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1.0 Purpose

This procedure describes a method for isoelectric focusing which allows for the separation of proteins based on their focusing point (pl).

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 The Director, PA is responsible for assignment of this procedure.

3.3 PA is responsible for training on this procedure and documenting this training to Biopharmaceutical Quality Assurance (BQA).

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

3.5 BQA is responsible for quality oversight of this operation.

4.0 Equipment

4.1 Isoelectric Focusing (IEF) Gels, (BDP PN 30013 or equivalent approved BDP PN).

4.1.1 Invitrogen IEF gels can be used for pl determination and confirmation of isoforms of purified products. Invitrogen IEF gels are 5% polyacrylamide, non-denaturing and do not contain urea. The pH 3-10 gels have a pl performance range of 3.5 – 8.3 and the pH 3-7 gels have a pl performance range 3.0-7.0.

4.2 Invitrogen PowerEase 500 XCELL II Mini-Cell Apparatus, Catalog number EI9001 (Includes lower buffer chamber, buffer core with platinum electrodes, gold terminals and silicone rubber gaskets, buffer dam, cell lid with cables, rear and front wedge).

4.3 Invitrogen PowerEase 500 Power Supply, catalog number El8600.

4.4 Calibrated Pipetor (10 µL, 20 µL, and 100 µL), Pipette tips, 1 µL to 250 µL (BDP PN 25006) capacity and Invitrogen Gel loading tips (LC 1001) BDP PN 20336 or equivalent approved BDP PN.

4.5 1.5 mL Micro-centrifuge tubes (BDP PN 20394).

4.6 Gel knife or spatula.

4.7 Invitrogen Gel Dryer, Catalog number NI2380 (consists of a bottom solid square, a plastic frame, and four plastic clamps), or equivalent.

4.8 Cellophane, PGC Scientifics (BDP PN 20596).

4.9 Shaker or rocker for agitation during incubations.

4.10 Staining trays (Glass and polypropylene).

5.0 Reagents

NOTE: When making up reagents, record steps in the BQC Solution Logbook, label each solution with BQC Number, initials, date prepared and expiration date. Refer to **SOP 22702, *Solutions Used in Process Analytics***.

5.1 For pH 3-10 IEF Gels

5.1.1 IEF Sample Buffer, pH 3-10 (2x), (BDP PN 30011 or equivalent approved BDP PN).

5.1.2 IEF Cathode Buffer, pH 3-10 (10x), (BDP PN 30012 or equivalent approved BDP PN).

5.1.3 IEF Anode Buffer (50x), (BDP PN 30015 or equivalent approved BDP PN).

5.2 For pH 3-7 IEF Gels

5.2.1 IEF Sample Buffer, pH 3-7 (2x) (Invitrogen catalog number LC5371 or equivalent).

5.2.2 IEF Cathode Buffer, pH 3-7 (10x) (Invitrogen catalog number LC5370 or equivalent).

5.2.3 IEF Anode Buffer, (50x) (Invitrogen catalog number LC5300 or equivalent).

5.3 IEF Standard: Serva IEF Marker, (BDP PN 30043) or equivalent approved BDP PN.

5.4 Fixing Solution: 0.5M Trichloroacetic Acid (TCA), (BDP PN 30252).

CAUTION: TCA is very toxic, please handle with care. Wear gloves and prepare in a hood.

5.4.1 Dissolve 81.7g of TCA in 1L of Direct-Q Water or equivalent. Stir at room temperature.

5.5 Coomassie Blue R-250 Staining Solution

5.5.1 Mix 2.5 g Brilliant Blue R-250 (BDP PN 30250), 460 mL of methanol and 460 mL of Direct-Q water. Stir at room temperature. Add 80 mL of glacial acetic acid and continue to stirring until mixed.

5.5.2 If Brilliant Blue R concentration (BDP PN 30610) is used, mix the contents of the bottle (473 mL) with 527 mL of Direct-Q water. Stir at room temperature.

5.6 De-staining Solution: 30% Methanol (BDP PN 10115), 10% Acetic Acid (BDP PN 10052), 60% Direct Q Water, [600 mL Methanol, 200 mL Acetic Acid and 1200 Direct-Q or equivalent].

5.7 Invitrogen Gel-Dry solution (BDP PN 30041 or equivalent approved BDP PN).

5.8 High purity water, Direct-Q water or equivalent.

6.0 Procedure

NOTE: Gloves must be worn both to protect the operator from the chemicals used and to protect the gel from proteins on fingers.

6.1 Sample and Buffer Preparation

6.1.1 For optimum staining, the loaded sample concentration will be between 1-5 µg. However, depending on the protein, a higher concentration can be loaded on the gel. The sample can be diluted with PBS prior to sample preparations if it is too concentrated.

6.1.2 Prepare the sample by adding one part sample to one part 2x IEF Sample Buffer and mix well. Typically, 10-20 mM salt concentration is optimum for isoelectric focusing. In some cases a higher salt concentration is required for protein solubility; however, this may interfere with isoelectric focusing.

6.1.3 Dilute the (10x) Invitrogen IEF Cathode Buffer 1:10 with high purity water before use. De-gas the IEF Cathode Buffer (1x working solution) under vacuum or purge 1 minute with nitrogen or helium gas. This reduces the possibility of bubbles from dissolved carbon dioxide forming during the gel run.

6.1.4 Dilute the 50x Invitrogen IEF Anode Buffer 1:50 with high purity water.

NOTE: When making up reagents, record steps in the BQC solution logbook. Label the solution with BQC number, initials, date prepared, and expiration date. Refer to **SOP 22702, *Solutions Used in Process Analytics***.

6.2 Assembly of XCELL Mini-Cell

6.2.1 Cut open the gel cassette bag and drain away the buffer contents.

6.2.2 Peel off the tape covering the slot near the bottom of the gel cassette.

6.2.3 In one continuous motion, pull the comb out of the cassette exposing the wells.

6.2.4 Use a pipette to wash the sample wells with cathode buffer. Leave the wells full of cathode buffer.

6.2.5 Orient the buffer core in the lower buffer chamber so that the (+) and (-) symbols on the crosspiece face the front.

6.2.6 Insert the front wedge so that its vertical face is toward the operator and its narrow taper is pointed up.

6.2.7 Insert the gel cassette(s) so that the notch that held the comb faces the buffer core. Two gels can be run simultaneously with one gel on either side of the buffer core. If only one gel is to be run, the buffer dam is used in place of the second gel cassette.

6.2.8 Insert the rear wedge with the screw hole at the top rear into the lower buffer chamber and apply gentle pressure to achieve a seal.

6.2.9 Pour anode buffer into the lower buffer chamber by pouring it in between the front of the lower buffer chamber and the gel cassette. Be sure the anode buffer fills the entire lower chamber.

6.2.10 Fill the upper buffer chamber with cathode buffer. The upper buffer chamber is the void formed between two gel cassettes (or one gel cassette and the buffer dam). The cathode buffer must cover the sample wells, approximately 150 mL.

6.3 Sample and Standard Loading

6.3.1 Using the Invitrogen gel-loading pipette tips, load the samples by placing the tip as close to the gel surface as possible without puncturing the gel, and slowly eject the sample into the well.

6.3.2 At least one lane must be reserved for the IEF Standard. If possible, reserve 2 lanes on either side. Apply 5 µL of standard to the selected well, or wells.

6.3.3 At least one lane must be reserved as a blank. Apply 20 µL of IEF sample buffer to the selected well, or wells.

6.3.4 Fill out the Lane Assignment Sheet, Form 22181-01.

6.4 Running Procedure

6.4.1 Place the lid on the buffer core ensuring that the electrodes are firmly seated with red to positive electrode (+) and black to negative electrode (-).

6.4.2 With the power OFF, connect the electrode wires to the power supply again with red to (+) and black to (-) electrodes.

6.4.3 Turn the power ON.

6.4.4 Press Select for choices of gel type. Then, select IEF Gel using the arrow keys.

6.4.5 Press Select for the number of gels to be run. Then, use the Up arrow key to select the specific number of gels.

6.4.6 Press Select for gel running conditions and then Start to begin.

6.4.7 IEF gels are run under the following conditions.

Voltage	100V constant – 1 hour
	200V constant – 1 hour
	500V constant – 30 minutes
Current	Start: 5 mA/gel
	End: 6 mA/gel
Run Time	Approximately 2.5 hours

6.5 Disassembly of XCELL Mini-Cell

6.5.1 Once the run is completed, continue pressing Stop to return to the first screen. Turn the power OFF and disconnect the electrodes. (Be sure the power is off before disconnecting the electrodes.)

6.5.2 Remove the lid and the rear wedge. The rear wedge can be removed by releasing the sure lock handle.

6.5.3 Carefully insert the knife's or spatula's beveled edge in the gap between the two cassette plates, and push down gently on the knife handle to separate the plates. Repeat on each side of the cassette until the plates are completely separated.

6.6 Staining Procedure

6.6.1 Remove the gel from the cassette and place it in a glass container (TCA will melt plastic). Pour the TCA fixing solution into the glass container. This should be done in the hood. Fix for 30 minutes to an hour in fixing solution. This step is important to fix the proteins and remove the ampholytes. Otherwise, a high background may result.

6.6.2 Discard the fixing solution in a red biohazard waste container and briefly rinse the gel with destain solution to remove salt.

6.6.3 Transfer the gel into a polypropylene staining tray. Add ~100 mL of Coomassie Blue R-250 staining solution and shake for 5 minutes.

6.6.4 Discard the stain and add ~100 mL of destain solution to the gel. Gently shake for ~30 minutes and repeat until the desired clarity has been achieved.

NOTE: All fixing, staining, and destaining should be done with gentle shaking in a covered staining tray.

6.7 Drying Procedure

6.7.1 When the desired clarity of the gel has been achieved. Decant the destain solution and add 25 mL of Invitrogen Gel-Dry solution. Gently shake in a covered staining tray for 15-20 minutes.

6.7.2 Place two pieces of cellophane per gel in a plastic container filled with high purity water.

6.7.3 Place the bottom, solid square of the gel dryer on a bench liner.

6.7.4 Lay one piece of cellophane on the bottom, solid square. Eliminate any trapped air bubbles by slowly rubbing a gloved finger over the surface.

6.7.5 Gently place the gel in the center of the cellophane sheet. Again, eliminate any trapped air bubbles by slowly rubbing a gloved finger over the surface.

6.7.6 Add 3-5 mL of Gel-Dry solution to the surface of the gel.

6.7.7 Carefully lay the second sheet of cellophane over the gel so that no air bubbles are trapped between the cellophane and the gel or around the edges of the gel.

6.7.8 Smooth out any wrinkles by rubbing gently over the surface with a gloved finger.

6.7.9 Place the plastic frame, beveled side up, on top of the cellophane.

6.7.10 Push the plastic clamps onto the three edges of the frame.

6.7.11 Tilt the frame on the remaining edge of the drain excess solution, then install the final clamp.

6.7.12 Set the assembly on a bench-top or in a drawer, gel-side up, to dry. Drying will take between 24 to 48 hours depending on the humidity and gel thickness.

6.7.13 When the gel is dry, remove it from the gel dryer, and trim off the excess cellophane

7.0 Densitometry

7.1 Densitometry, refer to **SOP 22906, *Operation of the Gel-Pro 4 Analyzer Software for Densitometry***.

8.0 Documentation

8.1 Attach the dried gel to Form 22181-01.

8.2 Attach the Densitometry printout with calculated pl value to the BQC Test Request, Form 22002-01.

8.3 Forms are checked by a PA analyst, reviewed by the PA Supervisor, and submitted for BQA review.

9.0 References and Related Documents

SOP 22702 *Solutions Used in Process Analytics*

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

Form 22181-01 *Process Analytics pH 3-10 IEF NOVEX Gel-Lane Assignment Sheet*

10.0 Change Summary

[redacted]