ORF65.1 Western Blot Protocol (Mini Gel)

1. **Timeline** (minimum times for each step given in parentheses)
   a. Day One
      i. cast gel and polymerize (30 min)
      ii. load samples (15 min)
      iii. run gel (2 h)
      iv. transfer gel to membrane (2 h)
      v. block membrane (1 h)
      iii. incubate membrane strips with patient sera overnight (12-14 h)
   c. Day Two
      i. wash membrane strips (1.5 h)
      ii. incubate membrane strips with secondary antibody (1.5 h)
      iii. repeat step i (1.5 h)
      iv. develop strips, dry, line up and score (1.5 h)

2. **Reagents**
   a. 30% (w/v) acrylamide solution, 200 ml

acrylamide	60.00 g
bis-acrylamide	1.60 g

   Make up to 200 ml with Milli-Q H_2O. Filter through 0.2 um bottle top filter. Store in foil-wrapped bottle at 4˚C.

   b. 10% (w/v) sodium dodecyl sulfate (SDS), 200 ml

SDS	20 g

   Make up to 100 ml with Milli-Q H_2O. Store at room temperature.

   c. 4X Stacking Gel Buffer, 50 ml

Tris base	3.03 g
10% (w/v) SDS stock solution	2.00 ml

   Make up to 50 ml with Milli-Q H_2O after adjusting ***pH to 6.8*.** Store at room temperature.

   d. 4X Separating Gel Buffer, 100 ml

Tris base	18.17 g
10% (w/v) SDS stock solution	4.00 ml

   Make up to 100 ml with Milli-Q H_2O after adjusting ***pH to 8.8*.** Store at room temperature.

   e. 10% (w/v) ammonium persulfate (APS), 50 ml

APS	5 g

   Make up to 50 ml with Milli-Q H_2O and store at -20˚C in 500 ul aliquots.

2. Reagents, cont'd

g. 2X SDS Sample Loading Buffer, 10 ml

bromphenol blue	0.0025 g
4X stacking gel buffer	2.50 ml
100% glycerol	2.00 ml
10% (w/v) SDS stock solution	2.00 ml

Make up to 9.5 ml with Milli-Q H_2O and add 5% (v/v) β-mercaptoethanol just prior to use. Store at room temperature.

h. 10X Running Buffer, 1.0 L

Tris base	30.00 g
10% (w/v) SDS stock solution	100.00 ml
glycine	144.00 g

Make up to 2.0 L with Milli-Q H_2O. Store at room temperature.

i. Transfer Buffer, 1.0 L

Tris base	3.02 g
glycine	14.40 g
methanol	200.00 ml

Make up to 1.0 L with Milli-Q H_2O. Store at room temperature.

j. 1% Skim Milk, 250 ml

Blotto	25 ml

Make up to 250 ml with Wash Solution 2/TBS (below). Store at 4˚C.

k. 5M NaCl, 1.0 L

NaCl	292.2 g

Make up to 1.0 L with Milli-Q H_2O. Store at room temperature.

l. 2M Tris, 1.0 L

Tris base	242.2 g

Make up to 1.0 L with Milli-Q H_2O after adjusting pH to 7.4 with HCl. Store at room temperature.

m. Wash Solution 1, 1.0 L

2M Tris, pH 7.4	25 ml
5M NaCl	40 ml
Triton-X	2 ml
Blotto (5% skim milk)	10 ml

Make up to 1.0 L with Milli-Q H_2O. Make fresh each time.

n. Wash Solution 2, 1.0 L

2M Tris	25 ml
5M NaCl	40 ml

Make up to 1.0 L with Milli-Q H_2O. Store at room temperature.

2. Reagents, cont'd

o. BCIP/NBT Buffer, 2.0 L

Tris base	24.22 g
NaCl	11.70 g
$MgCl_2$	20.30 g

Make up to 2.0 L with Milli-Q H_2O after adjusting pH to 9.5 with HCl. Store at room temperature.

3.1. Protocol for Pouring and Polymerizing Gel

a. Assemble plates with appropriate side spacers and clamps. Be sure to coat one plate with Sigmacote, to ease separation of plates after gel has been run. Place assembly into gel casting stand.

b. Prepare 15% SDS-PAGE separating gel to separate proteins using the following recipe:

Component	1X	2X
30% acrylamide	2.26 ml	4.52 ml
4X separating gel buffer	1.14 ml	2.28 ml
10% (w/v) APS	67.20 ul	134.40 ul
H_2O	1.04 ml	2.08 ml
TEMED	3.00 ul	3.00 ul
TOTAL VOLUME	4.51 ml	9.02 ml

b. Immediately syringe separating gel mixture into the gel plate sandwich. Carefully add a layer of Milli-Q H_2O using a syringe and a 22G needle. Polymerize at least 10 minutes.

c. Remove Milli-Q H_2O using a syringe and 22G needle and flush once with Milli-Q H_2O to remove unpolymerized acrylamide.

d. Prepare stacking gel according to the following recipe:

Component	1X	2X
30% (w/v) acrylamide	266.00 ul	532.00 ul
4X stacking gel buffer	180.00 ul	360.00 ul
10% (w/v) APS	16.80 ul	33.60 ul
H_2O	600.00 ul	1.20 ml
TEMED	3.00 ul	3.00 ul
TOTAL VOLUME	1.07 ml	2.13 ml

e. Immediately syringe stacking gel mixture onto separating gel. Quickly insert apprpriate comb as the stacking gel will polymerize within a minute. Make sure there are no air bubbles in the gel. Polymerize at least 5 minutes.

3.2. Protocol for Preparing and Loading Samples

a. Prepare ORF65.1 sample and molecular weight marker according to the following recipes:

ORF65.1	
2X Sample Buffer	237.5 ul
1X PBS	250.0 ul
β-mercaptoethanol	12.5 ul
ORF65.1 recombinant antigen	0.5 ul

Marker	
2X Sample Buffer	7.1 ul
1X PBS	7.5 ul
β-mercaptoethanol	0.4 ul
SDS-PAGE molecular weight marker	10.0 ul

b. Boil ORF65.1 and marker samples for 10 minutes.

c. Fill electrophoresis unit with 1X SDS-PAGE running buffer, remove sandwich from clamps, and pop into gel running portion of electrophoresis unit. Fill top reservoir with 1X SDS-PAGE running buffer, place plastic well marker on the outside plate to permit easy visualization of wells and remove comb carefully in order not to distort wells.

d. Once samples have finished boiling, load 400 ul of ORF65.1 and 25 ul of marker into appropriate wells.

h. Run gel at 200 V for 10 minutes at 4˚C to allow samples to enter gel; turn voltage down to a constant 100 V for 2 hours,. or until blue dye front reaches the bottom of the gel.

3.3. Protocol for Transferring Proteins and Blocking Membrane

(Prior to transfer, be sure that Bio-ice cooling unit has been filled with Milli-Q and frozen at -20˚C.)

a. Cut nylon membrane to the size of separating gel.

b. Thirty minutes prior to the end of the gel's running time, soak nylon membrane, two fiber pads, and four blotting pads in a shallow tray filled with transfer buffer.

c. Disassemble gel apparatus, carefully pry plates apart, and cut off stacking gel with a clean razor blade. Soak gel in transfer buffer for a few minutes, to allow it to equilibrate.

d. Open transfer apparatus gel cassettes with black panel lying flat on the bottom of the tray filled with transfer buffer; the clear panel should be against the side of the tray.

e. Prepare the transfer sandwich on the black panel in the tray filled with transfer buffer: *(smooth out bubbles at each stage)*
   i. one fiber pad
   ii. two blotting pads
   iii. equilibrated gel
   iv. equilibrated nylon membrane
   v. two blotting pads
   vi. one fiber pad

f. Cover the sandwich with the clear panel, fasten with the latch, and insert the gel cassette into the electrode module with the ***black panel facing the black cathode electrode panel***.

g. Insert the bio-ice cooling unit into the buffer chamber, fill the buffer chamber with transfer buffer, and transfer for 1-2 hours at 4C with stirring at a constant current of 190 mA.

3.4. Protocol for Blocking and Incubating Strips with Primary Antibody

a. Disassemble transfer apparatus and label lower left of membrane with pencil; dry membrane in 37˚C incubator for 20 minutes.

b. Block membrane in 10-12 ml of Blotto solution (5% skim milk) in a plastic bag for at least 1 hour on rocker platform.

c. Dilute primary antibody samples 1:100 in 1% skim milk: 990 ul 1% skim milk + 10 ul primary antibody, keeping a list of the strip number corresponding to each antibody.

d. Cut membrane into number of desired strips; number each in pencil along the bottom, numbering from the right to the left; and incubate each in a plastic bag with 1.0 ml primary antibody overnight on rocker platform (12-14 hours).

3.5. Protocol for Washing and Incubating Strips with Secondary Antibody

a. Wash strips in Wash Solution 1 three times for 15 minutes each time on rocker platform.

b. Wash strips in Wash Solution 2 four times for 10 minutes each time on rocker platform.

c. Dilute secondary antibody 1:2500 in 1% skim milk.

d. Incubate each strip with 1.0 ml secondary antibody in separate plastic bags on rocking platform for at least 1.5 hours.

e. Repeat steps (a) and (b).

3.6. Protocol for Developing and Lining Up Strips

a. Soak strips in BCIP/NBT buffer for ~5 minutes to allow for pH adjustment.

b. Prepare BCIP/NBT developer: 33 ul BCIP and 44 ul NBT for every 10 ml BCIP/NBT buffer. (This solution is light-sensitive, so keep in foil-wrapper tube throughout use.)

c. Develop strips individually in plastic bags by incubating with 1.0 ml BCIP/NBT developer for 2-10 minutes, depending on sensitivity of blot.

d. Soak strips in H_2O to stop development.

e. Dry strips on 3MM Whatman paper in 37˚C incubator for 20 minutes.

f. In numerical order, align bottom edge of strips along a line drawn on a piece of paper. Score subjects as positive, negative, or indeterminate, record in notebook.