Overexpression of Endoplasmic Reticulum (ER) proteins from *Arabidopsis thaliana* in baculovirus

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Abstract

The overproduction of proteins of the Endoplasmic Reticulum (ER) of plant cells in prokaryotic heterologue gene expression system remains a technical challenge. Recent advances in genetically modified insect cells technology and virus engineering methods have paved the way to produce recombinant ER plant proteins, including those harbouring post-translational modifications and therefore, to yield ER plant proteins that are natively folded and fully functional. The present contribution focuses on the baculovirus-expression system flashBac, which overcomes certain technical hurdles found in other insect cells-based expression systems such as the generation of a bacmid and the negative selection of recombinant clones.

Keywords: Endoplasmic reticulum (ER); heterologous gene expression; *Arabidopsis thaliana*; protein overproduction; flashBac; baculovirus; post-translational modifications.

1 Introduction

Insect cells are a widely used system for the overproduction of recombinant proteins of different origin, ranging from green plants to vertebrate and invertebrate animals. A key advantage over other expression systems such as bacteria cells, is the possibility to produce fully functional proteins that undergo postranslational modifications including glycosilation, phosphorylation and acetylation, to name a few. Indeed, early estudies showed that insect cells are a suitable system for the overexpression of endoplasmic reticulum (ER) plants proteins [1-2] and more recently, of proteins associated to the ER, paving the way to enhance our understanding of the molecular mechanisms underlying ER function [3].

Baculovirus are insect viruses that primarily infect insect larvae of the order Lepidoptera (e.g., butterflies and moths) [4] and constitute the most frequently used vehicle to produce recombinant proteins in insect cells. To this aim, a recombinant baculovirus is genetically modified to harbour a foreign gene of interest, which is subsequently expressed in a host insect cell line under control of a baculovirus gene promoter. The most extensively used baculovirus is based on the genetically modified genome *Autographa californica* Multicapsid Nucleopolyhedrovirus (AcMNPV). This is a double-stranded, supercoiled DNA genome of approximately 130kb in length that is packaged into a rod-shaped nucleocapsid [5-6]. Although this nucleocapsid can incorporate large gene inserts due to the property of expanding lengthways, the AcMNPV genome is considered to be too large for direct foreign gene insertion. For this reason, the gene of interest is usually cloned into a transfer plasmid that contain sequences flanking the *polh* gene in the virus genome. Following co-transfection (e.g., simultaneous introduction) of the virus genome and the transfer plasmid into host insect cells, exchange of DNA with the insertion of the gene of interest into the viral genome at the *polh* locus takes place through homologous recombination. Subsequently, a recombinant baculovirus is generated and the virus genome replicated. The recombinant virus can be easily harvested from the culture medium by centrifugation. The most frequently utilised lepidopteran insect cell lines for baculovirus expression system are derived from *Spodoptera frugiperda* (*Sf*9 and *Sf*21) and *Trichoplusia ni (T.ni).* These cell lines can grow in suspension or adherent cell culture in supplied or serum-free medium containg phosphates buffer to adjust the pH. These cell lines grow optimally at 28°C without the requirement of CO_2, rendering protein production scale-up feasible and economic [7].

The flashBAC™ baculovirus expression system is an advanced technology to produce recombinant baculoviruses. flashBAC™ builds on BacPAK6 technology in which a *lacZ* gene has been inserted at the AcMNPV *polh* gene locus and a Bsu361 restriction site introduced in each side of *lacZ* [8]. One important feature of the flashBAC™ system is the addition of a

Bacterial Artificial Chromosome (BAC) at the *polh* gene locus and the partial deletion of the essential *ORF1629* replication gene. The *polh* gene facilitates the maintenance of the virus genome in bacterial cells as a bacmid, while *ORF1629* prevents viral replication in insect cells. Production of recombinant virus in insect cells is attained through co-transfection of the AcMNPV genome (flashBAC™ DNA) with an appropriate transfer plasmid harbouring the foreign gene of interest. Homologous recombination between ORF603 and ORF1629 in flashBAC™ DNA and the transfer plasmid replaces the BAC replicon with the gene of interest under the polh promoter, while simultaneously restoring ORF1629, thus enabling replication of recombinant virus containing the gene of interest (**Figure 1**). An important advantage of the flashBAC system is that separation from the parental virus is not required because of the incapability of non-recombinant virus to replicate. Consequently, the production of recombinant virus is reduced to a single step procedure, turning it amenable to high-throughput gene expression platforms [9].

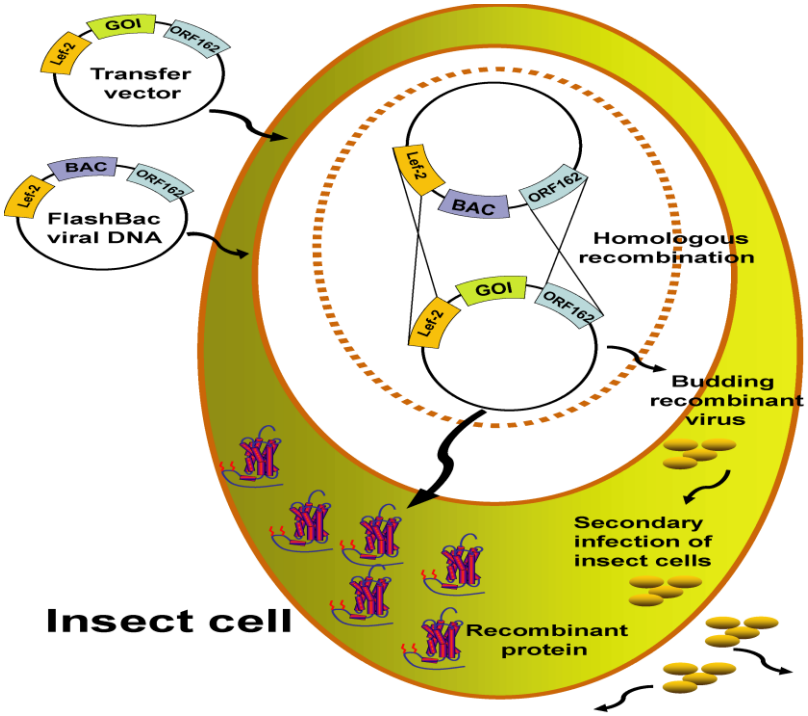

Figure 1. Schematic representation of the flashBAC™ system**.** The modified AcMNPV genome contains a BAC at *polh* gene locus with part of *ORF1629* gene deleted**.** Insect cells are co-transfected with *flash*BAC DNA and the transfer vector containing the gene of interest. Restoration of gene function and insertion of the foreign gene into the virus DNA under *polh* promoter takes place through homologous recombination. Baculovirus is produced as long as the recombinant virus replicates. Baculovirus is harvested and used to infect a new batch of cells to produce the recombinant protein of interest.

The baculovirus pOET5 vector (Oxford Expression Technologies, OET) is a dual promoter, compatible transfer plasmid for the flashBAC expression system (**Figure 2**). We used recently this vector and Sf9 and T. ni cells to overexpress proteins that control cell division in green plants [10]. The dual promoter feature of this transfer plasmid enables the simultaneous expression of two foreign genes under the strong AcMNPV polh promoter and the very late promoter p10**.** pOET5 contains a bacterial origin of replication and an ampicillin resistance gene, allowing plasmid propagation and selection in *E. coli* cells. The two multiple cloning sites (MCS) that have replaced the *polh* sequences harbour unique restriction sites that enables the insertion of a foreign gene in the correct orientation for its expression.

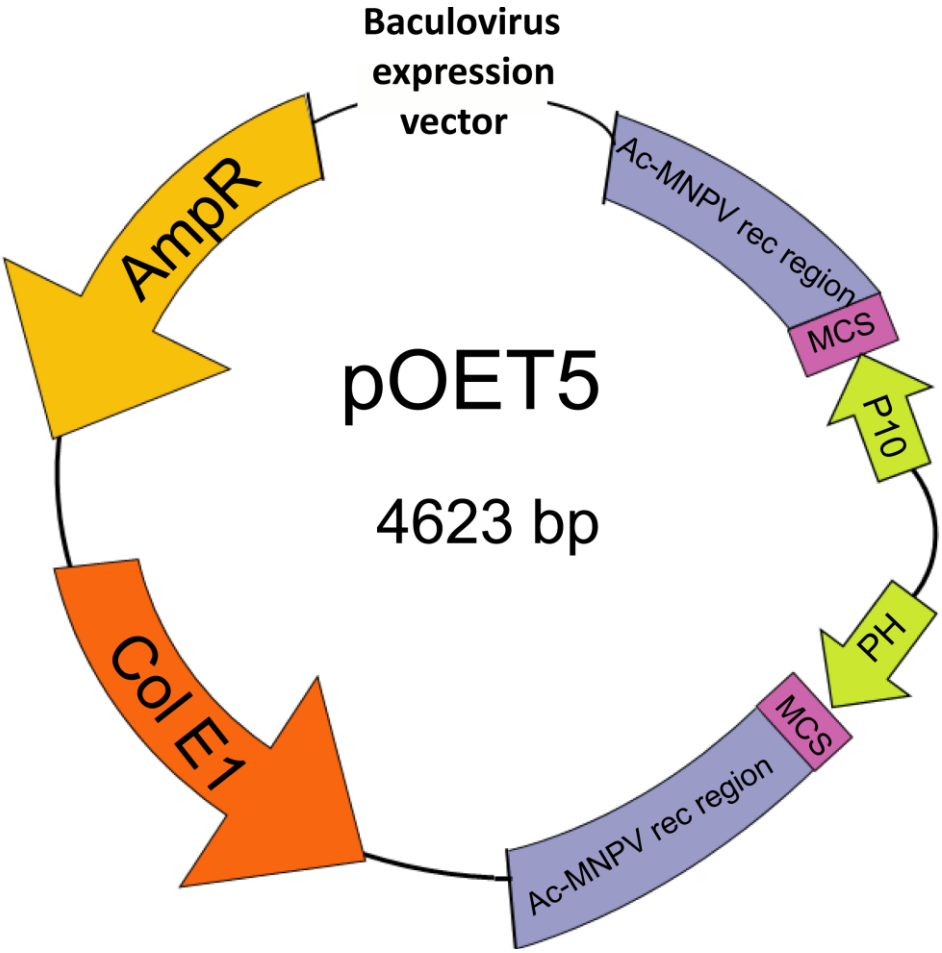

Figure 2. pOET5 vector map. The pOET5 transfer vector (4590bp) has a Col E1 bacterial origin of replication and an ampicillin resistant gene for selection in *E. coli.* Two multiple cloning sites (MCS) comprising unique restriction sites for foreign gene insertion in the correct orientation have replaced a segment the polyhedrin (*polh*) and p10 sequences. The *Autographa californica* Multicapsid Nucleopolyhedrovirus (AcMNPV) gene sequences flanking the gene in the transfer vector MCS enables homologous recombination with the viral DNA in insect cells facilitating expression cassette insertion into the locus.

2 Materials

All solutions are prepared at room temperature (unless indicated otherwise using ultrapure, deionised water (e.g., purified water with a conductivity of 18 MΩ-cm at 25 °C) and analytical grade reagents.

2.1 SDS (Sodium Dodecyl Sulphate) Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. 4-12% NuPAGE pre-cast gels 1.5 mm, 15 wells.
2. SDS-PAGE running buffer: MOPS 50mM,Tris base 50mM, SDS 3.46 mM, EDTA 1mM (*see* **Note 1**).
3. NuPAGE gel thank.
4. Plus Protein All Blue Molecular weight ladder or equivalent.
5. Leammli buffer 5x (Tris 125mM pH 6.8, glycerol 10% (v/v), SDS 4% (w/v), β-mercaptoethanol 2% (v/v), bromophenol blue 0.02% (w/v).

2.2 Immunobloting

1. Nitrocellulose membranes.
2. Western blot transfer buffer: Tris-HCl 25 mM, glycine 192 mM, SDS 0.01%, methanol 20 % (v/v).
3. PBS buffer: NaCl 150 mM, Na phosphates 50mM, pH 7.4.
4. PBS containing 0.05 % Tween-20 (PBST).
5. Blocking solution: 5 % milk in PBST. Store at 4 °C.
6. Thick blot filter paper for western blot, 9.5 x 15.2 cm.
7. Alkaline Phosphatase (AP) or horseradish peroxidase (HRP) anti-6x His tag antibodies. Secondary antibody that is HRP- or AP-conjugated.

8. Nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3- indolyl phosphate (BCIP) 1-Step™ ready-made mix or Clarity western ECL ready-made mix.

2.3 Insect cells work

1. 35mm (six well) plates for tissue culture.
2. Serum free TC100 medium.
3. SF 921 protein-free cell culture medium.
4. pOET5 vector.
5. baculoFECTIN II transfection reagent.
6. flashBAC^{TM} kit.
7. 5-bromo-4-chloro-3-indolyl-β-D galactopyranoside (X-gal).
8. N,N Dimethylformamide (DMF).
9. Fetal Bovine Serum (FBS).
10. Penicillin/streptomycin mix (10000IU and10000µg/ml respectively).
11. PBS buffer: NaCl 150 mM, Na phosphates 50mM, pH 7.4.
12. Neutral Red Dye solution diluted (1:20) in sterile Phosphate-buffered saline (PBS).

3 Methods

3.1 Cloning of heterologous plant ER genes

1. Add a 5’ sequence encoding for a hexahistidine-tandem after the start codon of the heterologue gene sequence to facilitate the purification of the gene expression product by immobilised metal affinity chromatography (IMAC). References [11-12] present

a revision of the chemical principles and applications underpinning IMAC.

2. Following gene amplification by PCR, the amplicon of interest is purified and cloned into the appropriate restrictions sites of the pOET5 vector (a combination we use when possibly is BamHI and SalI) using standard molecular cloning protocols.
3. Confirmation of appropriate cloning of the heterologue gene (e.g., in-frame, mutations-free) in pOET5 by DNA Sanger sequencing using appropriate pOET sequencing primers.

3.2 Insect cells transfection

Insect cells co-transfection to produce seed stocks (P0) of recombinant baculovirus. The entirety of the following technique is carried out under aseptic conditions.

1. Grow *Spodoptera frugiperda 9* (*Sf9*) cells in serum free TC100 medium as suspension cultures in shake flasks (28ºC, 120rpm).
2. At least one hour prior to transfection, use *Sf9* cells in the log phase of growth and with at least 95% viability to seed 35 mm cell culture dishes with 2 ml of Sf9 cells in serum free TC100 growth medium at a cell density of $0.5x10^6$ cells/ml.
3. Incubate at 28ºC for 1 hour.
4. Meanwhile, prepare the co-transfection mix of DNA and transfection reagent. For each co-transfection use sterile tubes and add 100µl of serum free TC100 medium, followed by 100ng virus DNA (as supplied in the *flash*BAC™ kit: 5µl at 20ng/µl).
5. Add 500ng of transfer vector containing gene of interest or 500ng of control plasmid (lacZ positive control as supplied in the *flash*BAC™ kit: 5µL at 100ng/µl).
6. Add 1.2µl of baculoFECTIN and gently mix to prevent shearing of the DNA.
7. Prepare a control (mock) transfection by omitting the addition of DNA to the mix.

8. Incubate the-transfection mix for 15 minutes at room temperature to allow the nanoparticle-DNA complex to form.
9. Gently remove 1ml of growth medium from each well of the 6-well plates and discard, leaving only 1ml of serum free TC100 medium in each well.
10. Add dropwise the co-transfection mix into the corresponding well, ensuring an even distribution of the mixture.
11. Incubate the plates overnight at 28°C.
12. Following overnight incubation, add 1ml of ESF 921 protein-free cell culture medium so each well had a total volume of 2ml.
13. Incubate the 6-well plates at 28°C for 4 more days.
14. Harvest the seed stock (P0) from each well into sterile tubes and store in the dark at 4°C.
15. For the lacZ positive control to confirm transfection efficiency, stain the infected cells using X-gal. To this aim, add 1ml of ESF 921 protein-free cell culture medium containing 15µl X-gal (2% w/v in DMF) to the well and incubate at 28°C for 5 hours.
16. Formation of a blue colour confirms lacZ expression in the recombinant virus.

3.3 Baculovirus amplification

1. The entirety of the following procedure are carried out under aseptic conditions
2. A Passage 1 (P1) virus stock is generated by taking 0.5ml of the P0 stock virus to infect 50ml of Sf9 cells at a density of $2x10^6$ cells/ml.
3. Incubate the cells for 5 days, 28°C, 120rpm.
4. Harvest by centrifugation (20 minutes, 4°C, 3000rpm). Recover the supernatant and store in the dark at 4°C. For long-term storage at -80°C, add 5% v/v of Fetal Bovine Serum (FBS).
5. Determine the titre of the virus P1 stock by plaque assay.

3.4 Plaque assay

1. The entirety of the following procedure is carried out under aseptic conditions.
2. 12-well or 6-well plates are seeded with either Sf9 in ESF 921 protein-free cell culture medium or *Spodoptera frugiperda* 21 (Sf21) insect cells in TC100 medium supplemented with 10% FBS. The recommended cell density is shown in the Table 1 below.

Insect cell line	Plate Type	Cell density per well (cells/ml)	Volume per well (ml)
Sf9	6-well	$0.35x10^6$	2
Sf9	12-well	$0.4x10^6$	1
Sf21	6-well	$0.75x10^6$	2
Sf21	12-well	$0.4x10^6$	1

3. Plates are incubated overnight at 28°C to obtain anticipated confluency ≥70%.
4. Then serial dilutions (1:10) of virus stock are performed by ten-fold steps (10^{-1}-10^{-7}) in TC100 medium+10% FBS using 50µl of virus and 450µl of growth medium as diluent at each step, ensuring to mix thoroughly.
5. Medium is aspired from wells and 100µl of virus dilutions gently added dropwise to the centre of each well.
6. Plates are incubated for 45 minutes at room temperature with agitation to allow the virus to adsorb. During the 45-minute incubation period the overlay is prepared as follows:
7. Low melting temperature agarose (Sigma-Aldrich 2% w/v solution in deionised water) is warmed to hand hot ~50-55°C and diluted 1:2 with pre-warmed (28°C) TC100 medium+10% FBS or ESF 921 protein-free cell culture medium (if using *Sf21* or *Sf9* cells respectively). Keep the mix in an oven set at 55°C to prevent setting.
8. Following the incubation period, the virus suspension is aspired and 1ml or 2ml of overlay added to 12-well or 6-well plates respectively, allowing the overlay to flow down

the side of the well and spread slowly over the cell monolayer.

9. Once the agarose has set at room temperature, 1ml of antibiotic mix (10000IU penicillin/10000µg/ml streptomycin) in TC100 medium+10% FBS or ESF 921 protein-free cell culture medium (if using *Sf21* or *Sf9* cells respectively) is added to the cells and the plate(s) incubated at 28ºC for 3/4 days for *Sf21/Sf9* cells, respectively.
10. In order to visualise the virus plaques, the cells are stained with a previously sterilised Neutral Red Dye solution diluted (1:20) in sterile PBS.
11. Plates are incubated overnight at 28ºC, then dye is tipped off and plaques counted. From the following equation virus titre was determined:

$$Average\ plaque\ count * Dilution\ factor * 10 = Virus\ titre\ (pfu/ml)$$

12. Where dilution factor is the inverse of the dilution and the multiplication by 10 is because 0.1ml of virus was added to each well.

3.5 Time course protein production

Once a virus titre of high infectivity (typically 10^7 to 10^8 pfu/ml) is obtained, time course expression tests are conducted in insect cell lines such as Sf9 and T.ni at three different multiplicity of infection (MOI) levels and for up to 96 h post infection.

1. For small-scale production trials of heterologue plant endothelium reticulum proteins, the cell lines Sf9 and *Trichoplusia ni* (T.ni) at a cell density of $1.5x10^6$ cells/ml and $1.0x10^6$ cells/ml, respectively, are infected with the P1 virus stock at 0.1, 1.0 and 5.0 Multiplicity of Infection (MOI) titres. Samples of 1 ml from the different MOIs are collected after 24 h, 48 h, 72 h and 96 h and harvested by centrifugation at 12,000 rpm for 10 min. The pellet and supernatant can be stored at -20°C for further analyses. To breakdown the insect cells, a lysis buffer solution

consisting of Tris 50 mM pH 8.0, β-mercaptoethanol5 mM, KCl 100 mM, Nonidet P-40 1% (v/v), cOmplete™, EDTA-free protease inhibitor cocktail tablets (Roche) is used (*see* **Note 2**).

2. Cells membrane fragments and other debris are removed by centrifugation at 12,000 rpm, at 2-8°C for 15 min. An aliquot of the supernatant (e.g., the soluble fraction) and the pellet (the insoluble fraction) for SDS-PAGE and Western Blotting analysis.
3. For large-scale expression, 1 to 4 L of Sf9 or T.ni insect cells in culture at $1.5x10^6$ cells/ml are infected and incubated at 28°C according to the optimised conditions found in the small-scale time-course expression tests.
4. Once the maximal expression hours is determined (usually, after 2-3 days incubation post-infection), the cells are harvested by centrifugation at 4,000 rpm, 2-8°C for 20 min (Beckman, J2-21) and the pellets stored at -20°C.
5. Cell lysis is carried out as described above. Intact cells, cell membrane fragments, and other debris are removed by centrifugation at 12,000 rpm, 2-8°C for 45 min (Beckman, J2-21).
6. The soluble fractions are recovered and used for SDS-PAGE and western blot analyses.

3.6 SDS-PAGE

1. Aliquots of the collected soluble fractions from the time-course expression tests are mixed with SDS-PAGE loading dye Leammli buffer 5x and heated at 95ºC for 5 minutes.
2. Assemble the protein gel system with 15-well precast NuPAGE 4-12% Bis-Tris gels (1.0mm thick). Fill the gel tank with the running buffer (e.g., MOPS 50mM,Tris base 50mM, SDS 3.46 mM, EDTA 1mM).
3. Gels are runs at 120V until the dye front reaches near the bottom of the gel (~40 minutes). Ensure the gel is running

during this time (bubbles should be visible coming off the bottom electrode) (*see* **Note 3**).

3.7 Western blotting

1. Once the SDS-PAGE run is completed, rinse the gel with deionised water and transfer to a small plastic box containing enough transfer buffer solution to cover the gel.
2. Cut a piece of polyvinylidene fluoride (PVDF) membrane (Bio-Rad®) of a size similar to that of the protein gel (*see* **Note 4**).
3. Soak the PVDF membrane in pure methanol for 1 minute (*see* **Note 5**).
4. Transfer the membrane to a small plastic box containing enough transfer buffer solution to cover the PVDF membrane and incubate for 5 minutes (*see* **Note 6**).
5. During the equilibration of the PVDF membrane in the transfer buffer, assemble the blotting sandwich as follows: Whatman filter-membrane-gel-Whatman filter. Place the gel on the PVDF membrane in such a way it cover the membrane completely. Use a 5 or 10 mL pipette to roll out air bubbles from the gel-membrane sandwich prior to placing in transfer cassette. This will prevent the trapping of air bubbles in between the gel and the PVDF membrane.
6. Use a semi-dry transfer blot to transfer the proteins from the gel to the PVDF membrane. Typically, this step performed at 25 V for 5 min per protein gel.
7. Following transfer, the membrane is blocked in PBS, 5% (w/v) skim milk powder, 0.1% Tween 20 overnight at 4°C with rotation.
8. The primary antibody is diluted according to supplier's recommendations in blocking solution. The membrane is incubated with the primary antibody diluted in PBST for 1-2 hours at 4°C with rotation. The use of Alkaline Phosphatase (AP) or horse radish peroxidase (HRP) anti-

6x His tag antibody is recommended to speed up the process.

9. The primary antibody is removed by six 5-minute washes with PBST followed by a 1 hour incubation (4°C with rotation) with secondary antibody that is HRP- or AP-conjugated (the dilution is according to supplier's recommendations) in PBST.
10. Following six 5-minute washes as in step 9 above, for HRP-conjugated antibody, the colorimetric 1-Step NBT/NCIP ready-made mix is applied to the membrane for the detection of proteins on the blots. For alkaline phosphatase-conjugated antibody use the 1-Step™ NBT/BCIP ready-made mix (*see* **Note 7**).
11. The blot images are captured on a ChemiDoc (BioRad) instrument or alike.

4 Notes

1. Unpolymerised polyacrylamide is a neurotoxin, so you need to wear gloves during this procedure.
2. Cell lysis solution can be irritant to skin, mucous membranes and eyes. In case of accidental contact, rinse cautiously with water for several minutes.
3. The electrophoresis and transfer apparatus uses high voltages.
4. Avoid touching the PVDF membrane with bare fingers as the proteins left on it (mainly keratin), may cross-react with the antibodies.
5. Methanol is a flammable and harmful solvent that may cause blindness or death if swallowed. Store in a cool, well ventilated area. Use PPE-based containers to handle it. Keep away from strong oxidising agents. Avoid heat/ignition sources.
6. It is very important that during the western blotting procedure that everything is kept damp. Pouring a small volume of transfer buffer onto the stack to keep it moist during assembling it should suffice. It is also essential

that there are no air bubbles between any of the layers otherwise transfer will not occur.

7. The NBT/BCIP staining solution is a flammable liquid and vapour. Harmful in contact with skin. Causes serious eye irritation.
8. The Tris-Glycine-Methanol blotting buffer solution must be disposed of as hazardous waste.
9. Western blotting bands fade away when stored for long time periods (e.g., a few days) even when the blot is covered with water. Therefore, it is best to image the blots immediately after performing the chemioluminescence reaction.

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