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Phostag™-gel retardation and *in situ* thylakoid kinase assay for determination of chloroplast protein phosphorylation targets

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The chloroplast phosphorylation network is important for posttranslational regulation of photosynthetic complexes, gene expression and metabolic pathways. In mass-spectrometric analyses a lot of putative phosphorylation targets have been found but these data need to be confirmed and brought into a physiological context. Here, we present a current protocol to quantify the phosphorylation state of thylakoid proteins and an *in situ* method to verify putative substrates for thylakoid associated kinases.

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Category: Technical note

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Abbreviations: BN: blue-native; STR: state transitions (short-term response); LHC: light harvesting complex; WT: wild type

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Introduction

Phosphorylation of chloroplast proteins plays an important role in regulation of light harvesting, assembly of photosynthetic complexes and adjustment of chloroplast gene expression. Phosphorylation of LHCII proteins induces antenna movement between the two photosystems to restore excitation balance (Allen et al. 1981; Allen 1992). The thylakoid intrinsic kinase STN7 is required for this process (Depege et al. 2003; Bellafiore et al. 2005). Its orthologue STN8 phosphorylates PSII core proteins (Bonardi et al. 2005; Vainonen et al. 2005) facilitating PSII remodeling (Dietzel et al. 2011) and probably repair (Tikkanen et al. 2008). Besides their major targets in PSII and its antenna complexes both kinases phosphorylate other thylakoid and stromal targets (Reiland et al. 2009, 2011). Other chloroplast kinases like CSK (Puthiyaveetil et al. 2008), PTK (Baginsky et al. 1999), TAK1 (Snyders and Kohorn 2001) and predicted kinases (Schliebner et al. 2008) constitute together with STN7 and STN8 a plastid regulatory network, which is far from being understood (for review see Lemeille and Rochaix 2010). Chloroplast phospho-proteomics and *in situ* experiments revealed that several hundred proteins are putatively phosphorylated (Steiner et al. 2009; Schoenberg and Baginsky 2012; Baginsky 2016) but data on substrate specificity and phosphorylation sites are rare.

Here, we present and evaluate two methods for investigation of the thylakoid kinase substrates. In Method 1 we determine and quantify the phosphorylation state using a gel retardation method exemplarily for Lhcb1 a major antenna protein of PSII. In Method 2 we describe an *in situ* thylakoid phosphorylation assay to verify possible substrates of thylakoid kinase. As example, we test the plastid glutamyl-tRNA-reductase binding protein (GBP) which has been described as spatial regulator of tetrapyrrole synthesis (Czarnecki et al. 2011).

Material and Methods

Method 1: Blue-native/phostag gel retardation assay

Principle

Phostag™ specifically captures and retains phosphomonoester dianions occuring as phosphorylation in proteins. In the gel approach the Phostag™ ligand is acrylamide-derivatized enabling covalent fixation in the gel matrix (Figure 1; Kinoshita et al. 2006, 2009, 2015). For activity the phostag ligand requires chelation of a divalent cation which usually is Mn^{2+} or Zn^{2+}. For separation of major

thylakoid phosphoproteins the Zn^{2+} at neutral pH appears to be the method of choice. This is in line with data obtained in a similar approach (Longoni et al. 2015). We determined the Lhcb1 phosphorylation state as a typical application of the method. We compared the phosphorylated Lhcb1 in WT, the LHC kinase mutant *stn7* and the kinase double mutant *stn7*/*stn8* in which thylakoid protein phosphorylation is almost abolished (Bonardi et al. 2005; Bellafiore et al. 2005; Wagner et al. 2008; Dietzel et al. 2011). We separated LHCII trimers and monomers from Arabidopsis thylakoids using blue-native PAGE (Schägger and Jagow 1991) and separated the respective complexes in a second dimension phostag gel (Figure 2A).

Materials and protocol

The gel run is carried out in a standard mini-slab-gel device. Make sure that gel slice from 1st dimension fits in the 2nd dimension. It is recommendable to use 0.7-0.9 mm gel spacers in the 1st and 1 mm in the 2nd dimension. The gel stock solutions should be sterile filtered (Table 1). These can be stored in the refrigerator or freezer for several days. Prepare the gel solutions for stacking and resolving gel fresh (Table 2) and degas them under nitrogen flow before adding APS and TEMED since some components are prone to oxidation. Allow the gel to polymerize overnight covered with 2-propanol or n-butanol.

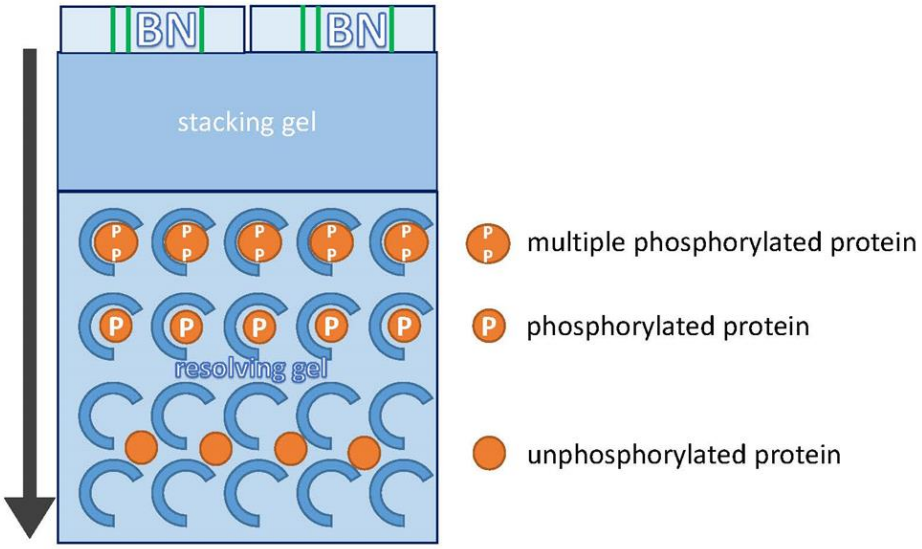

Figure 1: Principle of Phostag gel retardation. Proteins from 1st dimension gel slice (BN) are separated first in a Laemmli-type stacking gel. The actual phosphor-affinity electrophoresis takes place in the resolving gel where the Phostag™-compound is covalently fixed in the gel matrix. The method allows to distinguish between mono-phosphorylated and multiple phosphorylated proteins.

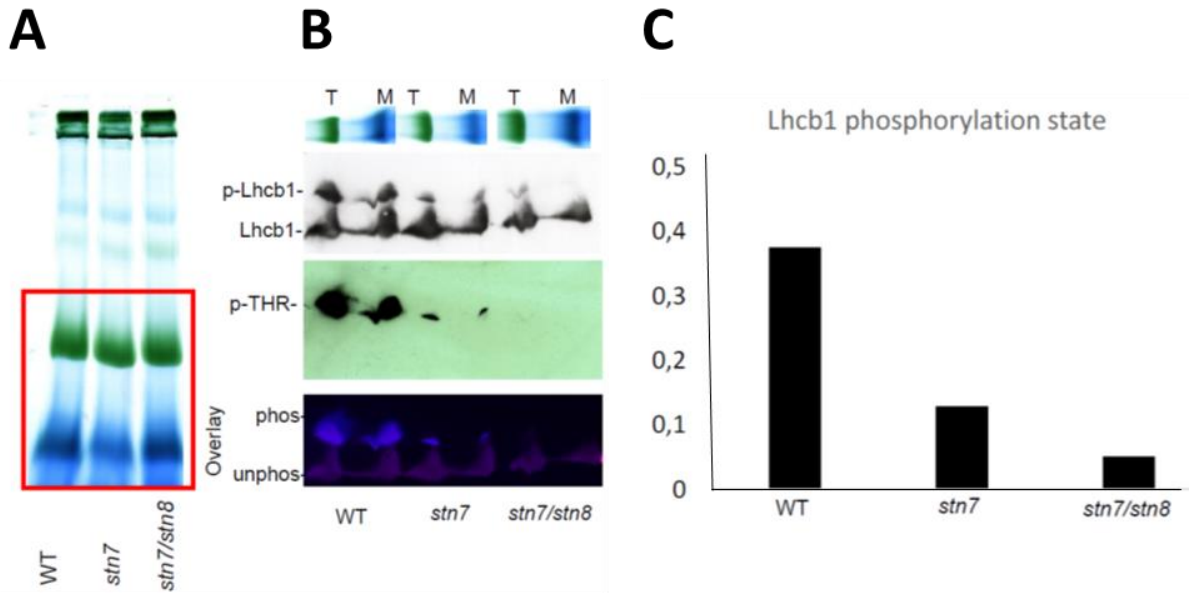

Figure 2: Determination of Lhcb1 phosphorylation state of WT, *stn7* and *stn7/stn8* from white light grown plants. (A) Thylakoid complexes were separated in the 1st dimension (BN). Gel slices marked in the red box were transferred on to the Phostag gel (B) and blotted. (B) Western immuno analysis of the separated proteins against Lhcb1 (upper panel) and against phosphorylated threonine residues (p-THR). The lower panel represents the overlay of both signals. "T" marks LHCII trimers and "M" marks LHCII monomers. (C) Quantification of Lhcb1 phosphorylation state from *B upper panel* (Lhcb1) *via* ImageJ (http://imagej.nih.gov/ij/).

Table 1: Stock solutions for Phostag gel shift assay

31% acrylamid solution	**100 ml**
Acrylamid	30 g
N,N-Methylenebisacrylamid	1 g
5 mM Phostag	**3.4 ml**
Phostag (Wako Chemicals)	10 mg
Methanol (for dissolving phostag)	100 µl
Ultrapure water	3.3 ml
5x Phostag running buffer	**500 ml**
Tris pH 7.8 (HCl)	0.5 M
MOPS	0.5 M
SDS	2.5 g
$NaHSO_3$ (for reducing conditions)	50 mM
3x sample buffer (-20°C)	**1 ml**
Tris pH 6.8	40 µl
Glycerol	300 mg
SDS	60 mg
Brom phenol blue	few grains
2-Mercapto-ethanol	150 µl
Ultrapure water	add to 1 ml
Phostag gel buffer	**100 ml**
BisTris pH 6.8 (HCl)	1.4 M
Blot-Transfer buffer	**100 ml**
Tris	25 mM
Glycin	192 mM
EDTA	1 mM
Methanol	20% (v/v)
Other solution	
Tris pH 6.8 (HCL)	0.5 M
$ZnCl_2$	10 mM
APS (-20°C)	10% (w/v)
TEMED	100%

Note: Ultrapure (metal ion-free) water is required.

Table 2: Composition of Phostag™ gel solutions for one mini gel

Stock	**Stacking gel [µl]**	**Resolving gel [µl]**
Acrylamid (30%)	300	1875
Gel buffer	500	1875
$ZnCl_2$	-	75
Phostag	-	37.5
H_2O	1190	3590
APS	10	25
TEMED	3	10
Total volume (rounded)	**~ 2000**	**~7500**

Note: Use freshly ultrapure water. Sterile filtration is recommended. Flow with N_2 to degas before adding APS and TEMED.

Carry out the 1st dimension (BN) as described in (Dietzel et al. 2011; see below for brief protocols). The gel slices (Figure 2A, B) are cut and allowed to soak the pre-chilled 3x sample buffer (1) for 5 min with gentle agitation. Then transfer the gel slices to the 1-well Phostag™ gel which was prepared the day before. Make sure to orient the bands in perpendicular direction to the gel surface in order to prevent broad bands and cover the gel with 1% Agarose made with 1x running buffer. If a protein weight marker is required, it is useful to embed a filter strip soaked with the marker. Run the gel for 2-3 h at a maximum current of 80 mA to allow maximum separation.

After the run Phostag gel can be treated, in principle, like "normal" Laemmli-type gels but a few points are important to consider:

Blotting: Incubate the gel for 10 min in transfer buffer containing 1 mM EDTA. Use the same buffer with EDTA for the protein transfer. Transfer times double with respect to normal blotting times.

Coommassie stain: Avoid staining longer than 2 hours since Phostag™ gels tend to destain very slowly. Long staining times will result in higher background.

Silver staining: It is possible, although the background is somewhat higher. This effect can be minimized by treating the gel with 1 mM EDTA for 10 min before applying the standard silver-staining protocol.

Example

In our example (Figure 2) we decided to transfer the proteins onto PVDF membranes because nitrocellulose membranes resulted in higher background. We incubated the blot with anti-Lhcb1 antibodies (Agrisera, Sweden) and after stripping with anti-phospho-threonine antibody. Lhcb1 separates in two bands in WT thylakoid extract. Thereby the upper band is phosphorylated (Figure 2B). In *stn7* this phosphorylation signal is strongly reduced which is the proof of principle since Lhcb1 is a known substrate of the LHCII kinase STN7. In the double mutant virtually no Lhcb1 phosphorylation was observed. This experiment nicely reflects results obtained by Bonardi et al. (2005) in standard western assays where only residual LHC phosphorylation was found in the *stn7* mutant. Quantification of the signals shows that under standard white light conditions approximately half of all Lhcb1 proteins is phosphorylated (Figure 2C). Future experiments will reveal the phosphorylation state of other photosynthetic proteins such as Lhcb2 and PSII core proteins (CP43) in order to investigate regulation of PSII *via* state transitions and PSII remodeling.

Method 2: *In situ* thylakoid kinase assay

Principle

Phosphorylation of thylakoid proteins is important for regulation of photosynthetic complexes but the kinase network extends to stromal proteins possibly linking photosynthetic performance with cellular homeostasis (*e.g.* gene expression). Here, we present a method to identify soluble stromal proteins as potential targets of thylakoid kinases. In our example we test whether the GBP (accession: AT3G21200; Czarnecki et al. 2011) is a putative substrate of the thylakoid kinases STN7, STN8 or other thylakoid kinases. For this purpose, we isolate thylakoids from WT, the single mutant *stn7* and the double mutant *stn7/stn8*. The kinases are activated by reduced plastoquinone bound to the cytochrome b_6f complex (Zito et al. 1999). In isolated thylakoids we apply dim illumination to activate photosynthetic electron transport and thus causing a reduced PQ-pool (Bellafiore et al. 2005). The potential target protein should carry a tag for affinity chromatography later

on. In our case we use GBP with a C-terminal 6 x histidine tag for the phosphorylation assay. We checked the Arabidopsis phosphorylation database PhosPh*AT* (Heazlewood et al. 2008) with a phosphosite prediction option (Durek et al. 2010; http://phosphat.uni-hohenheim.de/). GBP has a predicted Ser-phosphorylation site (S110) with a high score but experimental phosphoproteomic data is still lacking.

Materials and protocol

Prepare the required solutions and material (Table 3). Please mind local legal regulations on the use of phosphor isotopes. The highest kinase activity is achieved with fresh and gently isolated thylakoids (see below for a brief protocol). Therefore, it is recommended to prepare all solutions needed for the kinase assay in advance.

Table 3: Solutions required for *in situ* thylakoid kinase assay

Assay buffer	**10 ml**
HEPES pH 7.5 (KOH)	50 mM
Sorbitol	100 mM
$MgCl_2$	5 mM
NaCl	5 mM
ATP (add fresh)	0.4 mM
Protease inihibitor (EDTA-free!)	Manufacturer instruction
Wash buffer	**100 ml**
NaH_2PO_4 pH 8 (Na_2HPO_4)	50 mM
NaCl	300 mM
Imidazole	20 mM
Elution buffer	**100 ml**
NaH_2PO_4 pH 8 (Na_2HPO_4)	50 mM
NaCl	300 mM
Imidazole	200 mM
Other solutions and equipments	
γ-^{32}P-ATP (*e.g.* Easy Tides™)	111 Tbq/mol
Aceton	80% (v/v)
Ni-NTA-Agarose or sepharose	50 µl/sample
Non-phosphorylatable control protein (*e.g.* BSA)	same concentration as target
Phosphoimager + screen or X-ray film	
Spectrophotometer	
Optional: gel dryer	

At first, prepare the samples in 1.5 ml reaction tubes with assay solution containing the radioisotopes (Table 4) but omit the thylakoids. Place the tubes in a water bath of 25°C with a supplemental white light of 50-100 µmol photons*m^{-2}*s^{-1} using a fluorescent tube in approximately 20 cm distance.

Table 4: Typical assay volumes

Solutions	**1 Assay [µl]**	**5 Assays [µl]**
Assay buffer	85	425
Thylakoids	8 [1 µg chl/µl]	40
Protein (his-tagged)	6 [0.5 µg/µl]	30
γ-^{32}P-ATP	1	5
Total volume [µl]	**100**	**500**

Then start to prepare the thylakoids and keep the procedure as short as possible (30 min or less). Therefore, quickly add the required volume of thylakoid to the assay buffer that was prepared in advance. Dilute the thylakoids if necessary.

Place the samples back to the water bath and keep them for 30 min at approximately 80 µmol photons*m^{-2}*s^{-1}. Use negative controls (with kinase inhibitors or heat deactivated kinase and a non-phosphorylatable protein such as BSA or even better – a nonphosphorylatable 6 x His-tagged protein of your choice. The positive control is "built in" as the LHCII gets phosphorylated in WT but not in the kinase mutants. After the incubation time is over store the samples on ice.

Start to recover the target protein. Centrifuge 3 min at full speed. Transfer the supernatant to a fresh tube and keep the pellet - it's your positive control!

Incubate the supernatant with 50 µl Ni-NTA-Agarose-slurry (25 µl column volume) for 10 min with occasional shaking. Then transfer the all liquid and column material into a microfuge spin column and spin 30 s at 5000 rpm and then wash two times with 200 µl wash buffer.

Elute with 100 µl elution buffer (spin 1 min at full speed) - there should be some radioactivity in the tube and less radioactivity in the column material.

Add denaturing sample buffer to the eluate and the thylakoid pellet. Denature for 5 min at 70°C and centrifuge for 2 min at full speed in the microfuge.

Load not more than 1 µg target protein or 2 µg of total chlorophyll and run SDS-PAGE using a standard gel.

Afterwards dry the gel using a gel dryer or a drying frame. If you do not possess this equipment the gel can be transferred to membrane via western blotting. Expose the gel or the blot to a Phosphor-Imager™ screen for at least 3 days or X-ray film.

Example

In our experiment we added 3 µg of 6 x His-tagged GBP to thylakoids of WT and the kinase mutants *stn7* and *stn7/stn8* (8 µg total chlorophyll). For WT we expect phosphorylation of Lhcb-proteins and PSII core proteins as it is the case in our control treatment (Figure 3A). *stn7* is impaired in Lhcb phosphorylation and in *stn7/stn8* virtually all phosphorylation is lacking except one faint signal at ~25 kDa.

GBP (~34 kDa) shows a strong phosphorylation signal in the autoradiograph in WT and mutant lines (Figure 3B). There is some signal around 26 kDa which is probably residual phosphorylated Lhcb protein. But compared to the signal in Figure 3A the recapture of GBP by Ni-NTA worked fairly well. Neither the inactivated samples nor BSA are unspecifically phosphorylated indicating a true phosphorylation event by a thylakoid kinase.

GBP seems to get phosphorylated but not by STN7 or STN8. Hence, there must be another thylakoid associated kinase phosphorylating GBP, which in the future can be tested using the same approach with different thylakoid kinase mutant lines.

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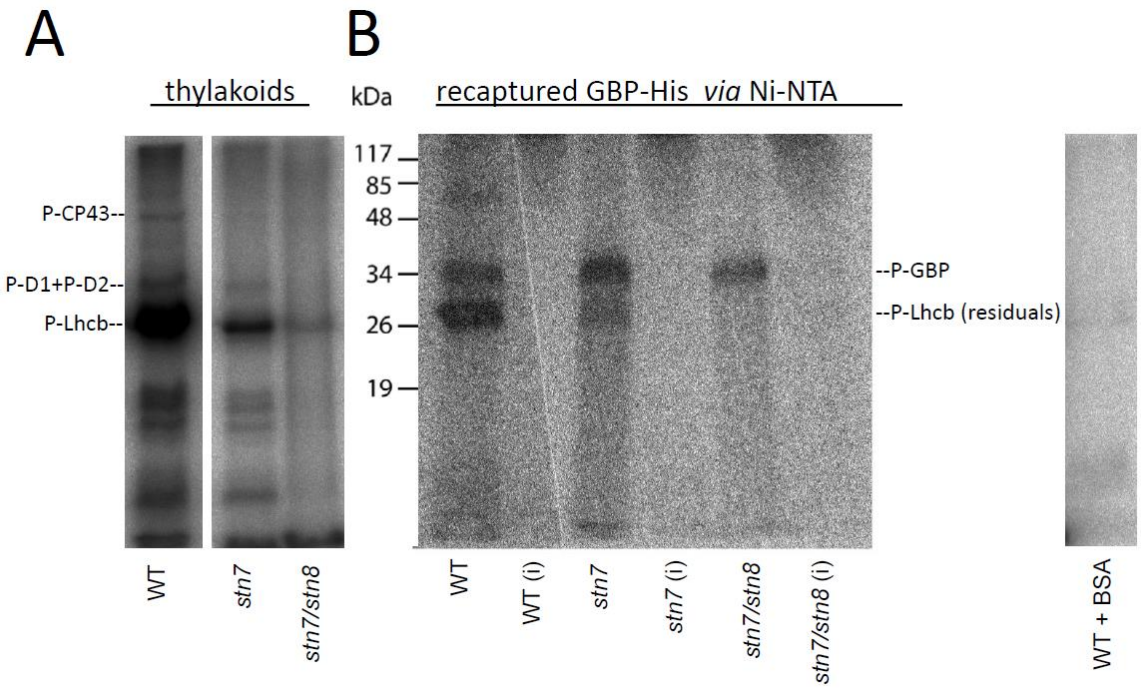

Figure 3: *In situ* kinase assay of thylakoids. (A) Autoradiograph of phosphorylated thylakoid intrinsic proteins of WT, the LHC-kinase mutant *stn7* and the double mutant *stn7*/*stn8* which additionally lacks PSII core phosphorylation. (B) Autoradiograph of recaptured GBP-His *via* Ni-NTA agarose and residual LHC-protein co-purified from the column material. BSA serves as control for a non-phosphorylatable protein. (i) marks the inactivated sample.

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Thylakoid preparation (in brief)

Homogenization buffer (HB):

0.33 M sorbitol
50 mM HEPES/KOH pH 7.6
5 mM $MgCl_2$
10 mM KCl
10 mM NaF (phosphatase inhibitor)

Hypotonic buffer:

Same as HB without sucrose

Hypertonic buffer:

Same as HB with double amount of sucrose (0.66 M)

- Harvest ~5 g of 2h-dark-adapted leaf material from young plant rosettes (*e.g.* 3 week old plants). Keep the samples all the time on ice and in the dark.
- For homogenization we use 40 ml pre-chilled homogenization buffer in a small size beaker for the waring blender and give 3 pulses of 5 seconds at highest speed.
- Filter the suspension through 4 layers of muslin and 1 layer of miracloth.
- Centrifuge the filtrate 5 min at 2000 x *g* in a prechilled falcon tube centrifuge.
- Gently, re-suspend the pellet with 500 µl HB using a fine brush or pipet slowly with a cut blue tip until all aggregates are re-suspended. Avoid shearing forces to keep thylakoid membranes intact!
- Add 9.5 ml hypotonic buffer mix and keep the chloroplasts for 5 min on ice during the osmotic shock.
- Add 10 ml of hypertonic buffer mix and centrifuge immediately for another 5 min at 2000 x *g*.
- Gently, re-suspend the pellet in the smallest volume possible (i.e. 1 ml or less).
- Determine chlorophyll content and proceed with the experiment (kinase assay).
- (For phostag gels it is possible to store the samples at -80°C with prior shock freezing in liquid nitrogen.)

Chlorophyll determination

For accurate chlorophyll determination, dilute 3 x 5 µl thylakoid suspension with 95 µl H_2O and then mix with 900 µl 90% acetone. Spin for 3 min in an Eppendorf tube centrifuge at maximum speed. Take the supernatant and measure absorption at 647 nm, 664 nm and 750 nm. Substract E750 nm from E664 nm and E647 nm.

Use the following formula for total chlorophyll content according to (Porra et al. 1989):

(1) Chl a+b [µg/ml] = 17.76*E647nm + 7.34*E664nm

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Blue native sample preparation (in brief)

(Protocol modified from Schägger and Jagow 1991 and Rokka et al. 2005)

1) Prepare the gel

3x gel buffer pH 7.0 (KOH)

150 mM BisTris, 1.5 M 6-amino caproic acid

BN-Gel (gradient from 5-12%)			
	Light solution (5%)	Heavy solution (12%)	Stacking gel
Acrylamid (30A:0.8BA)	1.36 ml	3.36 ml	2.73 ml
3x gel buffer	2.8 ml	2.8 ml	7.0 ml
Glycerol (75%)	0.56 ml	2.24 ml	-
Water (ultrapure)	3.6 ml	-	11.0 ml
Temed	15 µl	15 µl	40 µl
APS (10%)	20 µl	20 µl	60 µl

10x blue cathode buffer pH 7.0 (KOH)

150 mM BisTris
500 mM Tricine
0.1% w/v Coomassie blue G

10x cathode buffer pH 7.0 (KOH)

150 mM BisTris
500 mM Tricine

10x anode buffer pH 7.0 (KOH)

500 mM BisTris

2) Prepare and solubilize the thylakoids

Thylakoid wash buffer pH 7.0 (KOH)

330 mM sorbitol
50 mM BisTris

50B_40G pH 7.0 (KOH)

50 mM BisTris pH 7,0
40% (w/v) Glycerol

25B_20G

250 µl 50B_40G
20 µl protease inhibitor (*e.g.* Roche complete)
5 µl NaF (1M)
225 µl water (ultrapure)

2% BDM buffer

100 µl 50B_40G
40 µl β-dodecylmaltosid (10% w/v)
10 µl protease inhibitor
50 µl water (ultrapure)

2x BisTris ACA pH 7.0 (KOH)

50 mM BisTris
1 M 6-amino-caproic acid

10x BN loading buffer

500 µl 2x BisTris-ACA
400 µl sucrose (75% w/v)
50 mg Coomassie Blue G
add water to 1 ml final volume

empty well solution

135 µl 25B_20G
15 µl β-dodecylmaltosid (10% w/v)

- Wash 20 µg thylakoids (total chlorophyll) in 300 µl thylakoid wash buffer.
- Centrifuge at 6000 rpm for 3 min and 4°C afterwards discard the supernatant.
- Gently, resuspend the pellet in 17.5 µl 20B_25G using a cut tip.
- Add 2% BDM-buffer (20 µl) and mix the samples gently on ice (avoid foam).
- Allow solubilization for 10 min (on ice and darkened).
- (optional break point/storage: shock freeze samples in liquid N_2 and store them at -80°C).
- Centrifuge solubilized samples for 2 min in a microfuge (full speed, 4°C).
- Prepare in the meantime microfuge tubes with 3.7 µl BN loading buffer.
- Mix supernatant with loading dye (avoid foam).
- Load the gel and fill empty wells with empty well solution.
- Run the gel with blue cathode buffer at 100 V for the first 1h then rise voltage to 200 V (limit current to avoid overheating - we use 5 mA for a 90mm x 1 mm gel).
- Exchange blue cathode buffer with clear cathode buffer when the front reaches approx. half of the gel.
- Stop the run when the blue front reaches the end of the gel.

References

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