SOP: AAV2 GFP Transduction of CHO K1 Cells: Verification of Transgene Expression by Western Blot

Approvals:

Preparer: Hetal Doshi Date: 15 AUG2022
Reviewer: Dr. Maggie Bryans Date: 17AUG2022

1. **Purpose:**
   1.1. To describe the steps necessary for performing a Western blot from a pre-run SDS-Page gel containing lysates from CHO K1 cells transfected with AAV-CMV-GFP viral particles.

2. **Scope:** This SOP can be used to verify the presence of Green Fluorescence Protein (GFP) in CHO K1 cell lysates.

3. **Responsibilities:**
   3.1. It is the responsibility of the course instructor/lab assistant to ensure that this SOP is performed as described and to update the procedure when necessary.
   3.2. It is the responsibility of the students/technician to follow the SOP as described and to inform the instructor about any deviations or problems that may occur while performing the procedure.

4. **References:**
   4.1. iBlot 2 Dry Blotting System, User Guide, Invitrogen, Publication Number MAN0009112 Revision D.0
   4.2. Western Breeze Chromogenic Western Blot Immunodetection Kit Instruction Manual, Invitrogen, IM-1004 Version F
   4.3. SOP: SDS Page protein Gel Electrophoresis, Document # QCB 15 Rev 0

5. **Precautions:**
   5.1. Routine care should be exercised in the handling of buffers and samples of biological materials, which may have harmful biological activity in the case of accidental ingestion, needle stick etc.
   5.2. Gloves, a lab coat and protective eyewear should be worn when handling buffers and samples.
   5.3. Always wear gloves when handling polyacrylamide gels and PVDF blots.

6. **Materials:**
   6.1. iBlot 2 Dry Blotting System, Invitrogen Ref IB21001, including blotting roller.
   6.2. iBlot 2 Mini Transfer Stacks PVDF membrane, Invitrogen Ref 1824002, including iBlot 2 Absorbent Pads mini, and iBlot Filter paper mini.
   6.3. Sterile Filtered deionized water
   6.4. Forceps for handling the membrane
   6.5. A box or dish with a cover for incubating and washing the blot
   6.6. Fisher Open Air Rocker Ref # 0221765
   6.7. Western Breeze Chromogenic Immunodetection system Catalog # WB7103

6.8. Pre-run SDS PAGE gel of cell lysate of CHO K1 cells transduced with AAV2-CMV-GFP viral vector , IMAC purified EGFP expressed in E.Coli as positive control, and non-transduced CHO K1 cell lysate as negative control

6.9. Primary antibody mouse anti EGFP Monoclonal Antibody (F56-6A1.2.3), Invitrogen Ref MA1-952 (1:1000 dilution for e.g., 10µl in 10ml of prepared Antibody Diluent)

7. Procedure:

Note: Do not handle the membrane with bare or gloved hands, always use forceps. Do not allow the membrane to dry out. PVDF membranes dry quickly, when transferring the membrane between solutions have the next solution prepared before removing the membrane from its current solution.

7.1. If immunodetection is to be performed the same day as the blot is prepared prepare the following solutions before blotting the pre-run gel. These solutions should be used the same day they are prepared.

7.1.1. Prepare 10 ml of Blocking Solution for each blot as follows;

7.1.1.1. 5 ml of sterile filtered water

7.1.1.2. 2 ml of Blocker/Diluent (Part A)

7.1.1.3. 3 ml of Blocker/Diluent (Part B)

7.1.2. Prepare 10 ml of Primary Antibody Diluent for each blot as follows;

7.1.2.1. 7 ml of sterile filtered water

7.1.2.2. 2 ml of Blocker/Diluent (Part A)

7.1.2.3. 1 ml of Blocker/Diluent (Part B)

7.1.3. Prepare 160ml of Antibody Wash Solution for each membrane as follows;

7.1.3.1. 150ml of sterile filtered water

7.1.3.2. 10ml of Antibody Wash Solution (16X)

7.2. Running SDS PAGE gel

7.2.1. Run samples on 4-20% Tris-Glycine Gel, Invitrogen Novex WedgeWell (Reference # XP04200BOX) by following the “SOP: SDS Page protein Gel Electrophoresis, Document # QCB 15 Rev 0”

7.2.2. Sample preparation for loading the SDS-PAGE gel

To prepare the cell lysate follow “SOP: Transduction of CHO K1 cells with Viral vector AAV/DJ-CMV-GFP” XXX

7.2.2.1. 30µl of cell lysate from transduced and non-transduced CHO K1 cells was mixed with 30µl of 2X Laemmli Sample buffer in an appropriately labelled sterile 1.5 ml microfuge tube

7.2.2.2. For positive control add 8µl of IMAC purified EGFP expressed in E. Coli to an appropriately labelled sterile 1.5 ml microfuge tube add 22µl autoclaved milliQ water. Add 30µl of 2X Laemmli Sample buffer in an appropriately labelled sterile 1.5 ml microfuge tube

7.2.2.3. Heat each of the prepared sample tubes at 95°C in the heating block for 2 minutes

7.2.2.4. Place the sample tubes on ice until they are loaded on the gel

7.2.2.5. Just prior to loading give the sample tubes a quick spin in a tabletop centrifuge to collect the sample in the bottom of the tube

7.2.2.6. Load 7µl of Precision Plus Protein Kaleidoscope Ladder and 40µl of prepared samples in the appropriate wells

7.3. Preparation the SDS-PAGE gel for transfer

7.3.1. Prepare a container with 20ml deionized water for gel washing

7.3.2. Rinse the gel cassette with deionized water using a squirt bottle.

7.3.3. Open the cassette by inserting the gel knife at the bottom corner of the cassette being careful not to contact the gel. Angle the knife up and down to separate the two plastic plates. Insert the knife at the opposite bottom corner and separate the plates on that side. Carefully open the cassette. The gel will be attached to one of the two plates. Discard the plate not holding the gel.

7.3.4. With the gel exposed and still on one half of the cassette remove the gel foot and the well tabs. With the plastic plate and gel lying flat on bench top insert the gel knife vertically through the gel just above the foot lift the knife straight up and out of the gel. Reposition the knife to the side of the previous cut and insert through the gel continue until entire width of the gel has been cut. Do not pull the knife through the gel. Also remove the small tabs that define the wells from the top of the gel by cutting through them vertically with the gel knife.

7.3.5. Place the gel the container with 20ml of deionized water while preparing the iBlot 2 device.

7.4. Working with the iBlot 2 device;

7.4.1. Have ready; forceps, blotting roller, Mini Transfer Stack, mini–Stack Absorbent Pad and Mini Stack Filter Paper.

7.4.2. Put 10 ml of sterile filtered water in a container large enough to hold a membrane. To be used to wet a piece of Mini Stack Filter paper.

7.4.3. Open the Transfer Stack by carefully peeling back the foil top. Lay the foil inside up on the bench top.

7.4.4. The top half of the Mini Stack is separated from the bottom half of the stack by a white plastic divider. Carefully lift the top of the stack off the bottom by this divider and place it plastic divider up, on top of the foil. Leave the bottom of the stack in the tray.
It is possible for the membrane to stick to the bottom of the plastic divider. If the membrane is stuck to the divider use the forceps to carefully remove the membrane and place it back on the top of the bottom stack.

7.4.5. Place the plastic tray containing the Mini Stack in the well of the iBlot 2 device. The brown tab of the stack should be pointing to the back of the device and the plastic tray should fit securely in the well.

7.4.6. Make sure there are no bubbles between the membrane and the transfer stack. Remove any bubbles using the blotting roller. Be sure the roller is clean and damp before contact with the membrane.

7.4.7. Rinse the pre-run gel briefly, 1-10 seconds in deionized water to remove any small pieces of gel. Allow excess water to drip off gel. Position the gel on the membrane. Dip the roller in the deionized water and shake off excess water. Use the roller to gently remove any air bubbles between the gel and the membrane.

7.4.8. Place one piece of Mini Stack Filter paper in the water to wet.

7.4.9. Remove the presoaked filter paper from the water and allow excess water to drip off. Place the filter paper on top of the gel use the Blotting Roller to remove any air bubbles between the paper and the gel.

7.4.10. Remove and discard the white plastic separator from the top stack.

7.4.11. Place the top stack on top of the pre-soaked filter paper with the tan copper electrode surface facing up and the transfer gel layer facing down.

7.4.12. Remove any air bubbles using the blotting roller.

7.4.13. Place an iBlot 2 absorbent pad on top of the transfer stack. Make sure that the electrical contacts are aligned with the electrical contacts on the iBlot 2 device. Use the roller to flatten any protrusions in the stack.

7.4.14. Gently close the lid of the iBlot 2 device using one hand on each side of the lid.

7.5. Run the Transfer

7.5.1. On the control panel select “Saved methods”, select “GFP Transfer V1”

7.5.2. Confirm following parameters as mentioned below are displayed on the control panel

Step 1	Step 2	Step 3
20V	23V	25V
01:00	03:00	02:00

7.5.3. Select “Start Run” on the control panel

7.5.4. After the transfer is complete the current is shut off and the device beeps. Tap the “DONE” icon.

7.6. Disassemble the Stack

7.6.1. Open the iBlot 2 device. Discard the absorbent pad and the tops stack. Remove the filter paper and gel.

7.6.2. Using forceps remove the PVDF membrane from the stack and place in 20ml of sterile filtered deionized water. Wash for 3 minutes. Repeat this wash a second time.

7.6.3. At this point the blot can be removed from the wash, wrapped in plastic wrap and stored at 4°C until ready to perform immunodetection.

7.7. Immunodetection

7.7.1. If the blot was prepared previously and stored at 4°C

7.7.1.1. Prepare the blocking solution, antibody diluent and antibody wash solution detailed in section 7.1.

7.7.1.2. If membrane appears dry, rewet the membrane by placing it in dish containing 10 ml of methanol for 10 seconds and then washing twice in 20 ml sterile filtered water for 5 minutes per wash.

7.7.1.3.If the blot was washed and stored at 4˚C rinse the membrane briefly in a dish containing 20 ml sterile filtered deionized water.

7.7.2. Transfer the membrane from the water wash to the blocking solution. Cover the dish and place on a rotary platform and incubate at room temperature for 30 minutes.

7.7.2.1. During this incubation prepare the primary antibody solution,

7.7.2.1.1. Add 10µl of primary antibody, mouse anti EGFP monoclonal antibody, to the prepared antibody diluent as mentioned in step 7.1.2.

7.7.2.2. At the end of the 30-minute incubation with blocking buffer, decant the blocking solution and wash the membrane two times with 20 ml of sterile filtered deionized water for 5 minutes each wash.

7.7.3. Incubate the blot with primary antibody

7.7.3.1. Decant the water and add the prepared 10ml of 1:1000 dilution of primary anti body from step 7.7.2.1.1.Cover the dish and incubate on a rotary platform at room temperature for 1 hour.

7.7.3.2.At the end of the incubation decant the primary antibody solution, Wash the blot for 5 minutes on a rotary platform with 20 ml of the antibody wash solution. Repeat 3 times, (total of 4 washes).

7.7.4. Incubate the blot with 10 ml of prepared secondary antibody solution for 30 minutes at room temperature on a rotary platform.

7.7.4.1. Decant the secondary antibody solution and wash the blot for 5 minutes on a rotary platform with 20 ml of the antibody wash solution. Repeat 3 times, (total of 4 washes).

7.7.4.2. Rinse the blot with 20 ml of sterile filtered water for 2 minutes. Repeat this rinse 2 times, (total of 3 rinses).

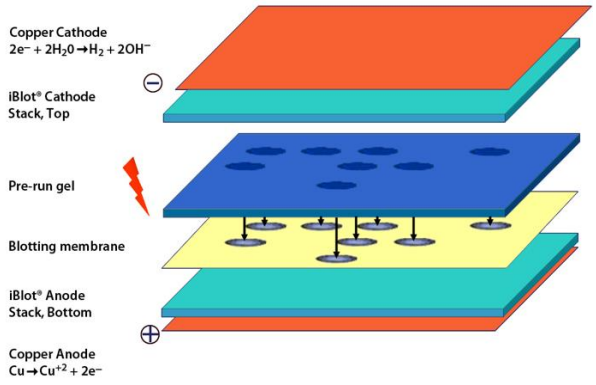
7.7.5. Return to rotary platform and incubate the blot in 5 ml of chromogenic substrate until purple bands develop. This may take from 5 to 15 minutes. Note the time required for color development.

7.7.5.1. Rinse the blot with 20 ml of sterile filtered water for 2 minutes. Repeat this rinse 2 times, (total of 3 rinses).

7.7.5.2. Place the membrane on a clean piece of filter paper to dry. Image the blot.

8. Attachments:

Schematic of iBlot™ 2 Transfer Stack showing the flow of current



9. History:

Revision Number	Effective Date	Preparer	Amendment
0	18AUG2022	Hetal Doshi	Initial release