# GVS North America Western Transfer Procedure for PVDF Membranes: chemiluminescent and colorimetric detection

## 1.0 Definitions:

1.1 PVDF: polyvinylidene fluoride

1.2 TBS: Tris buffered saline

1.3 T-TBS: Tris buffered saline with 0.05% Tween 20

1.4 GAR-AP: goat anti-rabbit IgG alkaline phosphatase conjugate

1.5 HRP-GAR IgG: horseradish peroxidase-goat anti rabbit conjugate

## 2.0 Equipment/Supplies/Reagents:

2.1 XCell *SureLock*™ Mini-Cell and XCell II Blot Module (Invitrogen Life Technologies)

2.2 12% precast, 1mm X 10well, acrylamide, Tris-glycine gels (Invitrogen)

2.3 Electrophoresis power supply (Invitrogen PowerEase 500)

2.4 10X Electrophoresis running buffer/10X Tris, glycine, SDS (Bio-Rad 161-0732)

2.5 10X Western transfer buffer/10X Tris/glycine (Bio-Rad 161-0734)

2.6 Pre-mix sample buffer (Bio-Rad 161-0737)

2.7 Beta-mercaptoethanol

2.8 Human serum

2.9 Prestained SDS PAGE molecular weight markers (Bio-Rad 161-0305)

2.10 Methanol

2.11 Heat block

2.12 Microcentrifuge

2.13 Blotting pads and paper

2.14 PVDF membrane

2.15 Rocker

2.16 10X TBS

2.17 Tween 20

2.18 Non-fat dried milk

2.19 Rabbit anti-human transferrin (Acris lot 33254)

2.20 GAR IgG-AP (Bio-Rad 170-6518)

2.21 AP Blotting Substrate Kit (Bio-Rad 170-6432)

2.22 HRP-GAR IgG (Jackson ImmunoResearch 111-035-045)

2.23 SuperSignal West Dura Luminol/Enhancer (Thermo Scientific #34075)

2.24 UVP BioImaging System

## 2.0 Procedure:

***Human serum is a biohazard, so use universal health precautions. Also, to avoid contaminating the membrane, clean anything which will come in contact with the membrane with water.***

2.1 Reagent Preparation

2.1.1 10X TBS (200mM Tris, 5M NaCl pH 7.5) To make 1 liter, add 800ml of deionized water to 1000ml graduated cylinder. Add 24.2gm of Tris and 292.2gm of sodium chloride. Stir to dissolve. Adjust pH to 7.5. Add deionized water to bring volume up to 1000ml.

For 1X TBS, add 100ml of 10X TBS to 900ml of deionized water. Final concentration is 20mM Tris, 500mM NaCl.

2.1.2 T-TBS (0.05% Tween in 1X TBS). To make 2 liters, mix 1800ml of deionized water, 200ml of 10XTBS, and 1ml or 1gm of Tween 20.

2.1.3 Blocking solution (0.5% non-fat dry milk in TBS): Add 0.5 gm non-fat dry milk to 100 ml TBS.

2.2 Preparation of Blots:

2.2.1 Prepare 1X electrophoresis buffer by mixing 540ml of deionized water + 60ml of 10X Tris/glycine/SDS (Bio-Rad 161-0732). Prepare 12% precast protein gels. Assemble the XCell Mini-cell according to Invitrogen's instruction manual.

2.2.2 Turn on heat block to 90ºC and prepare the final working solution of the reducing sample buffer by adding 50µl of beta-mercaptoethanol to 950µl of sample buffer. (Suggest working in hood when opening stock bottle of mercaptoethanol. It has a strong odor.)

2.2.3 Thaw a tube of heat inactivated human serum and prestained low molecular weight standards. Prepare the following protein samples, in order, in labeled 1.5ml microfuge tubes:

- 1: 175 human serum: 1µl of human serum + 175µl reducing sample buffer. Mix well.
- 1: 350 human serum: 75µl of the 1:175 dilution + 75µl of reducing sample buffer. Mix well.

- 1: 700 human serum: 75µl of the 1:350 dilution + 75µl of reducing sample buffer. Mix well.
- 1:1400 human serum: 75µl of the 1:700 dilution + 75µl of reducing sample buffer. Mix well.
- Prestained low molecular weight markers. Heat the solution to 40ºC for 1 minute to dissolve any solids. For two gels, pipet 25µl into a 0.5ml microcentrifuge tube.

2.2.4 Slide cap holders over the microfuge caps to ensure tubes remain closed during heating. Place microfuge tubes in heat block for 5 minutes.

2.2.5 Remove cap holders and quick spin tubes in centrifuge to bring down any liquid in on the slide of the tube or in the cap.

2.2.6 Load each gel as follows: Lane 1: 5µl of 1:175 human serum

Lane 2: 5µl of 1:350 human serum

Lane 3: 5µl of 1:700 human serum

Lane 4: 5µl of 1:1400 human serum

Lane 5: 5µl prestained standards

Lane 6: 5µl prestained standards

Lane 7: 5µl of 1:1400 human serum

Lane 8: 5µl of 1:700 human serum

Lane 9: 5µl of 1:350 human serum

Lane 10: 5µl of 1: 175 human serum

Set the power supply at 125V constant (30-40mA at the start, 8-12mA at the end) and run for approximately 60 minutes.

2.3 Blotting gels to PVDF

2.3.1 Wear gloves to avoid contaminating the PVDF membrane.

2.3.2 During the last 30 minutes of electrophoresis, prepare 1X Western transfer buffer by mixing 560ml deionized water, 160ml methanol, and 80ml 10X Tris/glycine (Bio-Rad 161-0734). Also, prepare the membrane(s) for blotting. Clean scissors or paper cutter with a water-saturated wipe, then a 70-100% ethanol-saturated wipe and let air dry. Cut membrane to 3 ¼ x 2 ¾ inch sheets. Also, cut 4 pieces of blotting paper to the same dimensions.

2.3.3 Visually inspect the membrane and check for sidedness. Membranes can be labeled using a pencil.

2.3.4 Wet the PVDF membrane in 100% methanol until the entire membrane is translucent. Briefly, rinse the membrane in deionized water and then equilibrate the membrane in Western transfer buffer for several minutes. The membrane will float on the surface of the buffer until it is completely equilibrated. After it is equilibrated, it can be easily submerged into the aqueous solution.

***NOTE: Complete wetting of the membrane is important to ensure proper protein binding. Abrupt wetting can lead to entrapment of air bubbles in the membrane, which can block transfer of proteins. Methanol is a liquid hazard waste and needs to be disposed in proper liquid hazard container.***

2.3.5 Prepare blotting pads by soaking pads in transfer buffer until saturated. Remove air bubbles by squeezing the pads while they are submerged in buffer or roll a test tube across pads until air bubbles removed. Removing of air bubbles is critical since they block protein transfer.

2.3.6 Refer to Invitrogen's XCell II™ Blot Module instructions for removing the gels after electrophoresis and transferring two gels.

2.3.7 If there is a sidedness to the membrane, place the smooth side of the PVDF next to the gel for the transfer.

2.3.8 Transfer at 25V constant for 1 hour (expected current is 100mA.).

2.3.9 Use a pencil to mark the blots. Use clean scissors to cut each blot in half between the lanes of prestained standards. Half the membrane will be assayed for chemiluminescent detection and the other half for colorimetric detection of human transferrin. At this point, the membranes can be air dried, placed between blotting paper and stored at room temperature until ready to continue.

2.4 Analysis of Blots

2.4.2 Immunoassay for Human Transferrin—chemiluminescent detection

2.4.2.1 If the PVDF membrane is dry, rewet the PVDF in methanol and then TBS buffer.

2.4.2.2 Decant the TBS, add 30 ml of blocking solution to each container and incubate for 1 hour with rocking.

2.4.2.3 Decant blocking solution and wash the blots, 3X with T-TBS, 5 minutes each. Prepare a 1:10,000 dilution of rabbit anti-human transferrin in T-TBS (6µl of rabbit anti-transferrin to 60 ml T-TBS).

2.4.2.4 Decant wash and add rabbit anti-transferrin to membranes, 30 ml/container, and incubate for 1 hour with rocking.

2.4.2.5 Decant solution and wash the blots, 3 times with T-TBS, 5 minutes each, with rocking. Prepare a 1:20,000 dilution of HRP-GAR IgG in T-TBS (3µl HRP-GAR IgG to 60 ml T-TBS).

2.4.2.6 Decant wash and add HRP-GAR IgG to membranes, 30 ml/container, and incubate for 1 hour with rocking.

2.4.2.7 Decant solution and wash the blots, 3X with T-TBS, 5 minutes each, with rocking. During the washes, prepare SuperSignal West Dura Luminol/Enhancer solution per manufacturer's instruction.

2.4.2.8 SuperSignal West Dura Luminol/Enhancer solution: Make fresh.

Add equal amounts of stable peroxide solution to Luminol Enhancer solution. (Add together 5 ml peroxide and 5ml Luminol). Mix thoroughly and protect from light.

2.4.2.9 Decant the final wash and add the SuperSignal West Dura Luminol/Enhancer solution. Incubate for 3 minutes.

2.4.2.10 Remove excess solution from the membranes and place membrane in plastic sheet. Ensure there are no air bubbles between the membrane and the plastic sheet.

2.4.2.11Capture Chemiluminescence using the UVP BioImaging System (suggested settings 1x1 bin, capture signal at multiple time points__10sec, 1minute and 5 minutes)

2.4.3 Immunoassay for Human Transferrin—colorimetric detection

2.4.3.1 If the PVDF membrane is dry, rewet the PVDF in methanol and then TBS buffer.

2.4.3.2 Decant the TBS, add 30 ml of blocking solution to each container and incubate for 1 hour with rocking.

2.4.3.3 Decant blocking solution and wash the blots, 3X with T-TBS, 5 minutes each. Prepare a 1:10,000 dilution of rabbit anti-human transferrin in T-TBS (6µl of rabbit anti-transferrin to 60 ml T-TBS).

2.4.2.4 Decant wash and add rabbit anti-transferrin to membranes, 30 ml/container, and incubate for 1 hour with rocking.

2.4.2.5 Decant solution and wash the blots, 3 times with T-TBS, 5 minutes each. Prepare a 1:3,000 dilution of GAR IgG-AP in T-TBS (20µl GAR IgG-AP to 60 ml T-TBS).

2.4.2.6 Decant wash and add GAR IgG-AP to membranes, 30 ml/container, and incubate for 1 hour with rocking.

2.4.2.7 Decant solution and wash the blots, 3X with T-TBS, 5 minutes each. Prepare alkaline phosphatase substrate solution during the washes.

2.4.2.8 Alkaline phosphatase substrate solution: Make fresh.

Add 3ml of 25X color development concentrate to 72ml deionized water and mix thoroughly. Immediately before use, add 0.75ml of AP color reagent A and 0.75ml of AP color reagent B to 75ml of 1X color development buffer. Mix.

***NOTE: Color reagent A and B in dimethylformamide (toxic). Dispose in appropriate liquid hazardous waste container.***

2.4.2.9 Decant the final wash and add alkaline phosphatase substrate solution. Allow bands to develop for 10 minutes at room temperature with rocking. Decant substrate solution and dispose as hazardous waste. Stop the reaction by 3-5 brief water rinses.

2.4.2.10 Use the UVP BioImaging System to capture data.