1 PURPOSE

The purpose of this assay is to determine viral identity by detecting the presence of AAV capsid proteins VP1, VP2, and VP3, their relative molecular weights and stoichiometry. This method uses polyacrylamide gel electrophoresis to separate the proteins that comprise the vector capsid. This assay is also used to determine protein purity by comparing the amount of viral protein to the amount of total protein present in the sample. Silver stain is used to detect nanogram quantities of proteins, lipids or nucleic acids in polyacrylamide gels following electrophoresis. SYPRO ruby stains only proteins, allowing the use of densitometry to assess protein purity. The protocol details the use of either NUPAGE gels/silver stain reagents from Invitrogen, or Tris-glycine gels /silver stain from Biorad.

2 MATERIALS

NOTE: Substitution of equivalent materials is at the discretion of the testing laboratory.

2.1 1.5 mL microcentrifuge tubes
2.2 Pipette tips, 0.1-20 µL, sterile, plugged, Ranin, Cat. # RT-L10F
2.3 Pipette tips, 20-200 µL, sterile, plugged, Ranin, Cat. # RT-L200F
2.4 Pipette tips, 200-1000 µL, sterile, plugged, Ranin, Cat. # RT-L1000F
2.5 Tube racks
2.6 Syringe
2.7 Needle, Blunt fill
2.8 Teflon coated stir bars
2.9 Glass staining tray
2.10 Pipettes, serological, sterile, individually wrapped, 5 mL, 10 mL, 25 mL, 50 mL
2.11 Centrifuge conical, 50 mL
2.12 Boil clips

3 EQUIPMENT

3.1 Pipettes-2, 20, 200, 1000 µl
3.2 Dri-Bath
3.3 Microcentrifuge
3.4 Milli-Q Water System
3.5 Electrophoresis Apparatus with Power Supply
3.6 Refrigerator
3.7 Waver / Shaker
3.8 Stir Plate
3.9 Pipet Aid
3.10 UV/white light box and imaging system
3.11 Imaging software to determine band densities

4 REAGENTS

Reagent	Source

Reagent	Source
Positive Control	Test Laboratory in-house Standard
Milli-Q Water	NA
1X DPBS-CMF	Mediatech 21-031-CM
NaCl, 5M solution	In House
Pre-cast, 10-Well Polyacrylamide Gel 4-12% Bis-Tris - or - 10% Tris-HCl Note- store gels at 4°C to minimize background development	Invitrogen #NP0321BOX - or - BioRad #161-1101EDU
Running Buffer 20X MOPS-SDS - or - 1X Tris-Glycine-SDS	Invitrogen #NP0001 - or - In House
Reducing Agent/Buffer NuPage Reducing Agent and NuPAGE LDS Sample Buffer (4x) - or - 10X Laemmli Sample Buffer with DTT	Invitrogen #NP0004 and Invitrogen #NP0007 - or - In House
BenchMark Protein Ladder	Invitrogen #10747-012
Silver Stain Reagents SilverXpress Silver Staining Kit - or - Fixative Enhancer Concentrate Silver Complex Solution Reduction Moderator Solution Image Development Reagent 5% Acetic Acid Stop Solution Development Accelerator Solution	 Invitrogen #LC6100 - or - Bio-Rad.#161-0461 Bio-Rad.#161-0462 Bio-Rad. #161-0463 Bio-Rad #161-0464 In House In House
Methanol, Reagent grade	Fisher #A412-1
Acetic Acid, Reagent grade	Fisher #A38-500
Sypro Ruby Protein Gel Stain	Invitrogen #S12000
Native Buffer NuPAGE LDS Sample Buffer (4x) - or - Native Sample Buffer 10% SDS, AccuGENE	Invitrogen #NP0007 Or Bio-Rad #161-0738 BioWhittaker Catalog #51213

5 SAFETY

5.1 Wear gloves, safety glasses, and protective clothing while preparing and working with all the silver stain solutions.

5.2 The Image Development Reagent should be used only in areas with good ventilation. Avoid breathing vapors. Avoid contact with skin.

6 POLYACRYLAMIDE GEL PROCEDURE

6.1 Prepare reducing buffer

6.1.1 For NuPage Bis-Tris gels thaw 1mL LDS Sample buffer (4X) and 400µL Reducing Agent (10X) before use.

6.1.2 For Tris-Glycine gels thaw 2X Laemmli Buffer with DTT in a 37ºC water bath for 30 minutes before use. Mix well by vortexing.

6.2 Prepare native buffer

6.2.1 For NuPage Bis-Tris gels use LDS Sample Buffer (4X)

6.2.2 For Tris-Glycine gels combine 450µL Native Sample Buffer and 50µL 10% SDS for a final volume of 500µL.

6.3 Prepare reduced samples

6.3.1 Prepare 2 Aliquots of the AAV8 RSM sample containing $1.5x10^{10}$ GC

6.3.2 Make (2) negative controls using an equivalent volume of DPBS-CMF each

6.3.3 For Nupage Bis-Tris gels Add LDS sample buffer and reducing agent as necessary

6.3.4 For Tris-Glycine gels add 10X Laemmli Buffer with DTT as necessary

6.3.5 Fill the wells of a dry bath with de-ionized water and heat the samples for 10 minutes

6.3.5.1 For Bis-Tris gels heat samples to 70ºC

6.3.5.2 For Tris-Glycine gels place a boil clip on each sample and heat to 90ºC

6.3.6 Remove the tubes from the heating block and allow them to cool to room temperature, approximately 5-10 minutes.

6.3.7 Pulse spin tubes in a microcentrifuge to bring the contents to bottom of tube.

6.4 Prepare native samples

6.4.1 Prepare 2 Aliquots of reference standard sample containing $1.5x10^{10}$ GC

6.4.2 Make (2) negative controls using an equivalent volume of DPBS-CMF each

6.4.3 Add native sample buffer

6.4.3.1 For Bis-Tris gels add 4X LDS Sample Buffer

6.4.3.2 For Tris-Glycine gels add 10X Native Sample Buffer with 1% SDS, without reducing agent

6.5 Prepare Protein Ladder Standards for Gel A (Silver Stain)

6.5.1 In a microcentrifuge tube labeled "Protein Ladder Dilution", combine 38µL of 1X DPBS-CMF and 2µL of protein ladder standard.

6.5.2 In a microcentrifuge tube labeled "Ladder A, Reduced" combine 1µL Protein Ladder Dilution from 6.6.1 above, 9µL 1X DPBS-CMF, and 4µL of reducing buffer.

6.5.3 In a microcentrifuge tube labeled "Ladder A, Native" combine 1µL Protein Ladder Dilution from 6.6.1 above, 9µL 1X DPBS-CMF, and 3µL of native buffer.

6.6 Prepare Protein Ladder Standards for Gel B (SYPRO Ruby Stain)

6.6.1 In a microcentrifuge tube labeled "Ladder B, Reduced" combine 1µL Protein Ladder Dilution from 7.6.1 above, 9µL of 1X DPBS-CMF and 4µL of reducing buffer.

6.6.2 In a microcentrifuge tube labeled "Ladder B, Native" combine 1µL Protein Ladder Dilution from 7.6.1 above, 9µL of 1X DPBS-CMF and 3µL of native buffer.

6.7 Assemble the gel apparatus.

6.7.1 Prepare two polyacrylamide gels and the electrophoresis unit according to manufacturer's directions

6.7.2 Fill the upper buffer chamber with running buffer until the wells are completely covered.

6.7.2.1 For Bis-Tris gels add 500µL Antioxidant to 200mL running buffer for use in the upper chamber

6.7.3 Fill the tank with 1X running buffer to the upper edge of the support beam in the tank.

6.8 Load the samples, protein ladders, and controls.

6.8.1 Using a syringe with needle, gently flush each well with running buffer (drawn from upper buffer chamber).

6.8.2 Load the entire volume of the protein ladders, samples, and controls according to the gel loading guide in Table 1, Gel A to be silver stained.

6.8.3 Load the entire volume of the protein ladders, samples, and controls according to the gel loading guide in Table 2, Gel B to be SYPRO ruby stained.

Table 1: Gel A Loading Guide, Gel A to be stained with Silver Stain

Lane	Sample
1	Benchmark Ladder A - Reduced
2	Negative Control - Reduced
3	AAV Reference Material - Reduced
4	
5	Marker/ Validation - Reduced
6	Benchmark Ladder A- Native
7	Negative Control - Native
8	AAV Reference Material - Native
9	
10	Marker/ Validation - Native

Table 2: Gel B Loading Guide, Gel B to be stained with SYPRO ruby stain

Lane	Sample
1	Benchmark Ladder B- Reduced
2	Negative Control - Reduced
3	AAV Reference Material - Reduced
4	
5	Marker/ Validation - Reduced
6	Benchmark Ladder B - Native
7	Negative Control - Native
8	AAV Reference Material - Native
9	
10	Marker/ Validation - Native

6.9 Carefully place the lid on the gel box. Avoid disturbing samples. Lid must be attached so that red and black power jacks on the safety lid and base line up.

6.10 Allow the gels to run for approximately 55 – 70 minutes until dye front has reached the bottom of the gel.

6.11 When electrophoresis is complete, open the gel cassette and cut an identifying mark in the bottom right corner of the gel under lane 12.

6.12 Proceed to Section 7 for gel A. Proceed to Section 8 for gel B.

7 GEL A SILVER STAINING PROCEDURE

Note: If using Invitrogen SilverXpress silver staining kit follow the manufacturer's recommended procedures.

7.1 Prepare Fixing Solution by combining reagents in the order shown in Table 3 in an appropriate container.

Table 3: Fixative Enhancer Solution

Reagent	Bis-Tris Gels	Tris-Glycine Gels
Milli-Q Water	90 mL	60 mL
Methanol	100 mL	100 mL
Acetic Acid	20 mL	20 mL
Fixative Enhancer Concentrate	0 mL	20 mL

7.2 Pour the Fixing Solution into a clean tray on a shaker and shake at about 50RPM.

7.3 Remove Gel A from the cassette plate; invert the gel and plate under fixative solution in the tray and gently agitate until the gel separates from the plate.

7.4 Fix on a waver/shaker plate.

7.4.1 For Bis-Tris gel fix for 10 minutes

7.4.2 For Tris-Glycine gel fix 20 minutes

7.5 Decant the Fixative Enhancer Solution from the staining tray.

7.6 **For Bis-Tris gels only**: wash gel in 100mL Sensitizing solution 2 x 30 minutes

Table 4: Sensitizing Solution

Reagent	Bis-Tris Gels	Tris-Glycine Gels
Milli-Q Water	105 mL	NA
Methanol	100 mL	NA
Sensitizer	5 mL	NA

7.7 Rinse the gel in 200 mL Milli-Q water for 2 x 10 minutes with gentle agitation.

7.8 Stain and Develop gel

7.8.1 For Bis-Tris Gels

7.8.1.1 Prepare the stain solution as listed in Table 5 and stain with gentle agitation for 15 minutes.

Table 5: Staining Solution for Bis-Tris gel

Reagent	Volume to Add
Milli-Q water	90 mL
Stainer A	5 mL
Stainer B	5 mL

Note: Do not prepare more than 5 minutes prior to use.

7.8.1.2 Wash the gel twice in Milli-Q water, 5 minutes each

7.8.1.3 Develop the gel with 95mL Milli-Q water and 5mL Developer

7.8.1.4 When bands are well defined add 5mL Stopper and continue shaking for 10 minutes

Note: Developing usually occurs quickly, but may take up to 15 minutes

7.8.2 For Tris-Glycine Gels

7.8.2.1 Prepare the stain solution as listed in Table 6 and stain 15 to 20 minutes

Table 6: Stain Solution for Tris-Glycine gel

Reagent	Volume to Add	Amount Added	Initials/Date
Milli-Q water	17.5 mL		
Silver Complex Solution	2.5 mL		
Reduction Moderator Solution	2.5 mL		
Image Development Reagent	2.5 mL		
Immediately before use:			
Development Accelerator Solution	25 mL		

Note: Do not prepare more than 5 minutes prior to use.

Note: It may take at least 15 minutes before the bands first become visible

7.8.2.2 Decant and appropriately discard Staining Solution from staining tray when bands are clearly visible.

7.8.2.3 Place gel A in 200 mL 5% Acetic Acid Stop Solution for 15 - 25 minutes.

7.9 Rinse the gel A in 200 mL of Milli-Q water for 5 minutes to overnight.

7.10 Proceed to Section 9 to scan gel.

8 GEL B STAINING PROCEDURE

8.1 Fix in 100mL of the solution outlined in Table 7 with gentle agitation for 2 x 30 minutes

Table 7: SYPRO Ruby Fixing Solution

Reagent	Volume to Add
Milli-Q Water	86 mL
Methanol	100 mL
Acetic Acid	14 mL

8.2 Decant the Fix solution and replace with 50mL SYPRO Ruby stain.

8.3 Wrap staining container in aluminum foil to stain protect from light and allow the gel to stain overnight.

8.4 The next day, prepare wash solution outlined in Table 8

Table 8: SYPRO Ruby Wash Solution

Reagent	Volume to Add
Milli-Q Water	83 mL
Methanol	10 mL
Acetic Acid	7 mL

8.5 Remove SYPRO Ruby stain from container and replace with wash solution
Note: SYPRO Ruby stain can be re-used a second time before discarding.

8.6 Wash covered gel for 30 minutes with gentle agitation followed by two 5 minute washed in Milli-Q water.

9 GEL DOCUMENTATION

9.1 View Gel A using a white light box and a suitable imaging system such that the reduced protein ladder appears on the left side of the image.

9.1.1 The image file name should include “Silver Stain” and the date in MMDDYY format.

9.2 View Gel B using a gel imaging system with 302nm UV-transillumination.

9.2.1 The image file name should include “SYPRO Ruby” and the date in MMDDYY format.

10 GEL B DENSITOMETRIC ANALYSIS

10.1 Using the densitometry features of a gel imaging or scanning system obtain a chromatogram measuring the background and intensity of all bands for each gel lane according to the software manual.

10.2 Integrate the areas under the peaks of the chromatogram.

10.3 Determine the intensity of VP1, VP2, VP3, and contaminating bands as a percentage of total area under all peaks.

10.4 Sample purity is expressed as the combined intensity of VP1, VP2, and VP3 as a percentage of total intensity of all peaks.

11 CRITERIA FOR A VALID ASSAY

11.1 For Viral Identity Assay (Gel A – Silver Stain) run on reduced samples to be considered valid all of the following need to be met:

11.1.1 The reduced protein ladder standard (Benchmark) must stain to show at least 12 bands at apparent molecular weights of 220, 160, 120, 100, 90, 80, 70, 60, 50, 40, 30, 25 kDa, respectively in Lane 1.

11.1.2 The reduced positive control (lane 2) must stain to show bands at approximately 89 kDa, representative of AAV VP1, approximately 75 kDa, representative of AAV VP2 and approximately 64 kDa, representative of AAV VP3, respectively, based on comparison to the protein ladder standard.

11.1.3 The reduced positive control (lane 2) must stain such that the approximate 64 kDa band representative of VP3 is darker than the approximate 89 kDa and approximate 75 kDa bands representative of VP1 and VP2, respectively.

11.1.4 The reduced negative control (lane 3) must show no stained bands.

11.2 For the Viral Identity Assay (Gel A – Silver Stain) run on native samples to be considered valid all of the following conditions must be met:

11.2.1 The native protein ladder (Benchmark) standard (lane 7) must stain to show at least 12 bands at apparent molecular weights of 220, 160, 120, 100, 90, 80, 70, 60, 50, 40, 30, 25 kDa, respectively in Lane 7.

11.2.2 The native positive control (lane 8) must stain to show the absence of bands representative of VP1, VP2 and VP3, at approximately 89 kDa, 75 kDa, and 64 kDa, respectively, based on comparison to the protein ladder standard.

11.2.3 The native negative control (lane 9) must show no stained bands.

11.3 For the Protein Purity Assay (Gel B – SYPTO Ruby Stain) run on reduced samples to be considered valid all of the following need to be met:

11.3.1 The fully reduced protein ladder standard must stain to show at least 12 bands at apparent molecular weights of 220, 160, 120, 100, 90, 80, 70, 60, 50, 40, 30, 25 kDa, respectively in Lane 1.

11.3.2 The reduced positive control (lane 2) must stain to show bands at approximately 89 kDa, representative of VP1, 75 kDa, representative of VP2, and 64 kDa, representative of VP3, respectively, based on comparison to the protein ladder standard

11.3.3 The reduced positive control (lane 2) must stain such that the 64 kDa band representative of VP3 is darker than the 89 kDa and 75 kDa bands representative of VP1 and VP2, respectively.

11.3.4 The fully reduced negative control (lane 3) must show no stained bands.

11.4 For the Protein Purity Assay (Gel B – SYPRO Ruby Stain) run on native samples to be considered valid all of the following conditions must be met.

11.4.1 The non-reduced protein ladder standard (lane 7) must stain to show 12 bands at apparent molecular weights of 220, 160, 120, 100, 90, 80, 70, 60, 50, 40, 30, 25 kDa, respectively in Lane 7.

11.4.2 The non-reduced UF11 positive control (lane 8) must stain to show the absence of bands representative of VP1, VP2 and VP3, at approximately 89 kDa, 75 kDa, and 64 kDa, respectively, based on comparison to the protein ladder standard.

11.4.3 The non-reduced negative control (lane 9) must show no stained bands.

12 SAMPLE SPECIFICATIONS

12.1 Viral Identity Assay (Gel A – silver stain)

12.1.1 Reduced Samples

12.1.1.1 For a test sample to pass, the electrophoretic pattern for the reduced sample(s) (lane 4 or 5) must contain bands at approximately 89, 75, and 64 kDa, respectively based on comparisons to the protein markers correct on either the silver stained gel or the SYPRO ruby stained gel.

12.1.1.2 The reduced test sample must stain such that the 64 kDa band representative of VP3 is darker than the 89 kDa and 75 kDa bands representative of VP1 and VP2, respectively on silver stained and sypro ruby stained gels.

12.1.1.3 A test sample fails if any, or all of the bands at positions approximate 89 kDa, 75 kDa, and 64 kDa are absent or the banding pattern is not stoichiometrically correct on either the silver stained gel or the SYPRO ruby stained gel.

12.1.2 Samples run in native lanes of gel

12.1.2.1 Any bands observed in the native sample lanes (lanes 10 and 11) of either the silver stained or SYPRO ruby stained gel will be noted and given a relative MW based on the protein ladder.

13 TEST RESULTS

13.1 Record observed results in the reference standard test record sheet.

14 APPENDICES

a) AAV8 Reference Standard Material Test Record Packet.