SOP: Batch Culture of Recombinant tPA Secreting CHO Cells

Approvals:

Preparer: Jason McMillan — Date 19MAR14
Reviewer: Dr. Margaret Byans — Date 20MAR14

1. **Purpose:**
   1.1. To produce a batch culture of mammalian cells.
2. **Scope:**
   2.1. Applies to the production of human tissue plasminogen activator (tPA) protein from recombinant Chinese Hamster Ovary (CHO) cells.
3. **Responsibilities:**
   3.1. It is the responsibility of the course instructor/lab assistant to ensure that this SOP is performed as described and to update the procedure when necessary.
   3.2. It is the responsibility of the students/technicians to follow the SOP as described and to inform the instructor about any deviations or problems that may occur while performing the procedure.
4. **References:**
   4.1. ATCC CRL9606 growth guidelines
   4.2. Bellco Spinner Flask (100mL) Cleaning and Autoclaving SOP
   4.3. Labconco Purifier Class 2 Biological Safety Cabinet (BSC) Operation SOP
   4.4. CO_2 Incubator SOP
   4.5. Oakton PC 700 Bench Series pH/ Conductivity/°C/°F Meter SOP
   4.6. spectrophotometer SOP
   4.7. Glucose Determination Assay SOP
   4.8. Lactate Determination Assay SOP
   4.9. Trypan Blue Assay SOP
   4.10. Human tPA Total Antigen ELISA SOP
   4.11. Human tPA Activity ELISA SOP
   4.12. Applikon ez-Control Bioreactor Controller Operation SOP
5. **Definitions:** N/A
6. **Precautions:**
   6.1. Use BL2 safety measures and discard waste in biohazard containers.
7. **Materials:**
   7.1. biological safety cabinet
   7.2. vial of CHO cells (ATCC 9606-CRL) recombinant for human tissue plasminogen activator (tPA)
   7.3. Ham's F12 Medium
   7.4. fetal bovine serum (FBS)
   7.5. 10X PBS
   7.6. 100mL vessel
   7.7. 1M $NaHCO_3$ (sodium bicarbonate)
   7.8. 200mM glutamine
   7.9. 10mg/mL gentamycin
   7.10. sterile 100mL Bellco spinner flasks
   7.11. sterile transfer pipets (2mL, 5mL, 25 ml, and 50mL) and pipette aid

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7.12. CO_2 incubator containing magnetic stir plate
7.13. UV-visible recording spectrophotometer
7.14. cuvettes for spectrophotometer
7.15. cuvette rack
7.16. 15ml conical tubes
7.17. Test tube rack
7.18. 1.5mL microfuge tubes
7.19. Microfuge tube holder
7.20. P20 and P1000 micropipette
7.21. microscope with 1000x magnification
7.22. cryogenic vials (1mL capacity) for storage of CHO cell master/working cell bank
7.23. sterile 250mL glass bottles for storage of CHO cell media
7.24. 100 mL glass bottle
7.25. 1L addition bottle
7.26. Male and female autoclavable connectors

8. Procedure:

8.1. **Initial Media Preparation:** Ham's F12 Medium, 90%; Fetal Bovine Serum, 10%:

8.1.1. Clean, assemble, and autoclave 100mL Bellco spinner flasks per SOP.

8.1.2. Gather the following items, spray with 70% isopropanol, and place in the biological safety cabinet:
(1) Pipette aid
(2) 5mL sterile pipettes
(2) 25mL sterile pipettes
(2) 100mL sterile Bellco spinner flasks
(1) 500mL bottle of pre-sterilized Ham's F12 Medium
(1) 50mL tube of pre-sterilized, heat inactivated fetal bovine serum (FBS)

8.1.3. Prepare biological safety cabinet (BSC) per Labconco Purifier Class 2 Biological Safety Cabinet (BSC) Operation SOP.

8.1.4. Sterilely remove 21.6mL of Ham's F12 Medium from a 500mL bottle of Ham's F12 and add to a sterile 100mL spinner flask.
8.1.4.1. Repeat with a second 100mL spinner flask.

8.1.5. Sterilely add 2.4mL of FBS to the sterile 100mL spinner flask.
8.1.5.1. Repeat with the second 100mL spinner flask.

8.1.6. Label one spinner flask as 90% Ham's F12, 10% FBS, [date], [group#], [operator initials]. Label the second spinner flask as **BLANK**, [date], [group#], [operator initials]

8.1.7. Place all spinner flasks containing CHO cell media in the CO_2 incubator. Set the speed of the magnetic stirrer to 60 rpm to ensure an even mixing of the culture without foaming.
8.1.7.1. Verify that the temperature is 37 ± 0.5°C and percentage of CO_2 is 5 ± 0.5%.

8.1.8. Check media for contamination after a minimum of 24 hours.

8.2. **Inoculation**

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8.2.1. Spray two 2mL sterile pipettes and a pipette aid with 70% isopropanol and place in the BSC.
8.2.2. Prepare Biological Safety Cabinet per BSC SOP.
8.2.3. Remove two vials of CHO cells from storage in the -80°C freezer and note in the ScienTemp -80°C Freezer Log.
8.2.4. Thaw contents rapidly by agitation in a 37°C ± 0.5°C water bath.
8.2.5. Spray vials with 70% isopropanol, and place in the biological safety cabinet.
8.2.6. Sterilely transfer the entire contents of both 1mL vials of thawed CHO Cells into the Bellco Spinner Flask labeled 90% Ham's F12, 10% FBS, [date], [group#], [operator initials] using a 2mL sterile pipet. **Do not add anything to the Bellco Spinner Flask labeled BLANK, [date], [group#], [operator initials].**
8.2.7. Swirl to mix.
8.2.8. Immediately after adding CHO Cells to Bellco Spinner Flask labeled 90% Ham's F12, 10% FBS, [date], [group#], [operator initials] using a 2mL sterile pipet (day 0) and at 1-day intervals the culture will be sampled to determine the OD, pH, viable cell count, analyte levels and tPA concentration. The culture will be scaled up just before the exponential phase of the growth curve begins to slow down, indicating the cell culture is moving into the stationary phase of the growth curve. The live cell concentration should be approaching 1 million cells/ml.

8.3. **Sampling the Culture**

8.3.1. Turn on the spectrophotometer to allow the lamp to warm up for use.
8.3.2. Collect the following items:
(4) microfuge tubes labeled "tPA" and "cells" and "trypan" and "microcentrifuge counterbalance"
(1) microfuge tube holder
(2) spectrophotometers cuvettes (1 labeled "Sample" and 1 labeled "Blank")
(1) cuvette holder
(1) P1000 pipette
(1) P20 pipette
(1) pipette aid
8.3.3. Prepare biological safety cabinet per Labconco Purifier Class 2 Biological Safety Cabinet (BSC) Operation SOP.
8.3.4. Collect the following items, spray with 70% IPA and place in Biological Safety Cabinet:
(1) 15ml conical tube
(1) test tube rack
(1) cuvette
(1) cuvette rack
(1) pipette aid
(1) 5mL pipette
(1) 2mL pipettes
8.3.5. Prepare pH Meter per Oakton PC 700 Bench Series pH/ Conductivity/°C/°F Meter SOP.
8.3.6. Prepare spectrophotometer per spectrophotometer SOP using media from the BLANK, [date], [group#], [operator initials] Bellco Spinner Flask to zero the machine.

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8.3.7. Spray BLANK, [date], [group#], [operator initials] Bellco Spinner Flask and 90% Ham's F12, 10% FBS, [date], [group#], [operator initials] Bellco Spinner Flask with 70% IPA and place in biological safety cabinet.

8.3.8. Using aseptic technique, remove 3 mL from 90% Ham's F12, 10% FBS, [date], [group#], [operator initials] Bellco Spinner Flask and place into a 15ml conical tube. Using aseptic technique, remove 1 mL from BLANK, [date], [group#], [operator initials] Bellco Spinner Flask and place into a cuvette.

8.3.9. Remove all items from the biological safety cabinet.

8.3.10. Return 90% Ham's F12, 10% FBS, [date], [group#], [operator initials] Bellco Spinner Flask and BLANK, [date], [group#], [operator initials] Bellco Spinner Flask to the CO2 incubator, making sure to loosen side arm caps once in incubator.

8.3.11. Cover the blank cuvette with Parafilm.

8.3.12. Remove 1ml of sample from 15ml conical tube and place in cuvette labeled "Sample."

8.3.13. Take OD Reading at 650nm per spectrophotometer SOP.

8.3.13.1. Mix CHO sample by inverting the cuvette several times before taking reading.

8.3.13.2. After reading return to 15ml conical tube for pH measurement.

8.3.14. Remove 100µl from 15ml conical tube and place in microfuge tube labeled "cells."

8.3.15. Determine cell count using the Trypan Blue SOP.

8.3.16. Using the 2.9ml in the conical tube take the pH reading per Oakton PC 700 Bench Series pH/ Conductivity/°C/°F Meter SOP.

8.3.17. Remove 1ml of sample and place in a 1.5mL tube and centrifuge in the benchtop mini centrifuge for 5minutes being sure to counterbalance the sample microfuge tube with the "microcentrifuge counter balance" microfuge tube containing 1ml of water. Remove the supernatant and place in the microfuge tube labeled "tPA," and label with Date, Group Name, and Vessel Name. Store at 2-8°C in a microfuge tube storage box labeled with Date, Group Name, Vessel Name until needed.

8.3.18. Record all sample data in batch record.

8.4. **Scale up to 100ml of media in 90% Ham's F12, 10% FBS, [date], [group#], [operator initials] Bellco Spinner**

8.4.1. Gather the following items, spray with 70% isopropanol, and place in the biological safety cabinet:
(1) Pipette aid
(2) 10mL sterile pipettes
(4) 50mL sterile pipettes
90% Ham's F12, 10% FBS, [date], [group#], [operator initials] Bellco Spinner Flask
(1) 500mL bottle of pre-sterilized Ham's F12 Medium
(1) 50mL tube of pre-sterilized, heat inactivated fetal bovine serum (FBS)

8.4.2. Sterilely remove 74.7mL of Ham's F12 Medium from a 500mL bottle of Ham's F12 and add to 90% Ham's F12, 10% FBS, [date], [group#], [operator initials] Bellco Spinner Flask.

8.4.3. Sterilely remove and add 8.3mL of FBS to 90% Ham's F12, 10% FBS, [date], [group#], [operator initials] Bellco Spinner Flask.

8.4.4. Return 90% Ham's F12, 10% FBS, [date], [group#], [operator initials] Bellco Spinner

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Flask to the CO2 incubator, making sure to loosen side arm caps once in incubator.

8.5. **Scale up to 1L bioreactor**

Note: When the 100mL suspension culture of CHO cells reaches a concentration of approximately 1,000,000 cells/mL, the entire contents of the 100mL spinner flask will be added to the bioreactor containing 1L of CHO cell growth media.

8.5.1. Prepare 1M $NaHCO_3$ (sodium bicarbonate)

8.5.1.1. Weigh out 21 ± 1 grams of $NaHCO_3$ and transfer to an Applikon bioreactor feed bottle.

8.5.1.2. Label the bottle as 1M $NaHCO_3$, [date], [initials], [group number], storage: room temp, disposal: drain.

8.5.1.3. Using a 250mL graduated cylinder, measure 250 ± 5mL deionized water and transfer into the feed bottle.

8.5.1.4. Add a magnetic stir bar and stir on a magnetic stirrer to dissolve.

8.5.1.5. Remove the stir bar and add lid and tubing per Applikon ez-Control Bioreactor Controller Operation SOP.

8.5.2. Prepare the Applikon bioreactor and 1L addition bottle with tubing and autoclavable male connector attached for autoclaving per the Applikon ez-Control Bioreactor Controller Operation SOP including calibrating the pH probe.

8.5.3. Autoclave the Applikon bioreactor with 100ml of 1X PBS and 1L addition bottle with tubing and autoclavable male connector attached per the Applikon ez-Control Bioreactor Controller Operation SOP.

8.5.4. Remove the Applikon bioreactor vessel from the autoclave and connect the DO probe to the controller.

8.5.5. Gather the following items, spray with 70% isopropanol, and place in the biological safety cabinet:
(1) Pipette aid
(1) 10mL sterile pipette
(2) 50mL sterile pipettes
(2) 500mL bottle of pre-sterilized Ham's F12 Medium
(1) 50mL tube of pre-sterilized, heat inactivated fetal bovine serum (FBS)
(1) 10mL bottle of 10mg/mL gentamycin
(1) empty 50ml tube
1L addition bottle with tubing and autoclavable male connector attached

8.5.6. Aseptically remove 50ml from one 500mL bottle of pre-sterilized Ham's F12 Medium and place in the empty 50ml tube.

8.5.7. Aseptically add the remaining 450ml and an additional 500mL bottle of pre-sterilized Ham's F12 Medium to the 1L addition bottle with tubing and autoclavable male connector attached.

8.5.8. Aseptically add 50ml of FBS to the 1L addition bottle with tubing and autoclavable male connector attached.

8.5.9. Aseptically add the 10mL bottle of 10mg/mL gentamycin.

8.5.10. Be sure the cap is on tightly and remove the 1L addition bottle with tubing and autoclavable male connector attached and bring it over to the Applikon bioreactor.

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8.5.11. Carefully remove the foil from the female connector on the addition port of the Applikon bioreactor.
8.5.12. Carefully remove the aluminum foil from the male connector on the 1L addition bottle and connect the male connector to the female connector on the addition port of the bioreactor.
8.5.13. Remove the clamp on the female connector on the addition port of the Applikon bioreactor.
8.5.14. On the Applikon touch screen select Menu > Manual Control > Acid Pump On
8.5.15. As the pump turns feed the tubing around it. Use care to avoid pinching fingers.
8.5.16. Once all of the media has transferred into the vessel turn off the acid pump. On the Applikon touch screen select Menu > Manual Control > Acid Pump Off
8.5.17. Disconnect the male connector of the addition bottle from the female connector on the addition port of the bioreactor. Bend the tubing of the addition port and reattach the clamp.
8.5.18. Connect the remaining parts of the bioreactor to the controller.
8.5.19. Input the setpoints and limits listed in the table below per the bioreactor SOP.

Parameter	pH	Temp (^{o}C)	%DO	Stirrer (rpm)
Set point	7.2	37	50	75
Upper Limit	7.3	38	52	76
Lower Limit	7.1	36	48	74

8.5.20. Allow the DO probe to polarize for a minimum of 6 hours.
8.5.21. Calibrate the DO probe per the Applikon ez-Control Bioreactor Controller Operation SOP.
8.5.22. Immediately (Day 0) and at 1-day intervals, sample the culture to determine OD, pH, viable cell count, analytes and tPA over time (see step 8.7).

8.6. When the cell count reaches approximately 1,000,000 cells/mL shut down the bioreactor per the Applikon ez-Control Bioreactor Controller Operation and harvest cells as described below.

8.7. **Bioreactor Sampling Instructions Days 0-2**

8.7.1. Log in as operator if not already done.
8.7.2. Raise the stirrer upper limit to 150 rpm.
8.7.3. Change the stirrer setting to 125 rpm.
8.7.4. Spray the headplate near the sampling tube with 70% IPA.
8.7.5. Remove the black clamp and set on the head plate.
8.7.6. Pull out the autoclavable female connector and set it next to the black clamp.
8.7.7. Place a 50ml pipette into the sampling tube and remove 50ml of sample and place in a 50ml tube.
8.7.8. Put the female autoclavable connector back into the sampling tube.
8.7.9. Bend the sampling tubing and place the black clamp back on the tubing.
8.7.10. Change the stirrer setting to 75 rpm.
8.7.11. Change the stirrer upper limit back to 76 rpm.
8.7.12. Testing preparation
8.7.12.1. Remove 1ml for OD and return back to the 50ml tube.

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8.7.12.2. Spin the 50ml tube at 900 rpm for 5 minutes.
8.7.12.3. Remove 1 ml of supernatant and store with previous tPA samples.
8.7.12.4. Remove all of the supernatant being sure not to disturb the pellet.
8.7.12.5. Re-suspend the pellet in 1ml of excess supernatant using a 5ml pipette. Discard the additional supernatant.
8.7.12.6. Perform the trypan blue assay per SOP.

8.8. **Bioreactor Sampling Instructions Days 3-EOR**
8.8.1. Log in as operator if not already done.
8.8.2. Raise the stirrer upper limit to 150 rpm.
8.8.3. Change the stirrer setting to 125 rpm.
8.8.4. Spray the headplate near the sampling tube with 70% IPA.
8.8.5. Remove the black clamp and set on the head plate.
8.8.6. Pull out the autoclavable female connector and set it next to the black clamp.
8.8.7. Place a 5ml pipette into the sampling tube and remove 5ml of sample and place in a 15ml tube.
8.8.8. Put the female autoclavable connector back into the sampling tube.
8.8.9. Bend the sampling tubing and place the black clamp back on the tubing.
8.8.10. Change the stirrer setting to 75 rpm.
8.8.11. Change the stirrer upper limit back to 76 rpm.
8.8.12. Perform testing per SOP's.

8.9. **Determine tPA Concentration**
8.9.1. Determine the tPA concentration at each time point per Human tPA Total Antigen ELISA SOP.
8.9.2. Determine the activity of the tPA at each time point per Human tPA Activity ELISA SOP.

8.10. Determine Lactate Concentration
8.10.1. Determine the lactate concentration at each time point per the Lactate Determination Assay SOP.

8.11. Determine Glucose Concentration
8.11.1. Determine the glucose concentration at each time point per the Glucose Determination Assay SOP.

8.12. **Prepare Growth Curves**
8.12.1. Plot OD, pH, viable cells, glucose, lactate, and tPA vs. time (use 2 y-axes).
8.12.2. Attach growth curve to Batch Record.
8.12.3. Determine growth rate and doubling time of the 100mL spinner flask and 1L bioreactor cultures.
8.12.4. Attach calculations to Batch Record.

9. Attachments:
9.1. Data table

10. History:

Name	Date	Amendment
Jason McMillan	18Mar14	Initial Release
Jason McMillan	26JUN15	Extensive modification for optimization

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The following parameters are recorded in the batch record doc #....

TIME (hours)	OD 650nm	pH	LIVE Cell Count	DEAD Cell Count	Viable Cells/mL	Percent Viability	GLU mg/dL	LAC mmol/L