Overcoming obstacles in a WB approach

Cowboy tricks

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Agenda

6
Trouble-shooting I
General handling procedures

7
Trouble-shooting II

8
Summary
Starting point of Western Blotting

Membrane & blocking reagent

Sample prep & sample load

Quality of 1st ab

1st ab species

Ab concentrations (1st & 2nd)

Target protein

Specific antibody

Detection type & exposure time
Careful attention to experimental protocols with optimization at key stages, but don’t forget the trivia...
Troubleshooting I-
General handling procedures

- Do not handle membranes with fingers, use forceps

- Set up blotting sandwich tight to avoid streaking
Do’s and Don’t’s

- Use HQ filter paper during transfer to avoid artifacts
Do’s and Don’t’s- Fluorescence

- Use powder-free gloves

- Use clean containers, no Coomassie™!
Do’s and Don’t’s- Fluorescence

- No contaminations & autofluorescent substances
  - Coomassie™
  - Triton™ X-100
  - BFB
  - Polyacrylamide gel fragments

Amersham™ ECL Plex™ Western Blot, Cy™5 channel

BPB running front, Cy™5 channel

CBB running front

Gel fragments
Do’s and Don’t’s- Fluorescence

- Do not write on the membrane with pen, use pencil/cut corners
Band Issues
No/Weak bands

Antigen related
→ Adjust exposure time
• Incorrect handling or storage of antigen/reagents/abs
• Target protein degradation
→ Improve storage conditions, use PIs
• Insufficient protein load, protein not expressed
→ Positive control, check protein concentration, enrichment
• Too much protein
→ Self association → Protein loss
Avoid overloading...
No/Weak bands

Transfer related

- Wrong MetOH concentration during transfer
  \[ \text{Small P} = +\text{MetOH}, -\text{SDS} \]
  \[ \text{Large P} = -\text{MetOH}, +\text{SDS} \]
- Small P (<10kDa) passed membrane
  \[ \text{Decrease pore size} \]
- Large P (>150kDa) retained in gel
  \[ \text{Increase mA, longer transfer, } +0.02\%\text{ SDS, no MetOH, Wet Blot} \]
- Check equipment & transfer efficiency by staining gel/membrane
Molecular Weight Markers helpful for Western Blotting

Pre-stained MW Markers, e.g. **Rainbow™ Markers** provide easy control of transfer efficiency & enable precise MW determination of target proteins.

Amersham™ **ECL DualVue™** WB Markers
- Visible on gel, blot & film
- Show separation quality, transfer efficiency & blot orientation
- pos. control of detection reaction & imaging for all HRP-based methods
- MW determination from image/film
No/Weak bands

Antibody/Buffer related

- $1^{st}$ ab not suitable for WB
- $1^{st}$ & $2^{nd}$ ab not compatible

→ Confirm host species and Ig type of $1^{st}$ ab
- Too low concentration of $1^{st}$ ab
- Too stringent/inappropriate washing or blocking conditions

→ Reduce concentration/change detergent, minimize washing/blocking steps
Unusual/Unexpected bands

Bands at lower MW

Antigen related

- Target protein degraded/digested, proteolysis, existing splice variants, other protein with similar epitope/novel proteins

→ Minimize time between sample prep & elpho, fresh sample kept on ice, use PIs, check literature, alternative abs
Changes in sample – post sampling
Collection/Extraction challenge

At sample harvest

*In vivo state*

“The true picture”

... a few minutes

ATP, glucose, pH
Lactate, Ca

... several minutes

Ca activated proteases
Loss of phosphorylation

Cell death

Necrosis / Apoptosis
Changes in sample – post sampling
Collection/Extraction challenge

Heat 95°C  TCA prec.  4°C 2 hours

Extensive degradation!

Post sampling strategy
- Snap-freeze or precipitate sample (stop degradation)
  If you can’t process samples immediately
- Add protective compounds (inhibitors, stabilizers)
  Stop proteases, phosphatases, protect against oxidation
- Work quickly (minimize damage)
  Avoid lengthy steps if possible during first steps in WF
Unusual/Unexpected bands

Band at higher MW
Antigen related
  • PTMs
  → Agents to remove modifications, check aa sequence

Band at much higher MW
Antigen related
  • Dimers, ..., IAs
  → Fresh DTT/BME, heating of sample
Unusual/Unexpected bands

Multiple bands with different MW

Antigen/Antibody related

• 1\textsuperscript{st}/2\textsuperscript{nd} ab conc. too high, cross-reactivity, non-specific binding

→ Use purified ab, optimize ab concentration, negative controls to check cross-reaction

Uneven band sizes in different lanes

• Problem with gel-cast
Unusual/Unexpected bands

Smile Effect

• Migration too fast/hot
  → Slower run, 4°C

• No mixing of gel reagents, empty lanes

• Too much salt in sample buffer

Ghost bands on film (ECL™)

• Sample/ab concentration too high (substrate depletion)
  → Load less target, decrease 2nd ab

Data courtesy of Mattias Vesterberg, Center for Molecular Medicine, Karolinska Institutet in Stockholm
Background Issues
High Background

Buffer/Membrane related

• Insufficient washing
• Blocking of membrane too short
• Inappropriate washing buffer
  → TBST for phospho abs & AP-abs
• Inappropriate detergent concentration
  → 0.1% Tween usually recommended
High Background

Antibody/Membrane related

- Concentration/incubation time of 1\textsuperscript{st} or 2\textsuperscript{nd} ab too high/long
  \[\rightarrow\text{Optimization of dilutions/incubation period}\]
- Inappropriate combination: ab, blocking reagent & membrane
  \[\rightarrow\text{Change blocking buffer & membrane, use detergents, negative controls, pre-absorbed 2}\textsuperscript{nd} ab\]

- Dependent on: Abs, application (Chemiluminescence or Fluorescence)
Uneven BG & Spots

Uneven BG

• Membrane dried partially
• Insufficient amount of liquid
• Solutions not evenly spread (Ab/ blocking/washing buffer/substrate)

→ Apply agitation

Uneven white spots on blot

• Air bubbles trapped during transfer
Unusual dots

Black Dots/Speckling (Starry sky)

- Ab binds to non-resolved blocking agent
  → Filter blocking agent and wash blot after blocking step
- HRP conjugate forms aggregates & precipitates on blot
  → Filter conjugate (e.g. Whatman™ PES/RC, 0.2µm)/new vial
- Cheap skim milk (in combination with rabbit 2nd ab)
- Contaminated sponges (transfer), use 2 Whatman filter papers
Finding the optimal...

1. Membrane

2. Blocking solution

3. Antibody concentration
GE Healthcare solutions for better Western blotting

Western blotting handbook
Imaging handbook
Discussion forum

Sample preparation
Electrophoresis
Blotting
Detection
Imaging and analysis

Sample preparation:
- Protein Markers

Electrophoresis:
- Electrophoresis equipment

Blotting:
- Transfer equipment
- Membranes and blocking agents

Detection:
- Amersham™ ECL™ Plex detection system
- Amersham™ ECL™ Prime detection system

Imaging and analysis:
- Amersham™ Imager 600
- Typhoon™ FLA9500
- IQTL 8.1
1. Optimal membrane

Choice of suitable membrane is critical for end result!

Number of unspecific bands varies with type of blocking solution and membrane
Membranes

Nitrocellulose membranes
- Low background with all commonly used detection methods
- Traditionally not recommended for stripping and reprobing
- Supported membranes can be used in stripping and reprobing applications

PVDF
- Higher protein binding capacity compared to nitrocellulose membrane
- Ideal for stripping and reprobing WB application
- Need to be pre wetted in either methanol or ethanol before use
Overview

Rebranding, Rationalisation and Modification

- 0.2µm Amersham™ Hybond™ PVDF
- ready-to-go membrane sandwiches
- Rolls, different sheet sizes

New names

<table>
<thead>
<tr>
<th>Nitrocellulose</th>
<th>Nitrocellulose</th>
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<tbody>
<tr>
<td>0.45 µm Whatman Protran BA85</td>
<td>0.45 µm Amersham Protran 0.45</td>
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<tr>
<td>0.2 µm Whatman Protran BA83</td>
<td>0.2 µm Amersham Protran 0.2</td>
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<tr>
<td>0.1 µm Whatman Protran BA79</td>
<td>0.1 µm Amersham Protran 0.1</td>
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<tr>
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<td>Nitrocellulose Supported</td>
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<td>0.45 µm Whatman Optitran BAS85</td>
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<td>PVDF</td>
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<tr>
<td>PVDF Low Fluorescence</td>
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<tr>
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<td>0.2 µm Amersham Hybond LF PVDF 0.2</td>
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<td>PVDF Sequencing</td>
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<tr>
<td>0.2 µm Whatman Westran S</td>
<td>0.2 µm Amersham Hybond SEQ PVDF 0.2</td>
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NO changes in physical or chemical composition
# Membrane selection guide

<table>
<thead>
<tr>
<th>Material</th>
<th>Material Type</th>
<th>Physical Strength</th>
<th>Binding Capacity</th>
<th>Pore Size</th>
<th>Minimal Protein Size</th>
<th>Recommended Detection</th>
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<tbody>
<tr>
<td>Amersham™ Protran Premium</td>
<td>Nitrocellulose</td>
<td>-</td>
<td>100 µg/cm²</td>
<td>0.2 or 0.45 µm</td>
<td>&gt;Mr 2000</td>
<td>Chemiluminescence, Fluorescence, Colorimetric</td>
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<td>Amersham™ Protran</td>
<td>Nitrocellulose</td>
<td>-</td>
<td>80-150 µg/cm²</td>
<td>0.1 to 0.45 µm</td>
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<td>Colorimetric, Chemiluminescence, Isotopic, Fluorescence</td>
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<tr>
<td>Amersham™ Protran Supported</td>
<td>Supported nitrocellulose</td>
<td>+</td>
<td>75-90 µg/cm²</td>
<td>0.2 or 0.45 µm</td>
<td>&gt;Mr 2000</td>
<td>Colorimetric, Chemiluminescence, Isotopic</td>
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<td>Fluorescence</td>
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<td>125 µg/cm²</td>
<td>0.45 µm</td>
<td>&gt;Mr 2000</td>
<td>Chemiluminescence, Chemifluorescence</td>
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<tr>
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<td>PVDF</td>
<td>+</td>
<td>&gt;200 µg/cm²</td>
<td>0.2 µm</td>
<td>&gt;Mr 2000</td>
<td>Colorimetric, Chemiluminescence, Isotopic</td>
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*New! 285 µg/cm², 0.2 µm*
2. Optimal blocking solution

- 5 blocking solutions evaluated

Number of unspecific bands varies with type of blocking solution and membrane
Optimal blocking solution

**Good-to-know: Low-fat milk**

- No milk blockers + Avidin/Biotin systems or Phospho abs → Contains endogenous biotin/phosphoepitopes
- No milk blockers for ab baths → Not stable for longer time periods
- Causes higher background on PVDF membrane
3. Optimal antibody concentration

Indications wrong ab concentration (ECL™)

- Inconsistent signal length/signal terminates quickly
- No signal/strange band shape → Signal faded/ended before detection started
- Dark BG with white bands/ghost bands on **film**
- High BG/Multiple bands
- Brown bands on **blot**
- Glowing bands on **blot**

Data courtesy of Mattias Vesterberg, Center for Molecular Medicine, Karolinska Institutet in Stockholm

Target protein
Optimal antibody concentration

Sensitivity, linearity, DR & S/N vary with antibody, membrane and blocking reagent

- Use dot blots or slot blots
- Use Western Blot (strips)
Optimal antibody concentration

Example:
- Set up of experimental design:

<table>
<thead>
<tr>
<th>secondary antibody 1: 10 000</th>
<th>secondary antibody 1: 25 000</th>
<th>secondary antibody 1: 50 000</th>
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<td>primary antibody</td>
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<tr>
<td>1:1000</td>
<td>1:1000</td>
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<td>1:2500</td>
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<tr>
<td>1:5000</td>
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<td>1:5000</td>
</tr>
<tr>
<td>1:10 000</td>
<td>1:10 000</td>
<td>1:10 000</td>
</tr>
</tbody>
</table>

1, 2, 3 = samples in three different amounts
Optimal antibody concentration

- Incubation of strips in different dilutions of 1\textsuperscript{st} ab

- Incubation of strips in different dilutions of 2\textsuperscript{nd} ab
Reminder: Use of phospho antibodies

Reminder:

- Washing buffer: TBS instead of PBS
- Blocking reagent: BSA instead of milk
- Use phosphatase inhibitors during sample prep & in all buffers
Summary

- Work accurately and avoid contaminations
- Use fresh solutions/reagents, appropriate storage
- Optimize protocol if needed
  → No signals/wrong signals, high background
    - Adjust incubation & washing times
    - Choice of membranes
    - Choice of blocking reagents
    - Choice of antibody & concentrations
Biomolecular Imagers

Amersham™ Imager 600

Typhoon™ FLA 9500

Typhoon FLA 7000/7000IP

ImageQuant™ LAS 500
Thank you for your attention!
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