PAGE analysis of mitochondrial electron transport chain complexes, protein samples dissolved in mitochondria extraction buffer require clarification by high-speed centrifugation (usually 8000 × g for 10 min) to remove any insoluble particles or mitochondrial debris. Otherwise, protein complexes may not enter the gel and could stack as a thick band on top of the gel with heavy Coomassie blue intensity, resulting in partial resolution of the protein complexes. This would affect both quantitation and qualification of enzyme activities. This gel technique usually works well for mitochondria isolated from animal tissues. The only potential problem is that there is no reliable method for complex III staining, so the method presented in this protocol and that published by Smet et al. (2011) should be tested. Additionally, complex II is the smallest complex among all the five complexes—it usually runs in the front line together with the Coomassie blue band; hence, a high-percentage gel such as 12% may be run just for complex II analysis. On the other hand, activity staining for complex I, DLDH, and complexes IV and V is always readily achievable. It should be pointed out that the non-gradient gel method could be problematic for mitochondrial proteins isolated from cultured cells, as often sufficient mitochondrial material is not available. If this is the case, it may be useful to pool more cells from several culture dishes or flasks to increase the yield of mitochondria.

For analysis of serum or plasma esterase activity, serum or plasma needs to be diluted so that the final protein concentration in the

loading sample is approximately 1 to 2 mg/ml. Overloading should be avoided, as it can prevent proteins from entering the gel. While it is easy to visualize esterase activity on a gel, it has been proven to be challenging to identify the nature of such an esterase, which may require prior partial purification or fractionation of the original samples.

Anticipated Results

For mitochondrial enzyme activities, it is expected that complex I and DLDH will each produce a distinct band. This is also true for complexes IV and V. Sometimes, complex III may escape detection due to its low content in a given mitochondrial sample. On the other hand, complex III could also appear as more than one activity staining band, which is likely due to the presence of other mitochondrial proteins in the bands such as cytochrome *c*. For complex II resolved on a 12% gel, the staining should be well defined. For low-percentage gels, such as 8% or 9% resolving gels, complex II activity could be masked by residual Coomassie blue. For cultured cells, complexes II, III, and V may not be visible even after prolonged staining, but complexes I and IV, as well as DLDH, should exhibit well defined activity staining bands. For esterase activity staining, it is expected there will always be detectable activity bands, for which protein identities or nature of the esterase may remain unknown.

Time Considerations

For mitochondrial complex analysis, it usually takes 2 days to complete one round of assays. On day 1, mitochondrial isolation and protein quantitation can be performed, and the samples, after mixing with gel loading buffer, can be stored overnight at −20°C for next-day gel analysis. Alternatively, the loading samples can be stored at −20°C for up to 3 months before gel analysis. On day 2, non-gradient gels should be cast and electrophoresis should be carried out. Running the gel usually takes approximately 2 hr at 250 V. This is followed by activity staining of the enzymes, which will take an additional 2 to 3 hr, including image documentation and data analysis.

For serum or plasma esterase activity staining, running the gel and activity staining can be completed within 1 day if the serum or plasma samples are already available. For mitochondrial esterase detection and quantitation, a 2-day schedule, similar to that of mitochondrial complexes, should be planned.

Conflict of interest

The authors have declared no conflicts of interest for this article.

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