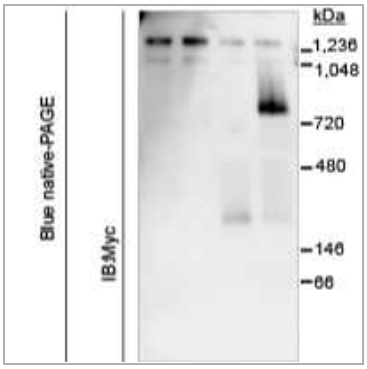

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We use this protocol and it's working

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Blue native-PAGE of protein complexes in plant cells

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ABSTRACT

Many protein complexes exist in cells, and these protein complexes are vital for cellular processes. However, much of our current analyses of protein-protein interaction mainly focus on binary interactions. Non-denaturing PAGE (polyacrylamide gel electrophoresis) provides an efficient method that enables analysis of multimeric protein complexes *in vivo*. In contrast to chromatography or sucrose fractionation methods, small amount of sample is required for analysis, and multiple combinations of samples can be tested simultaneously. Non-denaturing PAGE using the Coomassie G-250 dye, also known as blue native-PAGE resolves protein complexes according to their molecular weight and has been successfully used to identify protein complexes bound to membranes such as in mitochondria and chloroplasts. Recently, intracellular immune receptors in plants localizing in the cytoplasm, nucleus or plasma membrane were analyzed using the blue-native PAGE method. Here we share our method of resolving protein complexes from plant extracts or immunoprecipitated samples from plant cells on blue native-PAGE.

ATTACHMENTS

Blue native-PAGE figure_protocolsio.pdf

Last Modified: May 19, 2023

PROTOCOL integer ID: 79841

Keywords: Blue native-PAGE, BN-PAGE, BNP, Non-denaturing PAGE, intracellular immune receptors, NLRs, plants

GUIDELINES

* Although this protocol used the NativePAGE™ system from Thermo Fisher Scientific, similar set-ups from different suppliers, as well as setting up your own gel without SDS works just as well.
* Ensure samples are always kept on ice, and tissue samples are frozen immediately, to prevent dissociation or aggregation of protein complexes.
* Protein extraction buffer can significantly affect protein complex stability as well as extraction. Salt concentration of buffer, type and concentration of detergent used, as well as concentration of DTT are all critical factors that determin protein exractabiligy. Therefore, optimal extraction buffer must be empirically tested for each protein of interest.
One way to check extractability of buffer is to test whether any proteins remain in the pellet after centrifugation of cell debris. Protein extracts using different buffer conditions can be loaded onto blue-native PAGE to compare which buffer condition is ideal for extracting protein complexes.
However, not all buffer conditions ideal for extracting proteins will be ideal for preserving native states of protein complexes. The protein extraction buffer condition provided in this protocol is ideal for detecting oligomerization of NRC2, and related experimental data can be found in attachment.
* Concentration of plant extracts are critical for running blue native-PAGE. If bands appear smeary, it is likely due to high concentration of protein extracts leading to aggregation. Reducing the protein extraction concentration by dilution may resolve this issue. In particular, when using protein extracts, rubisco complex (~500kDa) should be clearly visible, in addition to bands at ~720kDa, and 1,000kDa for N. benthamiana extracts.
* Accumulation of protein complexes identified in BN-PAGE must correlate with protein accumulation observed on SDS-PAGE.
* 2 Dimensional SDS-PAGE should be performed to check that the protein bands observed on blue native-PAGE constitute of proteins that run on the same size on SDS-PAGE.
* The relevant position of the bands observed must be consistent throughout multiple replicates. This enables measuring the approximate size of protein complex with standard curve from the markers.

MATERIALS

Materials are divided into each section. Materials used throughout the procedure are listed as 'general materials'.
For materials denoted with asterisk, please refer to the Warnings section.
Check 'Buffers' section for detailed buffer recipes.

General materials

- Centrifuge (refrigerated model; Eppendorf 5415R)
- Rotator (kept cool; Stuart SB2 Fixed Speed Rotator)

- Microcentrifuge tubes
- Pipettes and tips
- Scissors
- Marker pen

Sample preparation

- Plants

  Nicotiana benthamiana plants are grown in 16 h light/8 h dark, at 22ºC, 45-65% humidity.

  Arabidopsis plants are grown in 8 h light/16 h dark, at 21ºC, up to 70% humidity. Light intensity was 100-200µmol.
- Cork borer (5 mm or 10 mm in diameter)
- 2 ml microcentrifuge tubes (cryogenic vials preferred)
- Tungsten beads
- Foil

Protein extraction

- Liquid nitrogen*
- GenoGrinder (SPEX®SamplePrep)
- Extraction buffer[1)]
- Vortex
- Microcentrifuge tubes (cryogenic vials if samples will be kept in -80ºC)

Immunoprecipitation

- Flag M2 beads (Sigma, A2220)
- Wash buffer[2)]
- Elution buffer[3)]
- Gel loading tip (or custom-made tapered tip)

  Using a flat steel appratus such as back of a spatula, slowly press the ends of the tip so that the tip end is narrowed.
- 3x Flag peptide (Sigma, F4799)
- Shaker

Blue native-PAGE sample preparation

- NativePAGE™ Sample Buffer (4X) (Thermo Fisher Scientific, BN2003)
- NativePAGE™ 5% G-250 Additive (Thermo Fisher Scientific, BN2004)

SDS-PAGE sample preparation

- 3x SDS sample buffer[4)] (or any other sample buffer used for SDS-PAGE)
- DTT (Melford, D11000)*

  Made to 1 M stock with distilled water
- Incubator (up to 70ºC)

Blue native-PAGE

- Anode buffer[5)]
- Cathode buffer[6)]
- NativePAGE™ 3-12% Bis-Tris protein gel, 1.0mm, 10well (Thermo Fisher Scientific, BN2001BOX)
- NativeMark™ Unstained protein standard (Thermo Fisher Scientific, LC0725)
- XCell SureLock™ Mini-Cell (Thermo Fisher Scientific, EI0002)
- Electrophoresis power supply (preferably in cold room; Bio-Rad 1645050EDU)

SDS-PAGE

- Custom-made (or pre-cast) gels
- SDS running buffer[7)]
- PageRuler™ Prestained Protein ladder, 10 to 180kDa (Thermo Fisher Scientific, 26616)

2D BN/SDS-PAGE

- Denaturation buffer[8)] for BN-PAGE gels
- Custom-made (or precast) gels for 2D SDS-PAGE (only resolution gel)
- 50 ml or 15 ml Falcon tubes
- 3X SDS sample buffer
- Distilled water

Protein transfer and fixation

- Transfer buffer[9)]
- Ethanol
- PVDF membrane (Bio-Rad, #1620177)
- Absorbent towel (WypAll x70, Kimberly Clark)
- Filter paper
- Trans-Blot®Turbo™ Transfer System (Bio-Rad, #1704150)
- 8% Acetic acid
- Western blot holder/box
- Distilled water
- TBS-T buffer[10)]
- 10x TBS buffer and Tween-20 (Sigma, P1379)
- Pencil

Immunoblotting

- Skimmed milk powder (Tesco)
- Antibody
- Western blotting detection reagent
  (Thermo Fisher Scientific, SuperSignalTM West Pico PLUS Chemiluminescent Sustrate, cat. 34580)

- Flat shaker

Buffers

1) Extraction buffer

50 mM	Tris-Cl, pH 7.5
50 mM	NaCl
5 mM	MgCl2
10% (v/v)	Glycerol
10 mM	DTT
0.2% (v/v)	NP-40 (alternative)
cOmplete™	Protease inhibitor cocktail (1 tablet/50 ml; Sigma, 11873580001)
2% (w/v)	PVPP (for larger samples)

2) Wash buffer

Extraction buffer, with 0.4% (v/v) NP-40 (alternative)

3) Elution buffer

Extraction buffer with 100-200 µg/ml 3x Flag peptide

4) 3x SDS sample buffer

100 mM	Tris-Cl, pH 6.8
30% (v/v)	Glycerol
3% (v/v)	SDS*
0.05% (w/v)	Bromophenol blue

5) Anode buffer

50 ml	20X NativePAGE^{TM} Running buffer (Thermo Fisher Scientific, BN2001)
950 ml	Distilled water (Milli-Q grade)

* Buffer should be made in advance and kept cold.

6) Cathode buffer (Light)

50 ml	20X NativePAGE^{TM} Running buffer (Thermo Fisher Scientific, BN2001)
5 ml	NativePAGE^{TM}Cathode buffer additive
945 ml	Distilled water (Milli-Q grade)

* Buffer should be made in advance and kept cold.

Optional)

Cathode buffer (Dark)

50 ml	*20X NativePAGE^{TM} Running buffer (Thermo Fisher Scientific, BN2001)*
50 ml	*NativePAGE^{TM}Cathode buffer additive*
900 ml	*Distilled water (Milli-Q grade)*

** Buffer should be made in advance and kept cold.*

7) 10x SDS running buffer (1 L)

30 g	Tris
144 g	Glycine
20% (w/v)	SDS*

* 10x stock buffers are made, and diluted to 1x before use.

8) Denaturation buffer

1x	SDS running buffer
10 mM	DTT*

9) Transfer buffer

Transfer buffer optimized for the protein transfer apparatus used in lab will work. We used the Trans-blot Turbo system from Bio-Rad.

200 ml 5x Trans-blot transfer buffer (Bio-Rad, #1704270)
200 ml Ethanol
600 ml Distilled water

10) TBS-T buffer

1x TBS buffer
0.1% Tween-20 (Sigma, P1379)

11) 10x TBS buffer (1 L)

24.2 g Tris
80g NaCl
Adjust pH to 7.6 with HCl*

SAFETY WARNINGS

Please follow lab safety procedures of each lab.

Liquid nitrogen may cause suffocation when used in enclosed spaces. Always be ware that the surrounding is well-ventilated.

For the following chemicals, please protect yourself with appropriate PPEs as well as read the Material Safety Data Sheet (MSDS) to be aware of the hazardous effect of these chemicals.
: DTT, SDS, HCl

BEFORE START INSTRUCTIONS

This protocol was developed over time by Hee-Kyung Ahn in the Jonathan Jones group. This method has been optimized mainly for detecting the oligomers of NRC NLRs in Solacaceae, but we have tested that this protocol also applies to NLRs in Arabidopsis seedlings and rosette leaves. For other proteins, additional optimization may be needed, and we are continuing to optimize the method for other NLR proteins. We hope this protocol will be useful, and help generate new discoveries in the plant biology community and beyond. If you have any suggestions or feedback regarding this protocol, please email hee-kyung.ahn@tsl.ac.uk.

Sample preparation

1 The following cases describe sample harvest methods using trasiently-infiltrated *Nicotiana benthamiana* (4-5 weeks old), *Arabidopsis* seedlings (~2 weeks old), or *Arabidopsis* leaves (4-5 weeks old.)

Step 1 includes a Step case.

Nb expression

At seedlings

At leaf extract

At leaves IP

Nb leaves IP

Protein extraction

step case 1m

Nb expression

1. Transiently infiltrate *N. benthamiana* (4-5 weeks old) with *Agrobacterium* carrying gene of interest.
2. Collect samples 2-4 days post infiltration.
3. Take 2-3 leaf disks using cork borer (diameter 10 mm), insert leaf disks into 2 ml tubes with 1 tungsten beads.
   - Add tungsten bead in 2 ml tubes before freezing.
4. Freeze the sample-containing tubes in liquid nitrogen, and store in -80°C until further use.

2 Grind samples in 2 ml tubes using a tissue homogenizer (GenoGrinder), at 1200 rpm for 00:01:00 .

Samples should be kept frozen at all times, and put sample tubes back into liquid nitrogen immediately after sample grinding is complete.

Note

Other equipments that enable rupturing frozen tissues with tungsten beads, for example, TissueLyser or other bead mills can all be used.

3 Open tube lids on ice, and add extraction buffer (see Materials, Buffer recipes).
The amount should be 200 µL per leaf disk , or 200 µL for 0.01g tissue weight .

4 Vortex for 00:00:15 . 15s

5 Incubate sample on rotator (must be kept cold) for 00:10:00 . 10m

6 Centrifuge at 4°C, 13,000rpm, for 00:15:00 . 15m

7 Transfer supernatant to clean new tube, centrifuge again at 4°C, 13,000 rpm for 00:05:00 . 5m

Note

Measure protein concentration using Bradford assay, and normalize protein concentration to the sample with lowest protein concentration.
However, using the same number of leaf disks during sampling, and adding same amount of extraction buffer ensures total protein concentration is similar across multiple samples.

8 Make Blue Native-PAGE samples from protein extracts.
It is advised to make master-mix of the 4x Native sample buffer, 5% Coomassie G-250 additive, and water.

For 40 µL of sample:
4x NativePAGE™ sample buffer 20 µL
5% Coomassie G-250 Additive 2 µL
Distilled water 18 µL
is added to make a total of 80 µL .

Note

Water is added to dilute the ion concentration of the protein extraction buffer to half strength. It is advised that the ion concentration of samples are below 50 mM. However, higher ion concentrations can be used, if all of the wells for blue native-PAGE will be loaded with buffers of similar strength.

Note

Do not boil samples, and keep the samples on ice at all times.

8.1 Samples can be frozen in liquid nitrogen, and stored in -70°C until further use.

9 Make SDS-PAGE samples from the same sample protein extracts.
This is to ensure same samples are used for both blue native-PAGE and SDS-PAGE.
For 40 µL of sample:
3x SDS sample buffer 20 µL
1 M DTT 8 µL
Distilled water 12 µL

9.1 Incubate samples at 70°C for 00:10:00 . 10m
After incubation, these samples are ready for SDS-PAGE.

Separation of samples by Blue Native-PAGE

10 NativePAGE™ 3 to 12% pre-cast gel is used with XCell*SureLock*™ Mini-Cell system.

> Note
>
> Although this method was optimized with pre-cast gel system from Thermo Fisher Scientific, other suppliers, as well as custom-made gels with varying gel percentages can be used for blue native-PAGE analysis as well.

11 Fill inner chamber with cold Cathode buffer (light) after removing comb.

> Note
>
> For some proteins, it is necessary to use the Dark Cathode buffer initially before exchanging for Light Cathode Buffer. However, for resolution of Rpi-amr3 and NRC2, light cathode buffer was sufficient. Light cathode buffer is preferred when immunoblotting, as Coomassie G-250 may interfere with immunoblotting steps.

12 Load samples. 10-15 µl (for 10-well gels). Add 5 µl of protein standard.

13 Fill outer tank with cold Anode buffer.

14 Gel running should be performed in cold room, or large refrigerator.

15 Running condition: 150 V, 45 min (1/3 run into gel)
250 V, 45 min-1 h (until dye front reaches end of the gel)
Stop before dye front runs through the gel.

Note

If using dark cathode buffer and subsequent immunoblotting will be performed, it is suggested to switch to light cathode buffer after the initial 45-minute run.

These gels can be used for protein transfer (next section), or separate strips can be used for 2 Dimensional blue-native/SDS-PAGE (see step case below).
Step 15 includes a Step case.
2D BN/SDS-PAGE

step case

2D BN/SDS-PAGE

After resolving different protein complexes according to their size, the protein complexes can be further dissociated into its individual components by applying a 2nd dimensional electrophoresis with SDS-PAGE.

16 Cut a strip of blue-native PAGE gel after electrophoresis is over, and carefully insert into 15 ml or 50 ml tube using gel holder or blunt/membrane forcep.

17 Add 3X SDS sample buffer with 10 mM DTT into the tube, for 00:15:00 . 15m

18 Rinse with water (at least three times).

Note

DTT and other reducing agents interfere with gel polymerization. Therefore, rinse thoroughly to remove excess DTT.

19 Prepare resolution gel of ideal percentage (for resolving Rpi-amr3 and NRC2, we used 8% gel).

Note

Alternatively, you can also use pre-cast gels from multiple suppliers that provide large wells in the gels to insert the gel strips.

Resolution gel recipe for 8% gel:

	A	B
	Water	4.6 mL
	1.5 M Tris (pH 8.8)	2.5 mL
	30% Acrylamide	2.7 mL
	10% SDS	0.1 mL
	10% APS	0.1 mL
	TEMED	0.006 mL
	Total volume	10 mL

20 Insert the denatured gel strip onto the prepared resolution gel.
Add stacking gel buffer to stack gel strip on top of the resolution gel.
Ensure no air bubbles are stuck in between the gel strip and the resolution gel.

Stacking gel recipe:

	A	B
	Water	1.4 mL
	1.0 M Tris (pH 6.8)	0.25 mL
	30% Acrylamide	0.33 mL
	10% SDS	0.02 mL
	10% APS	0.02 mL
	TEMED	0.001 mL
	Total	2 mL

21 Run with identical conditions for SDS-PAGE.

Protein transfer and immunoblotting of Blue Native-PAGE

1h 15m

22 After gels are run until dye fron reaches end of gel, gel cassette is pry open using cassette opening lever/gel knife.
Lift open all three sides of the cassette, and carefully cut both ends of the gel (side with wells and the opposite side where the dye front is).

23 Assemble transfer cassette. Avoid forming air bubbles between gel and membrane.

Note

If using NativeMark™ Unstained Protein Standard, a pink marker band will appear in the middle of the gel at this stage. Poke a small hole through the gel with a pencil to label the position of this marker on the membrane. (This marker, which is ~242 kDa may not be visible after transfer.)

24 Transfer is run with Transblot Turbo STANDARD method (20V constant, for 00:30:00). 30m

Note

Other types of semi-dry transfers can also be used, but methods must be optimized individually.
For wet-type transfer, transfer for 70 V (maximum 400 mA or lower) for 01:00:00 in cold room, using CAPS buffer (10 mM CAPS, calibrated to pH 11.0 with NaOH). The running time may need to be adjusted empirically.

25 After transfer is over, incubate the transferred membrane in 8% acetic acid for 00:15:00 . 15m

26 Rinse membrane with clean water two times briefly.

27 Place membrane on filter paper and let dry.
Up to 1 h in the fume hood, or overnight on bench.

28 After membrane is completely dry, re-activate membrane with ethanol.

29 As membrane is re-activated, Coomassie dye will wash out and markers will show up at this stage.
It is crucial to label all visible markers with pencil, as they will gradually disappear.

29.1 For some proteins, the epitope may be buried within the protein complex making it difficult to detect with antibodies.
To prevent this, incubate re-activated membrane with SDS running buffer (with up to 10 mM DTT) after re-activation 1 h to overnight. Wash thoroughly with TBS-T, 5 min 3 times.

30 After membrane is re-activated and Coomassie dye is significantly washed away, decant the ethanol, and immediately add TBS-T. Wash membrane with TBS-T, 3 times for 2 min each.

> Note
>
> When TBS-T is first added, re-activated membrane will tend to float, and as ethanol dries up, membranes may form blue patches. This can be prevented by adding excess amount of TBS-to submerge the membrane in buffer.

31 Add 5% skimmed milk in TBS-T to membrane, and block for 00:30:00 . 30m

32 Decant blocking solution, and add antibody in 5% skimmed milk in TBS-T for 1 h to overnight.

33 If using HRP conjugated antibodies, membranes can be washed with TBS-T 3 times (5 min each) and will be ready for detecting chemiluminescent signal.
If using non-conjugated antibodies, wash with TBS-T 3 times (5 min each), incubate with secondary antibody (in 5% skimmed milk in TBS-T), then repeat washing step before preparing

for chemiluminescent signal detection.