1.0 **Objective**

This procedure describes the identification of the major protein components of Ad, by antigen-antibody reaction.

2.0 **Scope**

The Western Blot of Ad is useful to identify the four protein bands recognised by the primary antibody, rabbit polyclonal Anti-Ad5. The major bands are the Hexon, Penton, Fiber and Core Proteins.

3.0 **Responsibilities**

3.1 It shall be the responsibility of the supervisor to:

3.1.1 Ensure that this procedure is followed by all Animal Cell Technology and Downstream Processing personnel performing the Western Blot analysis of Ad.
4.0 Material

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
<th>Catalogue #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gel Blot Paper (13 × 18 cm)</td>
<td>Schleicher &amp; Schuell</td>
<td>34640</td>
</tr>
<tr>
<td>Nitrocellulose Membrane Hybond™ ECL™</td>
<td>Amersham Pharmacia Biotech</td>
<td>RPN2020D</td>
</tr>
<tr>
<td>Rabbit polyclonal Anti-Ad5</td>
<td>Access Biomedicals</td>
<td>06281</td>
</tr>
<tr>
<td>Protein A HRP Conjugated</td>
<td>BIO-RAD</td>
<td>170-6522</td>
</tr>
<tr>
<td>ECL™ Western blotting detection reagents</td>
<td>Amersham Pharmacia Biotech</td>
<td>RPN2109</td>
</tr>
<tr>
<td>Timer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saran Wrap</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kodak X-Omatic cassette</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperfilm™ ECL™</td>
<td>Amersham Pharmacia Biotech</td>
<td>RPN2103K</td>
</tr>
</tbody>
</table>

5.0 Equipment

5.1 TRANS-BLOT® SD SEMI-DRY TRANSFER CELL (BIO-RAD) Cat # 170-3940

5.2 Power Supply Model 200 / 2.0 (BIO-RAD)

5.3 X-Omatic dev. Machine

6.0 Procedure

Once Ad samples are reduced with 2-Mercaptoethanol and heated at 100°C for 5 minutes, it is no longer mandatory to work in the Biosafety Level 2 Laboratory (BSL-2) for the Western Blot.
6.1 Solution Preparation

6.1.1 PBS 10× (1000 mL)

KCl
Supplier: _______________  Lot # _______________
Qty needed: 2.0 g    Weighed: _____________

KH₂PO₄
Supplier: _______________  Lot # _______________
Qty needed: 2.0 g    Weighed: _____________

NaCl
Supplier: _______________  Lot # _______________
Qty needed: 80.0 g    Weighed: _____________

Na₂HPO₄·7H₂O
Supplier: _______________  Lot # _______________
Qty needed: 21.6 g    Weighed: _____________

Add the above chemicals in 800 mL of Milli-Q H₂O. Mix until dissolved, using a stir bar. Complete to 1000 mL with Milli-Q H₂O using a 1000 mL volumetric flask. Filter through a 0.45 µm filter. Storage: 3 months at room temperature.

Filter pore size: ______________  Filter Co.: _____________
Filter Lot # _______________

Solution Lot # ______________  Expiry Date: ___________
Performed by: _______________  Date: _______________
6.1.2 PBS-T (0.1%) (1000 mL)

PBS 10x
Solution Lot #______________  Expiry Date: ____________

Tween-20
Supplier:__________________  Lot # ________________  Qty needed: 1.0 mL

Add 100 mL of PBS 10x concentrated solution to 900 mL of Milli-Q H₂O.
Add 1.0 mL of Tween-20 to this solution. Mix well.
Storage: 2 days at room temperature.

Solution Lot #______________  Expiry Date: ____________
Performed by:_______________  Date:_______________

6.1.3 5% Dried Skim Milk in PBS-T (0.1%) (100 mL)

Dried Skim Milk
Supplier:__________________  Lot # ________________
Qty needed: 5.0 g  Weighed:_____________

Add the skim milk powder to 100 mL of PBS-T(0.1%).
Mix well with a stir bar, until dissolved.
Storage: 1 day at room temperature.

Solution Lot #______________  Expiry Date: ____________
Performed by:_______________  Date:_______________
6.1.4 Towbin (Transfer Buffer) (1000 mL)
25 mM Tris, 192 mM Glycine, 20% methanol pH 8.3

Tris
Supplier: ____________________ Lot # __________________
Qty needed: 3.03 g Weighed: __________________

Glycine
Supplier: ____________________ Lot # __________________
Qty needed: 14.41 g Weighed: __________________

Methanol
Supplier: ____________________ Lot # __________________
Qty needed: 200 mL Weighed: __________________

Add the above chemicals in 700 mL of Milli-Q H$_2$O.
Add the methanol and mix until dissolved, using a stir bar.
Complete to 1000 mL with Milli-Q H$_2$O using a 1000 mL volumetric flask.
Check the pH: ______ at ______ °C. Do not adjust the pH.
Storage: 3 months at 2-8 °C

Solution Lot # ____________________ Expiry Date: ____________
Performed by: ____________________ Date: ____________
6.1.5 0.1% Ponceau Red in 5% Acetic Acid  (500 mL)

Ponceau Red
Supplier:___________________  Lot #_________________
Qty needed: 0.5 g  Measured:____________

Acetic Acid
Supplier:___________________  Lot #_________________
Qty needed: 25 mL  Measured:____________

Add the Ponceau red and the acetic acid in 400 mL of Milli-Q H\textsubscript{2}O.
Mix until dissolved, using a stir bar.
Complete to 500 mL with Milli-Q H\textsubscript{2}O.
Filter through Whatman # 1 filter.
Storage: 3 months at room temperature.

Solution Lot #_______________  Expiry Date:___________
Performed by:________________  Date:________________

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6.2 Blotting

6.2.1 Perform the gel electrophoresis on the required samples according to the DOP: SDS-PAGE of Adenovirus (Ad).

Samples: _____________________________________________________

Date of Electrophoresis: ___________ Performed by: ___________
Date of Western Blot: ________________ Performed by: ____________

6.2.2 Prior to the end of the electrophoresis run, soak the blot paper and nitrocellulose membrane, all in one container with Towbin transfer buffer for 15-30 minutes (keep it at 4°C), in the following order:

2 × Blot Paper
1 × Nitrocellulose membrane (NC)*
2 × Blot Paper

Blot Paper; Supplier: ______________ Lot # __________________
NC membrane; Supplier: ____________ Lot # __________________

* Before soaking, carefully cut the membrane to the proper dimensions of the gel (wear gloves to avoid contamination of the membrane).
* Carefully slide the NC membrane into the transfer buffer with a 45° angle, using forceps, to prevent air bubbles.

Soaked for: _______ minutes
Towbin Transfer Buffer Lot # __________________

6.2.3 After the electrophoresis, remove the gel and quickly rinse the gel in transfer buffer. Keep the equilibration time short to prevent diffusion of low molecular weight proteins out of the gel. Adequate gel equilibration is done by changing the equilibration buffer several times during a short period. (This will help to limit diffusion of low molecular weight macromolecules while providing efficient salt reduction).

1 × 5 min with at least 3 buffer changes

6.2.4 Place in the following order, on top of the anode of the TRANS-BLOT® SD SEMI-DRY TRANSFER CELL. Carefully, roll out
bubbles with a test tube after each layer is laid down (except on the gel):

- 2 × Blot Paper
- 1 × NC membrane
gel *
- 2 × Blot Paper

* Avoid moving the gel against the NC membrane once it is laid down.

6.2.5 Carefully place the cathode assembly onto the stack.

6.2.6 Run at 10 V for 60 minutes. (Do not exceed 25 V on this machine)
A current of 5.5 mA/cm² for mini gels is recommended to prevent excessive heating.
Monitor the current during the run:

Time start: _________ Voltage: _________ Current: _________
Time end: __________ Voltage: _________ Current: _________

6.2.7 Once the transfer is completed, turn off and disconnect the unit.
Remove the cathode assembly. Discard the blot paper.

6.2.8 If desired, stain the gel with Coomassie Blue to monitor the transfer efficiency.

Coomassie Blue staining done: Yes ☐ No ☐

6.2.9 Remove the NC membrane and label protein side up, with a soft pencil or by cutting a corner, for easy identification.

6.2.10 Let the membrane dry on a tissue for 5 minutes.
The membrane can be stained with 0.1% Ponceau Red in Acetic Acid solution, to visualise the transfer:

Soak the membrane in the Ponceau Red solution, for 5 min.
Pour out the solution and rinse with water 10-20 sec, to clear the background.
Mark all visible bands with a pen.
Remove the Ponceau Red by washing the NC membrane in PBS-T (0.1%).

Ponceau staining done: Yes ☐ No ☐
Ponceau red solution lot # ______________________

6.2.11 Block in 5% dried skim milk / PBS-T (0.1%) for 1 hour, at room temperature, with shaking.

5% dried skim milk / PBS-T (0.1%) solution lot # ______________________
1 Hour block ☐

6.2.12 Wash the NC membrane with PBS-T (0.1%) with shaking:

1 × 15 min ☐
1 × 5 min ☐
1 × 5 min ☐

6.2.13 Add the primary antibody: Rabbit polyclonal Anti-Ad5 at the appropriate dilution, in PBS-T (0.1%).

Incubate overnight with shaking at room temperature.

Rabbit polyclonal Anti-Ad5 Dilution: 1/5000 ☐
Supplier: ____________________________
Lot #: ____________________________

6.2.14 Wash the NC membrane with PBS-T (0.1%) with shaking:

1 × 15 min ☐
1 × 5 min ☐
1 × 5 min ☐

6.2.15 Add the conjugated second antibody: Protein A HRP Conjugated at the appropriate dilution, in PBS-T (0.1%).

Incubate for 1 hour, while shaking at room temperature.
6.2.16 Wash the NC membrane with PBS-T (0.1%) with shaking:

1 × 15 min.
1 × 5 min.
1 × 5 min.
1 × 5 min.
1 × 5 min.

6.3. Detection

6.3.1 The following items are needed:

- Gloves (non-latex)
- Paper towels
- Forceps
- NC membrane
- 20 mL scintillation vial
- 1-5 mL Finnpipette and tips
- ECL™ Western blotting detection reagents
- Timer
- Saran Wrap
- Scissors
- Kodak X-Omatic cassette
- Hyperfilm™ ECL™

Perform the following steps on the bench

6.3.2 Mix 1.5 mL of reagent 1 with 1.5 mL of reagent 2 in a 20 mL scintillation vial.

6.3.3 With protein side up, blot the NC membrane with tissue and add drop by drop the mixed reagents covering the entire NC membrane.
surface.

6.3.4 Incubate the NC membrane for 1 minute at room temperature.

6.3.5 Remove excess liquid by blotting again with tissue paper.

6.3.6 Put the membrane face-down on a sheet of Saran Wrap and carefully fold the wrap around the membrane so as to prevent any liquid from leaking into the X-ray cassette.

Perform the following steps in the dark room (under red light)

6.3.7 Put wrapped membrane face-up in X-ray cassette and add a piece of Hyperfilm™ ECL™. Close the cassette and time for a 15 second exposure as a first trial. Record the exposure time

6.3.8 Immediately after the exposure, develop the film in the X-Omatic developing machine.

6.3.9 If the film was overexposed, develop again with another film, with a shorter exposure time.

<table>
<thead>
<tr>
<th>Film #</th>
<th>Exposure time:</th>
</tr>
</thead>
<tbody>
<tr>
<td># 1</td>
<td></td>
</tr>
<tr>
<td># 2</td>
<td></td>
</tr>
<tr>
<td># 3</td>
<td></td>
</tr>
</tbody>
</table>

Prepared by:__________________ Date:__________________

7.0 Acceptance Criteria

7.1 The Western Blot of Ad must show four bands recognized by the primary antibody (rabbit polyclonal Anti-Ad5).

7.2 The four bands must correspond to the four bands of the CsCl purified Ad std.

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7.3 These four bands are Hexon, Penton, Fiber and Core proteins.

8.0 Results

9.0 References

9.1 Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (BIO-RAD) Cat # 170-3940, Instruction Manual