Table of Contents

1.0 Purpose

The purpose of this method is to determine the approximate molecular weight distribution of the protein(s) present in the sample. Depending on the protein detection procedure used, the method can also be used for assessment of the purity, concentration, and identity of protein(s) in the sample.

2.0 Scope

Process Analytics (PA) personnel will perform this procedure. Other Biopharmaceutical Development Program (BDP) personnel may use this protocol for development or in-process analysis.

3.0 Authority and Responsibility

3.1 The Director, Process Analytics (PA) has the authority to define this procedure.

3.2 PA is responsible for training laboratory personnel and documenting this training to Biopharmaceutical Quality Assurance (BQA).

3.3 PA personnel are responsible for the performance of this procedure.

3.4 PA is responsible for reviewing the data and documentation of the results of this procedure.

3.5 BQA is responsible for quality oversight of this procedure.

4.0 Materials and Equipment

4.1 Novex Power Ease 500 power supply (Novex EI8600) or equivalent.

4.2 Novex Xcell Mini-Cell Apparatus (Novex EI9001) or equivalent.

4.3 Novex Pre-cast Gels - NuPAGE™ 4 - 12% Bis-Tris Gels (Novex NP0321, BDP PN 30054 or equivalent).

4.4 Sample Buffer (4X) NuPAGE™ LDS Sample Buffer (Novex NP0007, BDP PN 30047). Denaturing buffers contain SDS or LDS. NuPAGE™ SDS Sample Buffer (Novex NP 0003) can no longer be purchased and can be prepared (see procedures).

4.5 Molecular Weight Standards: Run molecular weight standards with each gel as an internal calibration standard. Novex Mark 12 Molecular weight standard (LC5676), BDP PN 30028, Novex See Blue Plus2 (LC5925) BDP PN 30027, Pharmacia High or Low molecular weight calibration kits or equivalent.

4.6 Gel Running Buffer: 20X MES/SDS Buffer, BDP PN 30078 (Novex NP0002) or equivalent. 20x running buffer is diluted before use. Prepare the 1X SDS Running Buffer by adding 950 mL of Direct Q water to 50 mL of the 20X NuPAGE MES Running Buffer. 1X SDS Running Buffer can also be prepared from scratch. Final concentration of a 1x solution is 50 mM MES, 50 mM Tris Base and 3.5 mM SDS, pH 7.3. Do <u>not</u> add acid or base to adjust the pH.

Buffer Composition:
97.6 g MES [2-(N-morpholino) ethane sulfonic acid]
60.6 g Tris Base
10.0 g SDS
3.0 g EDTA
Direct Q Water, or equivalent, to 500 mL

<u>NOTE</u>: EDTA can have a detrimental effect on certain proteins. The buffer composition can be followed and the EDTA can be omitted. Record the solution preparation in the BDP Solution Logbook as per **SOP 22702, *Solutions Used in BQC***.

4.7 Reducing Agent: 10X NuPAGE Sample Reducing Agent (BDP PN 30077, Novex NP0004 or equivalent). Reducing agent is added to the sample buffer for a final concentration of 1X when samples need to be run reduced. Example: If the total sample volume is 40 μL (sample and sample buffer), then 4 μL of the reducing agent (10X) is added to the sample.

4.8 Antioxidant: NuPAGE™ Antioxidant (BDP PN 30076 Novex NP0005 or equivalent). When running reducing samples, 500 μL of the antioxidant is added to 200 mL of the diluted 1 X MES/SDS running buffer to keep the proteins denatured. The 200 mL of running buffer and antioxidant then get poured in the upper/inner chamber.

<u>NOTE</u>: If the gel being run has reduced and non-reduced samples on the same gel, the antioxidant could affect the non-reduced samples; therefore, the antioxidant is not added to the running buffer.

4.9 Novex Gel Loading Tips (BDP PN 20336, Novex LC1001) or equivalent and a calibrated pipetor.

4.10 High purity water, MilliQ, Direct Q water, or equivalent.

4.11 Novex Gel-Dry Solution (BDP PN 30041, Novex LC4025 or equivalent), Novex Mini-Cellophane (Novex NC2380 or equivalent) and a gel drying frame (Novex Mini-Gel Drying Frame, Novex NI2380 or equivalent).

4.12 Microfuge tubes, gel knife or spatula and assorted containers for staining.

5.0 Procedure

NOTE: Gloves must be worn to protect the operator from the chemicals used and to protect the gel from protein on fingers which will show up on the gel using silver stain.

5.1 Preparation of 4X NuPAGE™ LDS or SDS sample buffer (from the NuPAGE™ Electrophoresis System Bis-Tris Gel Instruction Booklet).

Buffer Composition:
4.00 g Sucrose
0.682 g Tris Base
0.666 g Tris HCl
0.800 g SDS or LDS
0.75 mL of a 1% Bromophenol Blue Solution
0.006 g EDTA
(0.25 mL of a 1% Phenol Red Solution) *Optional
Direct Q Water to 10.0 mL

For Reducing Buffer: Add 0.308 g DTT or 1 mL BME or Novex Reducing Agent (see 2.7). Record the solution in the BDP Solution Logbook with the name of the solution, the preparer's initials and date, and the expiration date.

NOTE: The buffer compositions found in the NuPAGE™ Instruction Book call for EDTA in both the sample and running buffers; however, through experimentation, it was discovered that EDTA has detrimental effects on certain proteins (i.e., HSV-863). Whether or not the EDTA should be omitted will be determined on an individual basis.

Since Novex no longer makes SDS Sample buffer, the buffer composition can be followed from above. Mix until all the SDS is dissolved and store in aliquots at -20 ± 4°C.

5.2 Sample preparation is recorded on NuPAGE™ 4-12% Bis-Tris Gel Lane Assignment Sheet, **Form 22176-01** or **Form 22176-02**. Samples are diluted to a protein concentration appropriate for the staining protocol to be used. A 1-5 μg/μL concentration will allow detection of impurities using Coomassie Blue staining, while lower amounts (.1-.5 μg/μL) may be used for Silver Stain. (Depending on the product, a higher amount of protein may need to be loaded on the gel.)

Prepare the samples by adding an appropriate volume of 4X sample buffer to the sample. For reduced samples, add the appropriate volume of 10X reducing agent to the sample (see 4.7).

NOTE: The 4X sample buffer can be diluted with Direct Q H_2O, or equivalent, (1:1 dilution) to achieve a 2X sample buffer. This allows for the sample preparation to be one part sample and one part 2X sample buffer.

Example: To make a 2X sample buffer.

(5 mL) 4X NuPAGE LDS sample buffer
+ (5 mL) Direct-Q H_2O
_____
(10 mL) 2x NuPAGE Sample Buffer
Sample Preparation (1:1 dilution with the 2X Sample Buffer)
(20 µL) 2X NuPAGE Sample Buffer
+ (20 µL) Sample
(40 µL) Sample (1:1 Dilution)

*The Sample protein concentration will be determined using the 2x NuPAGE sample buffer.

Mix well. If sample requires heating, heat sample at 70°C for 10 minutes or 100°C for 4 minutes. Vortex the samples for at least 5 seconds following heating. The heating temperature and time depend on the sample.

5.3 Dilute the 20X MES/SDS Buffer 1:20 with Direct-Q H_20 or equivalent before use (see 4.6).

5.4 Cut open the gel pouch with scissors, and remove the gel cassette.

5.5 Drain away the packaging buffer.

5.6 Peel the tape off the bottom of the cassette.

5.7 In one steady motion, pull the comb out of the cassette.

5.8 Orient two gels in the Mini-Cell so that the notched, “well” side of the cassettes faces the buffer core. If running only one gel, the square plastic buffer dam replaces the second gel cassette.

5.9 Use a pipet to wash the sample wells with 1X running buffer. Leave the wells full of running buffer.

5.10 Load the samples into the wells by underlying the sample beneath the running buffer. For the best results, load the sample buffer in all of the blank wells to ensure uniform band width in all lanes.

NOTE: When running non-reduced and reduced samples on the same gel, separate the samples with one lane of non-reduced sample buffer and then one lane of reduced sample buffer.

5.11 Fill the upper buffer chamber of the Mini-Cell with 200 mL of 1X NuPAGE SDS Running Buffer and the lower buffer chamber with 600 mL of 1X NuPAGE SDS Running Buffer.

When running reduced samples, the antioxidant needs to be added to the running buffer and poured into the upper chamber (see 4.8).

5.12 With the power OFF, place the lid on the buffer core and connect the electrode wires to the power supply. Turn on the power supply and run the gel(s). An appropriate running condition for 4-12% gradient Bis-Tris gels is 200V at constant voltage for 35 minutes; the run is complete when the bromophenol blue tracking dye reaches the bottom of the gel.

NOTE: Running conditions may be altered for the needs of specific projects. Record the running conditions on the appropriate Lane Assignment Sheet (**Form 22176-01** or **Form 22176-02**). Also record the running conditions in the appropriate Power Supply Equipment Logbook.

5.13 When the run is complete, turn off the power, disconnect the electrodes, and remove the gel(s) from the apparatus.

5.14 The two plates of the cassette are fused on three edges. The notched ("well") side of the cassette should face up. To separate the plates, a gel knife or spatula is inserted into the gap between the plates. Pushing down on the handle will gently separate the plates.

5.15 Carefully remove and discard the top plate. Remove the gel from the cassette plate by the following method.

Use the sharp edge of the gel knife to remove the bottom lip of the gel. Hold the cassette plate and gel over a container with the gel facing downward and use the knife to carefully loosen one lower corner of the gel and allow the gel to peel away from the plate.

5.16 Proceed with detection of the proteins in the gel. The gel can be stained with Coomassie blue (**SOP 22175, *Staining of Gels with Coomassie Blue R-250***), SilverXpress Silver Staining (**SOP 22177, *SilverXpress Silver Staining***), or Sypro Ruby Protein Stain (**SOP 22134, *Staining of Gels with Ruby Protein Stain***) or proteins can be electroluted, electroblotted onto a polyvinylidene (PVDF) membrane for subsequent staining or sequence analysis, or transferred to a membrane for immunoblotting, Western Transfer Blot *(***SOP 22179, *Western Transfer Blot***).

5.17 Following staining of the gel, if requested, the relative electrophoretic mobility of the standard proteins and unknowns is determined by densitometry using (**SOP 22906, *Operation of the Gel-Pro 4 Analyzer Software for Densitometry***). Print the densitometry report that includes a scanned photograph of the gel and the molecular weights of the standards and the unknowns.

5.18 Following analysis, the gel is preserved by incubation with a dehydrating solution such as Novex Gel-Dry Solution, which decreases the amount of gel cracking, then dried by passive evaporation between two sheets of porous membrane (Novex Mini-Cellophane or equivalent) on a gel drying frame (Novex Mini-Gel Drying Frame or equivalent) (**SOP 22161, *Invitrogen Gel Drying***). Dried gels are suitable for densitometry if required. Once dried, the gel is taped to the appropriate Lane Assignment Sheet, **Form 22176-01** or **Form 22176-02**, and attached to the BQC Test Request **Form 22002-01**.

6.0 Documentation

6.1 Record the results on the BQC Test Request, **Form 22002-01**.

6.2 Attach the appropriate NuPAGE™ 4-12% Bis-Tris Lane Assignment Sheet, **Form 22176-01** or **Form 22176-02**, and the densitometry report (if requested) to the Test Request Form and submit for PA and BQA review.

7.0 References and Related Documents

SOP 22134 *Staining of Gels with Sypro Ruby Protein Stain*

SOP 22161 *Invitrogen Gel Drying*

SOP 22175 *Staining of Gels with Coomassie Blue R-250*

SOP 22177 *SilverXpress Silver Staining*

SOP 22179 *Western Transfer Blot*

SOP 22702 *Solutions Used in BQC*

SOP 22906 *Operation of the Gel-Pro 4 Analyzer Software for Densitometry*

Form 22176-01 *NuPAGE™ 4-12% - Bis-Tris Lane Assignment Sheet MARK 12 MW Standard*

Form 22176-02 *NuPAGE™ 4-12% - Bis-Tris Lane Assignment Sheet, See Blue Plus 2 Pre-Stained Standard*

Laemmli, U.K. 1970. *Nature* 227: 680-85

Current Protocols in Immunology.

Electrophoresis Chapter 10 in Current Protocols in Protein Science, 1995

Novex Xcell II Mini-Cell Instructions Manual.

Novex Pre-cast Gel Instructions.

Novex Power Ease 500 power supply User Manual.

BioDesign Instructions for Gel Drying with BioDesign Gel Wrap.

NuPAGE Electrophoresis System Bis-Tris Gel Instruction Booklet

8.0 Change Summary

[redacted]