

Title: Batch Culture of Recombinant tPA Secreting CHO Cells SOP

Approvals:

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Reviewer: Bob O'Brien Date 04Apr08

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. 100mL Spinner Flask SOP

4.3. Biological Safety Cabinet SOP

4.4. CO₂ Incubator SOP

4.5. pH Meter SOP

4.6. spectrophotometer SOP

4.7. Biolyzer SOP

4.8. Biolyzer Pipet SOP

4.9. Trypan Blue Assay SOP

4.10. tPA ELISA SOP

4.11. tPA Activity Assay SOP

4.12. Applikon Bioreactor 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 NaHCO₃ (sodium bicarbonate)

7.8. ProCHO4 media (manufactured by Cambrex/Biowhittaker)

7.9. 200mM glutamine

7.10. 10mg/mL gentamycin

7.11. sterile 100mL Bellco spinner flasks

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- 7.12. sterile transfer pipets (50mL, 10mL, and 2mL) and pipettor
- 7.13. CO₂ incubator containing magnetic stir plate
- 7.14. UV-visible recording spectrophotometer
- 7.15. cuvettes for spectrophotometer
- 7.16. 1.5mL microfuge tubes
- 7.17. microscope with 1000x magnification
- 7.18. cryogenic vials (1mL capacity) for storage of CHO cell master/working cell bank
- 7.19. sterile 250mL glass bottles for storage of CHO cell media
- 7.20. computer and Microsoft Excel for Windows
- 7.21. 100 mL glass bottle
- 7.22. Sigma 2K15 refrigerated centrifuge
- 7.23. biolyzer
- 7.24. biolyzer pipet

8. Procedure:

8.1. 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:
 - automatic pipettor
 - 10mL sterile pipettes
 - 50mL sterile pipettes
- 8.1.3. Prepare biological safety cabinet (BSC) per SOP.
- 8.1.4. Spray the outside of the following with 70% isopropanol then place in the biological safety cabinet:
 - 100mL sterile Bellco spinner flasks
 - 500mL bottles of pre-sterilized Ham's F12 Medium
 - 100mL of pre-sterilized, heat inactivated fetal bovine serum (FBS)
- 8.1.5. Sterilely remove 90mL \pm 1mL of Ham's F12 Medium from a 500mL bottle of Ham's F12 and place in a sterile 100mL spinner flask.
 - 8.1.5.1. Repeat with a second 100mL spinner flask.
- 8.1.6. Sterilely add 10mL \pm 1 of FBS to the Ham's F12 bottle.
 - 8.1.6.1. Repeat with the second 100mL spinner flask.
- 8.1.7. Label all spinner flasks as 90% Ham's F12, 10% FBS, [date], [group#], [operator initials].
- 8.1.8. Place all spinner flasks containing CHO cell media in the CO₂ incubator. Set the speed of the magnetic stirrer to the maximum setting that ensures an even mixing of the culture without foaming.
 - 8.1.8.1. Verify that the temperature is $37 \pm 0.5^{\circ}\text{C}$ and percentage of CO₂ is $5 \pm 0.5\%$.
- 8.1.9. Check media for contamination after a minimum of 24 hours.
- 8.1.10. Store media in refrigerator.

8.2. Inoculation

- 8.2.1. Pre-warm the spinner flasks containing CHO cell culture medium at $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$.

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- 8.2.2. Spray a minimum of four 2mL sterile pipettes with 70% isopropanol and place in the BSC.
- 8.2.3. Prepare Biological Safety Cabinet per BSC SOP.
- 8.2.4. Remove 2 vials of CHO cells from storage in the -86°C freezer.
- 8.2.5. Thaw contents rapidly by agitation in a 37°C ± 0.5°C water bath (Belly Dancer).
- 8.2.6. Spray vials with 70% isopropanol, and place in the biological safety cabinet.
- 8.2.7. Sterilely transfer the entire contents of each 1mL vial of thawed CHO Cells into each of the previously prepared Bellco Spinner Flask containing 100mL CHO Cell Culture Medium using a 2mL sterile pipet.
- 8.2.8. Swirl to mix.
- 8.2.9. Immediately after adding CHO Cells to Bellco spinner flask (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. Collect the following items, spray with 70% IPA and place in Biological Safety Cabinet:
 - 2 microfuge tubes labeled “tPA, Tn” and “cells”
 - 1 microfuge tube holder
 - 3 spectrophotometers cuvettes
 - 1 cuvette holder
 - 4 2mL pipets
 - pipet pump
 - 8.3.2. Prepare biological safety cabinet per BSC SOP.
 - 8.3.3. Prepare pH Meter per pH Meter SOP.
 - 8.3.4. Prepare biolyzer and biolyzer pipet per biolyzer and biolyzer pipet SOPs.
 - 8.3.4.1. Remove lactate and glucose Biolyzer slides from the -20° C freezer.
 - 8.3.5. Prepare spectrophotometer per spectrophotometer SOP using media to zero the machine.
 - 8.3.6. Spray blank and culture spinner bottle with 70% IPA and place in biological safety cabinet.
 - 8.3.7. Using aseptic technique, remove 2 – 2.2mL sample of culture and place into a cuvette.
Note: Do not mix blank and sample cuvettes.
 - 8.3.8. Remove all items from the biological safety cabinet.
 - 8.3.9. Return suspension culture and blank to the CO₂ incubator, making sure to loosen caps once in incubator. Set the speed of the magnetic stirrer to the maximum setting that ensures an even vortexing of the culture without foaming.
 - 8.3.10. Cover the sample cuvette with parafilm.
 - 8.3.11. Take OD Reading at 650nm per spectrophotometer SOP.
 - 8.3.11.1. Mix CHO sample by inverting the cuvette several times before taking reading.
 - 8.3.12. Take readings for glucose and lactate using the Biolyzer per the Biolyzer SOP.
 - 8.3.13. Determine cell count using the Trypan Blue SOP.

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- 8.3.14. Take pH reading per pH meter SOP.
8.3.14.1. Transfer the sample to a test tube to measure the pH per the pH meter SOP.
8.3.15. Remove the sample to a 1.5mL tube and centrifuge at high speed for 5minutes.
Remove the supernatant and store at 2-8°C until needed.

8.4. Scale up to 1L bioreactor

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

- 8.4.1. Prepare 1M NaHCO₃ (sodium bicarbonate)
8.4.1.1. Weigh out 21 ± 1 grams of NaHCO₃ and transfer to an Applikon bioreactor feed bottle.
8.4.1.2. Label the bottle as 1M NaHCO₃, [date], [initials], [group number], storage: room temp, disposal: drain.
8.4.1.3. Using a 250mL graduated cylinder, measure 250 ± 5mL deionized water and transfer into the feed bottle.
8.4.1.4. Add a magnetic stir bar and stir on a magnetic stirrer to dissolve.
8.4.2. Prepare 1X PBS.
8.4.2.1. Using a 10mL pipet, measure 10 ± 0.5mL of 10x PBS and dispense into a 100mL vessel.
8.4.2.2. Using a 100mL graduated cylinder, measure 90 ± 5mL of deionized water and transfer into the 100mL vessel. Swirl to mix.
8.4.2.3. Label vessel as 1X PBS, [date], [initials], [group number], storage: room temp, disposal: drain.
8.4.3. Set up Applikon bioreactors per the bioreactor SOP including calibrating the pH probe.
8.4.4. Autoclave the bioreactors with 1X PBS per the bioreactor SOP for 20 minutes.
8.4.5. Remove the bioreactor vessel from the autoclave and connect the DO probe to the controller.
8.4.6. Turn on the controller.
8.4.7. Allow the DO probe to polarize for a minimum of 6 hours.
8.4.8. In the biological safety cabinet prepare the media.
8.4.8.1. Add ~6mL of 200mM glutamine (1.2mM final concentration) and 10mL of 10mg/mL gentamycin (0.1mg/mL final concentration) to the 1L bottle of ProCHO4 media.
8.4.9. Aseptically pour the media into the bioreactor through the inoculation port using a sterile funnel.
8.4.10. Connect the remaining parts of the bioreactor to the controller.
8.4.11. Input the setpoints and limits listed in the table below per the bioreactor SOP.

| Parameter | pH | Temp (°C) | %DO | Stirrer (rpm) |
|-------------|-----|-----------|-----|---------------|
| Setpoint | 7.2 | 37 | 50 | 75 |
| Upper Limit | 7.3 | 38 | 52 | 76 |
| Lower Limit | 7.1 | 36 | 48 | 74 |

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- 8.4.12. When the 100mL suspension culture of CHO cells reaches a concentration of approximately 1,000,000 cells/mL, aseptically transfer the culture to the bioreactor.
- 8.4.13. Start BioXpert Lite per bioreactor SOP.
- 8.4.14. Immediately and at 2-day intervals, sample the culture to determine OD, pH, viable cell count, analytes and tPA over time (see step 8.3).
- 8.5. When the cell count reaches approximately 1,000,000 cells/mL shut down the bioreactor per the bioreactor SOP and harvest cells as described below.
- 8.6. **Harvest and Preparation of Working Cell Bank**
 - 8.6.1. Gather the following, spray with 70% IPA and place in the BSC:
 - 30mL sterile centrifuge tubes (40)
 - 25mL sterile pipets (10)
 - 2mL sterile pipets (20)
 - cryovials (55)
 - 250mL sterile glass bottles (4)
 - 8.6.2. Prepare the biological safety cabinet per SOP.
 - 8.6.3. Using a 25mL sterile pipet, transfer 25mL of the culture into sterile 30mL centrifuge tubes.
 - 8.6.4. Centrifuge tubes for 10min. at 3000xg per centrifuge SOP.
Note: Always balance the test tubes in the centrifuge.
 - 8.6.5. Prepare storage menstrem
 - 8.6.5.1. Combine the following item into a container capable of holding >50mL and mix well.
 - 40mL± 1.0mL of Ham's F12
 - 5mL± 0.5mL of FBS
 - 5mL± 0.5mL of glycerol
 - 8.6.5.2. Filter sterilize.
 - 8.6.5.3. Label bottle as CHO storage menstrem with the date.
 - 8.6.5.4. Spray with 70% IPA and place in the biological safety cabinet.
 - 8.6.5.5. Remove filter unit and place cap on bottle.
 - 8.6.6. Following centrifugation of the culture, decant tPA containing medium into sterile 250mL bottles.
 - 8.6.7. Label bottles as unpurified tPA in ProCHO4 [date} and [group #].
 - 8.6.8. Store in the refrigerator at 2-8°C.
 - 8.6.9. Add about 1mL of storage menstrem to each centrifuge tube to resuspend the pelleted CHO cells.
 - 8.6.10. Sterilely dispense 1mL± 0.1mL aliquots into sterile 1.5mL cryovials.
50 cryovials for the working cell bank are expected.
 - 8.6.11. Label in the following manner using a cryopen: CHO (ATCC CRL-9606) BT220-[day or evening], DATE.
 - 8.6.12. Place in a styrofoam tube rack. Label container same as cryovials.
 - 8.6.13. Store at -85°C.
- 8.7. **Determine tPA Concentration**
 - 8.7.1. Determine the tPA concentration at each time point per tPA ELISA SOP.

