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1. PURPOSE

1.1. The purpose of this procedure is to describe the maintenance of HEK293TT (293TT) cells.

2. SCOPE

2.1. This procedure applies to the Vaccine, Immunity and Cancer Directorate.

2.2. This procedure describes general maintenance and culturing techniques for HEK293TT cells that are used in other procedures for the production of HPV L1 or L1/L2 virus-like particles (VLPs), HPV L1/L2 pseudovirions (PsV), and for cell-based assays.

3. REFERENCES

3.1. 10010: Lot Number and Test Run Number Assignment

3.2. 10009: General Record Review

3.3. 10023: Good Documentation Practices

3.4. 15000: Waste Disposal at the Advanced Technology Research Facility

3.5. 15006: Reagent Preparation for the HPV Serology Laboratory

3.6. 15008: Biosafety Manual and Laboratory Standard Operating Procedures

3.7. 26000: Biosafety Cabinet (BSC) Use and Maintenance

3.8. 26001: Operation, Use and Maintenance of C02 Incubators

3.9. 26004: Use and Maintenance of the Cellometer Auto 2000

3.10. 26005: Use and Maintenance of a 2-8°C Refrigerator

3.11. 26006: Use and Maintenance of the Liquid Nitrogen Freezer

3.12. 26007: Use and Maintenance of the Fisher Scientific Isotemp GDP10 Water Bath

3.13. 26009: Use and Maintenance of Pipettes

3.14. 26015: Use and Maintenance of an Inverted Microscope

3.15. 26019: Controlled-Rate Cell Freezing Using a CoolCell Device

3.16. 26030: Use and Maintenance of -80°C Freezers

3.17. 26033: Use and Maintenance of the Thermo Scientific Sorvall XTR Centrifuge

4. RESPONSIBILITIES

4.1. The Research Associate, hereafter referred to as Analyst, is responsible for reviewing and following this procedure.

4.2. The Scientific Manager or designee is responsible for training personnel in this procedure and reviewing associated documentation.

4.3. The Quality Assurance Specialist is responsible for quality oversight and approval of this procedure.

4.4. Trained personnel perform assay record review per “10009: General Record Review.”

5. DEFINITIONS

Term	Definition
DMEM	Dulbecco’s Modified Eagle’s Medium
DPBS	Dulbecco’s Phosphate Buffered Saline
FBS	Heat-inactivated Fetal Bovine Serum
SDS	Safety Data Sheets
293TT	HEK293TT cells
293TT TM	Thawing Media
293TT MM	Maintenance Media
293TT FM	Freezing Media

6. REAGENTS, MATERIALS AND EQUIPMENT

6.1. Reagents

6.1.1. Bleach, Concentrated (FNLCR Warehouse, Cat # 68100251 or equivalent)

6.1.2. DMEM with: High Glucose, L-glutamine, Phenol Red; without: Sodium Pyruvate, HEPES (Life Technologies, Cat # 11965-126)

6.1.3. 1X Dulbecco's Phosphate-Buffered Saline (DPBS), Sterile (Life Technologies, Cat # 14190-136 or equivalent)

6.1.4. Fetal Bovine Serum (FBS), Heat-Inactivated (Hyclone, Cat # SH30070.03HI or equivalent)

6.1.5. Glutamax-I (Life Technologies, Cat # 35050-061)

6.1.6. HEK293TT Cells (stored in liquid nitrogen (LN_2)

6.1.7. Hygromycin B 50 mg/mL (Life Technologies, Cat # 10687-010)

6.1.8. MEM Nonessential Amino Acids (Life Technologies, Cat # 11140-050)

6.1.9. Primary Disinfectant (Cavicide, FNLCR Warehouse, Cat # 79300360 or equivalent)

6.1.10. Ster-ahol (VWR, Cat # 14003-358 or equivalent)

6.1.11. Trypan Blue 0.4% (Life Technologies, Cat # 15250-061)

6.1.12. Trypsin-EDTA (0.05%), Phenol Red (Life Technologies, Cat # 25300-054)

6.1.13. ViaStain AOPI Staining Solution (Nexcelom, Cat # CS1-0106-5mL)

6.2. Consumables

6.2.1. Absorbant Wipe, Wypalls Low-Lint Paper Towels (FNLCR Warehouse, Cat # 79300335 or equivalent)

6.2.2. Biohazard Benchtop Keeper (VWR, Cat # 11214-711 or equivalent)

6.2.3. 150 mL Bottle, Media Storage (Thomas Scientific, Cat # 8600B13 or equivalent)

6.2.4. 250 mL Bottle, Media Storage (Thomas Scientific, Cat #1743D05 or equivalent)

6.2.5. 500 mL Bottle, Media Storage (Thomas Scientific, Cat # 1743D10 or equivalent)

6.2.6. 1 L Bottle, Media Storage (Thomas Scientific, Cat # 1743D15 or equivalent)

6.2.7. 2 L Bottle, Media Storage - Corning Polystyrene Roller Bottle (VWR, Cat # 89184-640 or equivalent)

6.2.8. 250 mL Filter Bottle, 0.2 µm PES Membrane - Nalgene (Thomas Scientific, Cat # 1234K60 or equivalent)

6.2.9. 500 mL Filter Bottle, 0.2 µm PES Membrane - Nalgene (Thomas Scientific, Cat # 1234K58 or equivalent)

6.2.10. 1000 mL Filter Bottle, 0.2 µm PES Membrane - Nalgene (Thomas Scientific, Cat # 1234K59 or equivalent)

6.2.11. T-150 Flask (Thomas Scientific, Cat # 9381J33 or equivalent)

6.2.12. T-225 Flask (Thomas Scientific, Cat # 9381M60 or equivalent)

6.2.13. 5-layer Flask (VWR, Cat # 89204-478 or equivalent)

6.2.14. 8-layer Flask CELLdisk (Greiner Bio-one, Cat # 678108 or equivalent)

6.2.15. Hemocytometer, Disposable (Nexcelom, Cat # CP2-002)

6.2.16. Ice Pan (Thomas Scientific, Cat # 1200R42 or equivalent)

6.2.17. Laboratory Marker, Alcohol-Resistant (FNLCR Warehouse, Cat # 66400058)

6.2.18. Pipette Tips (Rainin)

6.2.19. 5 mL Serological Pipets (FNLCR Warehouse, Cat # 66401365 or equivalent)

6.2.20. 10 mL Serological Pipets (FNLCR Warehouse, Cat # 66401370 or equivalent)

6.2.21. 25 mL Serological Pipets (FNLCR Warehouse, Cat # 66401361 or equivalent)

6.2.22. 50 mL Serological Pipets (FNLCR Warehouse, Cat # 66401363 or equivalent)

6.2.23. 100 mL Serological Pipets (Thomas Scientific, Cat #7536R26 or equivalent)

6.2.24. 50 mL Tubes, Conical (FNLCR Warehouse, Cat # 66401493 or equivalent)

6.2.25. 1.5 mL Tube, Polypropylene (Cryotube) (VWR, Cat # 87003-296 or equivalent)

6.2.26. Protection Sleeves, Particle (Fisher Scientific, Cat # 17-981-41D or equivalent)

6.2.27. Terminal Pipet Keeper (VWR, Cat #11214-715 or equivalent)

6.2.28. Wet ice

6.3. Equipment

6.3.1. Automated Cell Counter (Nexcelom Cellometer Auto 2000 or equivalent)

6.3.2. Centrifuge (Benchtop)

6.3.3. Class II Biosafety Cabinet (BSC)

6.3.4. CO_2 Incubator

6.3.5. CoolCell Devices (VWR, Cat # 75779-724 or equivalent)

6.3.6. Hemocytometer (Improved Neubauer 0.1mm deep)

6.3.7. Inverted Light Microscope (Nikkon TMS, Nikkon LBOPHOT or equivalent)

6.3.8. Freezers (-20°C, -80°C, LN_2)

6.3.9. Pipettes (Rainin)

6.3.10. Serologic Pipettor

6.3.11. Timer

6.3.12. Waste Container, White, HDPE Plastic, with Screw Top Lid (Amphorea, Cat # 250MLPHARMA or equivalent)

6.3.13. Water Bath

7. HEALTH AND SAFETY CONSIDERATIONS

7.1. Proper safety precautions must be taken while working in a laboratory setting. This includes, but is not limited to, proper protective equipment such as lab coats, safety glasses, closed-toe shoes, and non-latex gloves.

7.2. Refer to the respective Safety Data Sheet (SDS) when working with any chemicals.

7.3. Refer to “15000: Waste Disposal at the Advanced Technology Research Facility,” for waste disposal procedures at the respective location.

7.4. All work is performed inside a Class II Biosafety Cabinet (BSC).

7.5. All surfaces are decontaminated with Cavicide or equivalent disinfectant following manufacturer instructions before and after each experiment, and then may be followed with Ster-ahol.

7.6. All contaminated BSL-2 level liquid waste must be decontaminated using 10% Clorox bleach (final concentration) with a minimum contact time of 30 minutes in a waste container before sink disposal.

8. PROCEDURE PRINCIPLES

8.1. The cell culture lot number is assigned per “10010: Lot Number and Test Run Number Assignment.”

8.2. Analyst may wear protective sleeves over lab coat when cell culturing.

8.3. When working with large numbers of 293TT flasks, 5 or more, it is suggested that the flasks be split up into batches while processing for ease of handling and to minimize time flasks are removed from the CO_2 incubator (37±2°C, 5±2% CO_2).

8.4. Before working with 293TT cells prepare a waste container with bleach and place it in the BSC. The final volume of waste may be estimated and the amount of bleach necessary

to achieve a final 10% solution can be added. Additional bleach may be added after procedure if necessary. Refer to 15000.

8.5. Acceleration and Deceleration using centrifuges

8.5.1. When centrifuging conical tubes using the Sorvall XTR centrifuge per 26033, you can use maximum ramp up/acceleration speed and break speed of 6 or higher.

8.6. If no expiration date is listed on ViaStain AOPI Solution bottle, manufacturer validates product up to one year of receipt.

9. REAGENT PREPARATION

9.1. All cell culture media store at 2-8°C should come to room temperature prior to use.

9.2. Thawing Media (293TT TM)

9.2.1. Refer to “15006: Reagent Preparation,” Section 17.

9.3. Maintenance Media (293TT MM)

9.3.1. Refer to 15006, Section 18.

9.4. Freezing Media (293TT FM)

9.4.1. Refer to 15006, Section 19.

10. PREPARING A NEW CELL LINE FROM FROZEN CELLS

10.1. In BSC, prepare a 150 cm^2 (T-150) flask with 30 mL of Thawing Media (293TT TM) and store at room temperature. Label the flask with the cell culture lot number. Refer to Attachment 3: 293TT Cell Flask Label and Cryovial Label.

10.2. Remove one vial of 293TT cells from the liquid nitrogen freezer, and store on dry ice if not immediately processed at step 10.3.

Note: Update LN_2 vial inventory to maintain cell line vial tracking and select “Freezer Inventory Updated” on “20001-02: 293TT Cell Thaw Form.”

Note: Once the thawing process has begun, the cells should be processed immediately to maximize recovery.

10.3. Immerse frozen vial in a 37±2°C water bath being careful to not immerse the cap of the tube, since water may inadvertently enter tube.

Note: Do not leave the vial in water bath by placing it in a floating foam tube rack.

10.4. Swirl tube in water and continually inspect the tube to verify how much the cells have thawed.

10.5. Remove the tube from the water bath once cells are mostly thawed and only a small frozen pellet remains.

10.6. Dry off tube with paper towel. Spray a piece of paper towel with Ster-ahol and use it to wipe the tube. Dry tube with paper towel.

10.7. Place the thawed vial of cells inside the BSC.

10.8. Invert tube to mix then assess viability per “26004: Use and Maintenance of the Cellometer Auto 2000” or “Attachment 1: Counting Cells with a Hemocytometer” for manual counting instructions.

10.9. Record unrounded cell counts and viability of the thawed cells on 20001-02. Continue with culturing the cells if the viability is ≥60%; otherwise, a new vial of cells needs to be thawed.

Note: Record calculated average cell counts to the nearest tenth.

10.10. Pipette all the thawed cells directly into the Thawing Media in Flask from step 10.1. Gently mix cells to evenly cover flask.

10.11. Incubate flask(s) in a CO_2 incubator (37±2°C, 5±2% CO_2). This will be “Thaw” or “Passage 0.”

10.12. After 72 hours, inspect cell culture for adherence by using a microscope per “26015: Use and Maintenance of an Inverted Microscope.”

10.13. Once the cells are adherent and confluency reaches 70%-95% (~every 3-4 days), split cells per Section 11. This will be “Passage 1” and will begin the cell passage count.

10.14. Record maintenance on “20001.03: 293TT Cell Culture Maintenance Form.”

11. MAINTAINING CELL CULTURE

Note: 293TT cells should be split when confluency reaches 70%-95% (approximately every 3-4 days) after adherence and should be maintained for use no more than 30 passages. Any deviations require approval by the Scientific Manager.

11.1. Inspect the confluency of the 293TT cells by using a microscope per 26015. Once the cells are adherent and confluency reaches 70%-95% (~every 3-4 days), split cells.

11.2. Place flask in BSC.

11.3. Discard media from the flask.

11.3.1. Place the flask upright and tilted so media collects into a corner which does not contain cells to minimize loss of cells.

11.3.2. Remove media in flask using a sterile serological pipet or by decanting into waste container.

11.3.3. For the multi-layer flasks, decant the media into waste container.

11.4. Using a sterile serological pipet, add DPBS per Table 1. Do not dispense DPBS directly onto cells, they will become detached.

Table 1: Volume of DPBS to add to each flask

Flask Type	DPBS (mL)
T150 and T225	5 - 15
5-Layer	20 - 30
8-Layer CELLdisk	40 - 60

11.5. Gently rinse cells with DPBS by slowly rotating the flask so that DPBS washes over the cells.

11.6. Discard DPBS into waste container.

11.6.1. Tilt flask so the DPBS collects into a corner of the flask where there are no cells adhering to flask surface.

11.6.2. Remove the media in flask using a sterile serological pipet or by decanting into waste container.

11.6.3. For the multi-layer flasks, decant into a waste container.

11.7. Wash with DPBS one additional time per steps 11.4 to 11.6 if needed.

11.8. Add trypsin to each flask per Table 2. Gently rotate flasks to distribute trypsin evenly over cells and incubate for 3-5 minutes in the CO_2 incubator (37±2°C, 5±2% CO_2). Use a timer during this step to minimize time cells are exposed to trypsin, since prolonged exposure is toxic to cells.

Note: Do not let trypsin sit on the cells longer than 25 minutes.

Table 2: Volume of Trypsin-EDTA to add to each flask

Flask Type	Trypsin-EDTA (mL)
T150 and T225	3 - 5
5-Layer	12 - 20
8-Layer CELLdisk	27 - 45

11.9. After incubation, take flasks out of incubator and gently rock trypsin back and forth over the cell attachment surface to detach cells completely. Continue rocking flasks until all cells have detached; you can verify detachment by using a microscope per 26015.

Note: Some gentle taps against the side of the flasks may be helpful for cell detachment.

11.10. Place flasks into BSC. Using a sterile serological pipet, add 293TT MM per Table 3 and gently rinse the cell attachment surface with the media to neutralize trypsin.

Table 3: Volume of 293TT MM to add to each flask

Flask Type	293TT MM (mL)
T150 and T225	10
5-Layer	40
8-Layer CELLdisk	90

11.11. Using a sterile serological pipet, remove media and cells from all flasks and pool into an appropriately sized bottle or conical tube. For multi-layer flasks, decant the cells into bottle or conical tube.

11.12. Repeat step 11.4 and 11.11, if necessary, to remove adherent cells. If cells remain in flask, but are not adherent, the flask can be rinsed with a minimum volume of 293TT MM.

11.13. Ensure cap on bottle or conical tube is secured. Gently mix cells by inverting several times. If large clumps of cells remain at the bottle of the bottle, they can be disassociated using a serological pipette.

11.14. Count cells per 26004 or Attachment 1.

11.15. Capture Cell Counts and Viability on 20001-03; refer to Attachment 2.

11.15.1. Viability for each Cell Count must be ≥ 80% for Cell Count to be used for Cell Concentration. If the Viability fails, the Cell Count is not used to calculate Cell Concentration. Repeat steps 11.14 and 11.15 as needed; refer to Attachment 2.

11.15.2. The Percent Difference of Cell Concentration 1 and Cell Concentration 2 counts need to be ≤ 25% for the counts to be considered valid. If the results are not ≤ 25%, repeat steps 11.14 and 11.15 as needed; refer to Attachment 2.

11.15.2.1. To calculate Percent Difference:

C1 = Unrounded Cell Count 1
C2 = Unrounded Cell Count 2
Average Count = (C1+C2) ÷ 2

% Difference = $\frac{CC1-CC2}{\text{AAAAAAAA}\ \ \text{CCCCCCCCCCCCC}} \times 100\%$

Note: Record Average Cell Counts to the nearest tenth.

Note: Record Percent Difference as a whole number.

11.16. Generating daughter flasks

11.16.1. Inoculate daughter flasks per Table 4 by adding cells to flask containing medium.

Table 4: Recommended Flask Inoculation (Expected Growth Rates)

Flask Type	3 Day Incubation (million cells)	4 Day Incubation (million cells)	Total Volume (mL)
T150	9 (± 2)	4 (+ 2)	25-35
T175	10 (± 2)	4 (+ 2)	35-40
T182	10 (± 2)	4 (+ 2)	35-40
T225	10 (± 2)	4.5 (+ 2.5)	45-55
5-Layer	40 (± 5)	18 (+ 7)	180-220
8-Layer CELLdisk	90 (± 10)	40 (+ 20)	420-480

Note: If culture is growing slower than expected, or if advised by Scientific Manager, inoculate daughter flasks per Table 5.

Table 5: Recommended Flask Inoculation (Slower than Expected Growth Rates)

Flask Type	3 Day Incubation (million cells)	4 Day Incubation (million cells)	Total Volume (mL)
T150	12 –18	6 – 9	25-35
T175	13 – 19	7 – 10	35-40
T182	13 – 19	7 – 10	35-40
T225	14 – 20	7 – 10	45-55
5-Layer	50 – 100	25 – 50	180-220
8-Layer CELLdisk	120 – 200	60 – 100	420-480

11.16.2. To determine volume of cells to inoculate per flask, use the following calculation:

(Desired inoculation cell number [cells/flask] from Table 4 ÷ Cell Concentration [cells/mL] from 11.15) = volume of cells to be added to flask

For example, if inoculating a T150 for 4 days of growth at an Expected Growth Rate: $4x10^6$ cells/flask (final concentration) ÷ $1x10^6$ cells/mL (calculated Cell Concentration) = 4 mL of cells per flask.

11.16.3. To determine amount of media required per flask, subtract the volume of cells added to each flask from the total volume of cell culture media to be added to each flask.

For example, if seeding a T150, subtract the 4 mL of cells from 30 mL total volume to get 26 mL media.

11.17. Label flasks with lot number, passage number, cell concentration, current date, and analyst initials. The first split after thaw is considered P1. See Attachment 3 for flask label.

11.18. Incubate flasks in a CO_2 incubator (37±2°C, 5±2% CO_2)for 3-4 days until 70-95% confluent. Do not overgrow.

11.19. Record generated flasks for cell maintenance on 20001-03.

11.20. Repeat steps 11.1 to 11.19 to culture cells to desired passage. Proceed to section 12 if freezing cells, or discard culture per 15000.

12. FREEZING OF 293TT CELLS

Note: In order to generate a new lot of frozen 293TT cells, the cells should be frozen from passage 2 and up to passage 4. The use of higher passage numbers may introduce more mutations into the cell line and lead to poorer performance in other assays such as VLP production and neutralization assays. Any deviations require approval by the Scientific Manager.

Note: It is important to chill all components needed to freeze down cells prior to starting.

12.1. Capture cell counts and viability on 20001-03: 293TT Cell Culture Maintenance Form see attachment 6, and Cell Culture Freeze Information on 20001-04: 293TT Cell Culture Freezing Form, see Attachment 7.

12.2. Chill a rack with labeled cryotubes to 2-8ºC. See Attachment 3 for vial label.

12.3. Chill the Freezing Media (293TT FM) on wet ice inside the BSC.

12.4. Chill the CoolCell device to 2-8ºC.

12.5. Chill the Centrifuge to 2-8°C per 26033.

12.6. Remove the flasks from the CO_2 incubator (37±2°C, 5±2% CO_2) and place into BSC.

12.7. Inspect cell culture for confluency by using a microscope per 26015.

Note: Only utilize 293TT cells which have grown to 70-95% confluency.

12.8. Collect Conditioned Media from flasks.

12.8.1. Place the flask upright and tilt it so that media collects into a corner of the side of the flask which does not contain cells to minimize loss of cells.

12.8.2. Using a sterile serological pipet, or by decanting, pool the Conditioned Media into an appropriately sized conical container (e.g., 50 mL or 500 mL conical tube).

12.9. Using a sterile serological pipet, add volume of DPBS per Table 1 to flask containing cells. Do not dispense DPBS directly onto cells.

12.10. Gently rinse cells with DPBS by slowly rotating the flask, so that DPBS washes over the cells.

12.11. Discard DPBS into waste container.

12.11.1. Tilt flask so the DPBS collects into a corner of the flask where there are no cells adhering to flask surface.

12.11.2. Using a sterile serological pipet, or by decanting, discard DPBS into waste container.

12.12. Wash flasks one additional time per steps 12.9 to 12.11 if needed.

12.13. Add volume of trypsin per Table 2. Gently rotate flasks to distribute trypsin evenly over cells and incubate for 3-5 minutes in the CO_2 incubator (37±2°C, 5±2% CO_2). Use a timer during this step to minimize time cells are exposed to trypsin, since prolonged exposure is toxic to cells.

Note: Optimal incubation temperature for trypsin is 37±2°C. Analyst writes comment on 20001-04 if another incubation temperature used (e.g., Room Temperature).

Note: Do not let trypsin sit on the cells longer than 25 minutes.

12.14. After incubation, take flasks out of incubator and gently rock trypsin back and forth over the cell attachment surface to detach cells completely. Continue rocking flasks until all cells have detached; can verify detachment by using a microscope per 26015.

Note: Some gentle taps against the side of the flasks may be helpful for cell detachment.

12.15. Using a sterile serological pipet, add 293TT MM to each flask as defined in Table 3 and gently rinse the cell attachment surface with media to neutralize trypsin.

12.16. Process Conditioned Media from step 12.8.

12.16.1. Centrifuge the Conditioned Media at 300±10 x g, 2-8°C, 5 minutes per 26033.

12.16.2. Avoiding the cell debris at the bottom of the tube, transfer the Conditioned Media to a new 50 mL conical tube, labeled "**Conditioned Media**", and dispose of the original tube with cell debris.

12.16.3. Store Conditioned Media on wet ice.

12.17. Using a sterile serological pipet or decant 293TT MM and cells from flasks in step 12.15 and transfer into a new 50 mL conical tube. Ensure cap on conical tube is secured. Gently mix cells by inverting several times.

12.18. Count cells per 26004 or Attachment 1.

12.19. Capture cell counts and viability on 20001-03: 293TT Cell Culture Maintenance Form, see attachment 6.

12.19.1. Viability for each Unrounded Cell Count must be ≥ 80% for Cell Count to be used for Cell Concentration. If the Viability fails, the Cell Count is not used to calculate Cell Concentration. Repeat steps 12.18 and 12.19 as needed; refer to Attachment 2.

12.19.2. The Percent Difference of Cell Concentration 1 and Cell Concentration 2 counts need to be ≤ 25% for the counts to be considered valid. See step 11.14.2.1 for Percent Difference calculation. If the results are not ≤ 25%, repeat steps 12.18 and 12.19 as needed; refer to Attachment 2.

Note: Record Average Cell Counts to the nearest tenth.

Note: Record Percent Difference as a whole number.

Note: Record initial volume of cell suspension for calculations in 12.22 on 20001-04: 293TT Cell Culture Freezing Form.

12.20. Centrifuge 293TT cells from step 12.19 at 300±10 x g, 2-8°C, 5 minutes per 26033.

12.21. Decant the supernatant in waste container and suspend cells at $10x10^6$ cells/mL using Conditioned Media harvested in step 12.16.3.

12.21.1.1. To calculate Conditioned Media Volume

If Average Cell Count = 4 x 10^6 (cells/mL)
Determine initial volume of cell suspension before centrifugation.
Initial Volume of cells = 30 (mL)

Multiply Average Cell Count by Initial Volume of cells.

4 x 10^6 (cells/mL) x 30 (mL) = 120 x 10^6 (cells) total

Divide total number of cells counted by desired concentration of cells needed for freezing to calculate mL of conditioned media needed.

120 x 10^6 (cell) / 10 x 10^6 (cells/mL) = 12 (mL) of conditioned media needed.

12.22. Keep conical tube with cells on wet ice inside BSC.

12.23. Add equal volume of chilled 293TT FM, making a 1:2 ratio (for example, 10 mL of 293TT cells + 10 mL of 293TT FM), for a final concentration of 5x10^6 cells/mL.

12.24. Aliquot 1.0 mL culture into each chilled cryotube.

12.25. For controlled-rate freezing, follow “26019: Controlled-Rate Cell Freezing Using a CoolCell Device.”

12.26. After the control-rate freezing procedure, transfer tubes into designated box and store in LN_2 freezer. See Attachment 4 for box label and label placement.

12.27. Record final aliquot storage location on 20001-04 and update LN_2 freezer inventory file.

13. ATTACHMENTS

13.1. Attachment 1: Counting Cells with a Hemocytometer

13.2. Attachment 2: Process Steps For % Viability

13.3. Attachment 3: 293TT Cell Flask Label and Cryovial Label

13.4. Attachment 4: Box Label Example

13.5. Attachment 5: 20001-02: 293TT Cell Thaw Form

13.6. Attachment 6: 20001-03: 293TT Cell Culture Maintenance Form

13.7. Attachment 7: 20001-04: 293TT Cell Culture Freezing Form

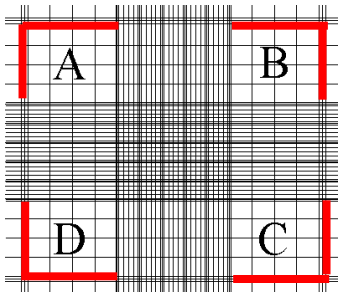
14. REVISION HISTORY

Version	Change	Reason
6.0	1. 1.1 added abbreviation 293TT 2. 2.1 removed ATRF location from scope 3. 3.1 new SOP numbers 4. 4.4 added 10009 reference 5. Include Definitions in section 5 6. Include more reagents in section 6 7. Include equivalent disinfectant in 7.5 8. Added 8.2 to include protective sleeves 9. Added 8.3 to process flasks in batches 10. Added 8.4 to prepare 10% bleach 11. Added 8.5 for centrifuge settings 12. Added wording “you can” in 8.5.1 13. Added section 9.1 14. Updated steps referenced in 11.7 15. Updated 11.13 flasks can be rinsed 16. Updated step referenced in 11.21 17. Added note for passage number 18. Added 12.1 for documentation references 19. Added 12.7 to inspect cells 20. Changed 12.8 from remove to collect 21. 12.9 Clarify where DPBS is added 22. 12.10 remove redundant wording 23. 12.12 added if needed 24. 12.12 updated reference steps 25. 12.13 update reference HSL_LAB_001.04 26. 12.16 update reference 27. Deleted 12.16 to bring volume up to 50mL 28. 12.16.3 Add step for media storage 29. 12.17 add decant media 30. Added 12.20.2 Record cell volume 31. 12.21 Added example calculation 32. 12.22 change chill to keep 33. 13.2 move Table to Attachment	1. Clarification 2. Clarification 3. Updated SOP numbers 4. Reflect current practices 5. Clarification. 6. Reflect current practices 7. Reflect current practices 8. Reflect current practices 9. Reflect current practices 10. Reflect current practices 11. Reflect current practices 12. Clarification 13. Clarification 14. Clarification 15. Reflect current practices 16. Clarification 17. Clarification 18. Ease of use 19. Reflect current practices 20. Clarification 21. Clarification 22. Clarification 23. Consistency between procedures 24. Clarification 25. Clarification 26. Clarification 27. Reflect current practices 28. Clarification 29. Clarification 30. Reflect current practices 31. Clarification 32. Clarification 33. Clarification

Version	Change	Reason
7.0	1. Version history for revisions 1-5.1 are linked to the prior numbering scheme for this SOP. 2. SOP was renumbered from HSL_LAB_001. 3. Form number 20001-01 was inadvertently skipped. 4. Add guidance for passage number for freezing cells 5. Updated guidance for rounding	1. Explain the gap in version history prior to last revision. 2. Reflect SOP numbering requirements per 10000. 3. Author oversight. 4. Clarification for addressing freezing cells. 5. Clarification of using unrounded values.

Attachment 1: Counting Cells with a Hemocytometer

- Count cells with a hemocytometer using trypan blue. Use equal volumes of trypan blue and cells.
- Record the number of live cells (trypan blue negative) and dead cells (trypan blue positive) cells in a QA Issued Laboratory Notebook.
- Refer to Notebook Number and Page Number on assay form and/or logbook forms where counts are used. Analyst is responsible for having notebook entry reviewed by Scientific Manager.
- To count cells, add 10 µL of trypan blue/cell mixture to hemocytometer.
- Count cells in quadrants A, B, C, and D (refer to diagram below). Approximately 80-200 cells are expected to be present from the combined cell counts from the four quadrants. If significantly different from this, check cell stock or perform a different dilution if needed. Only count cells that fall on two of the four outer edges of each of the four quadrants, as defined by the red lines depicted in the diagram below.



- To calculate cell concentration, take the average of all the cell counts (total cells counted/ # of quadrants counted [A, B, C, and D]). Multiply this number by the dilution factor, then, multiply by 10,000. This will provide cell number per mL.
- For example, if you dilute your sample 1:2 with trypan blue and count 100 live cells in all four quadrants, then the cell concentration would be the following:

$$(100 \div 4) \times 2 \times 10{,}000 = 500{,}000 \text{ cells/mL}$$

Attachment 2: Process Steps For % Viability

Process steps when % Viability passes for Counts 1 and 2.

% Viability		Avg Cell Count*	% Difference*	% Viability		Avg Cell Count*
Count 1	Count 2			Count 3	Count 4	
P	P	(Count 1 + 2) ÷ 2	P			
P	P	(Count 1 + 2) ÷ 2	F	P	P	(Count 1 + 2 + 3 + 4) ÷ 4
P	P	(Count 1 + 2) ÷ 2	F	P	F	(Count 1 + 2 + 3) ÷ 3
P	P	(Count 1 + 2) ÷ 2	F	F	P	(Count 1 + 2 + 4) ÷ 3

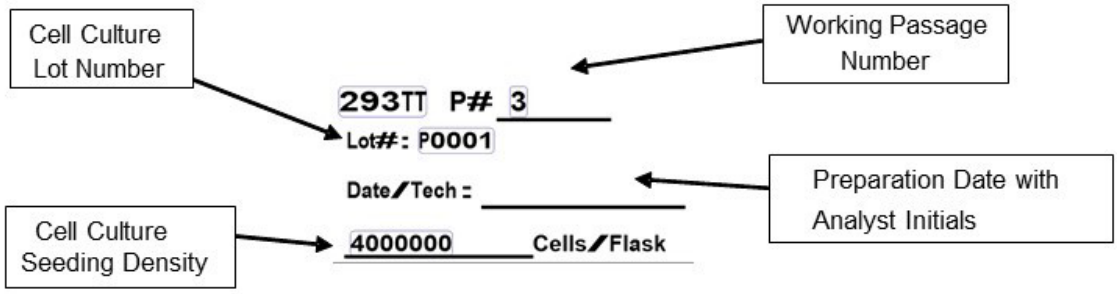
Process steps when % Viability fails for Count 1 and/or 2.

% Viability				Avg Cell Count*	% Difference*	Additional Actions
Count 1	Count 2	Count 3	Count 4			
P	F	P		(Count 1 + 3) ÷ 2	P	
P	F	F	P	(Count 1 + 4) ÷ 2	P	Alert Scientific Manager
P	F	P		(Count 1 + 3) ÷ 2	F	Perform Count 4, and average cell count is (Count 1 + 3 +4) ÷ 3
P	F	F	P	(Count 1 + 4) ÷ 2	F	Alert Scientific Manager, Scientific Manager will perform two counts.
F	F	P	P	(Count 3 + 4) ÷ 2	P	Alert Scientific Manager
F	F	F				Perform manual Trypan Blue exclusion count and discuss with Scientific Manager, Scientific Manager will perform two counts.
F	F	P	P	(Count 3 + 4) ÷ 2	F	Alert Scientific Manager, Scientific Manager will perform two counts.
F	P	P		(Count 2 + 3) ÷ 2	P	
F	P	F	P	(Count 2 + 4) ÷ 2	P	Alert Scientific Manager
F	P	P		(Count 2 + 3) ÷ 2	F	Perform Count 4, and average cell count is (Count 2 + 3 +4) ÷ 3

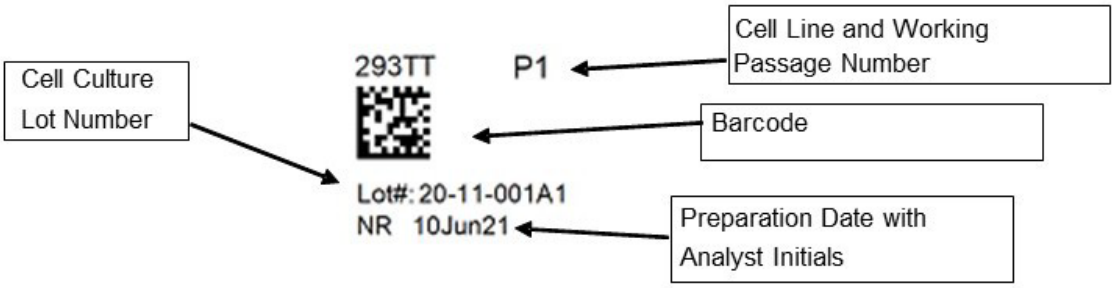
* Passing cell counts used in calculation only.

Attachment 3: 293TT Cell Flask Label and Cryovial Label

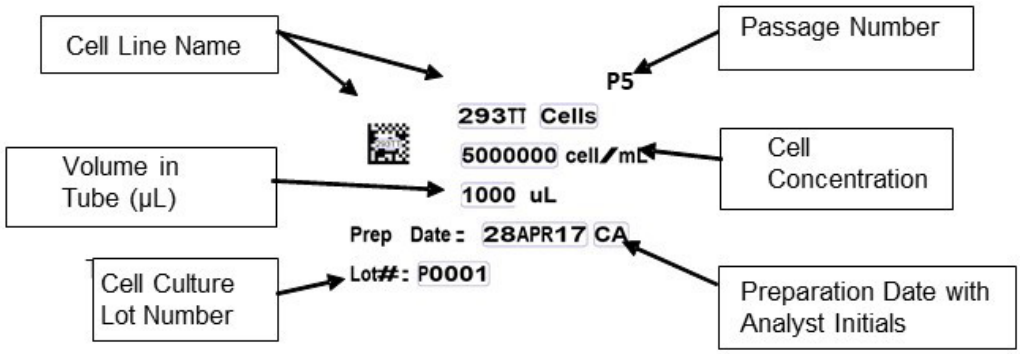
293TT Cell Flask Label and Cryovial Label (Manual)



293TT Cell Flask Label and Cryovial Label (LIMS)



Note: A template can be used to print labels for the flasks, for ease of labelling.



Attachment 4: Box Label Example

P0001
Study: 293TT Cells LOT# LAB2017xxx
Sample Type: Human Cell Line
Date: 22JUN17
Initials: TK
Box 1 **of** 2

Attachment 5: 20001-02: 293TT Cell Thaw Form

Frederick National Laboratory for Cancer Research sponsored by the National Cancer Institute		Vaccine, Immunity and Cancer Directorate Standard Operating Procedure Form	
Form Title: 293TT Cell Thaw Form			
Document ID: 20001-02		Version:	6.0
Associated SOP: 20001		Effective Date:	13Aug21
Supersedes Version:	5.1	**Page 1 of 1**	

Equipment

Equipment Name	Equipment ID	Calibration Due Date
Water Bath	☐ HSL_010 ☐ Other:	
BSC	☐ HSL_007 ☐ HSL_008 ☐ HSL_076 ☐ Other:	
Cellometer Auto 2000	☐ HSL_019 ☐ HPV_005 ☐ Other:	☐N/A
CO_2 Incubator	☐HSL_026 ☐HSL_027 ☐HSL_023 ☐HSL_024 ☐ Other:	
Pipette: µL	PIP_	
☐N/A Pipette: µL	PIP_	

Reagents

Reagent Name	Lot Number	Expiration Date
Thawing Media (293TT TM)		
Vita Stain AOPI Staining Solution		

Viability Check

Initial Thawed Vial		Repeat Viability Check on new vial if 1st Fails ☐ N/A	
Cell Count 1 (x 10^6 Cells/ mL)	Viability 1 (%) (≥ 60%)	Cell Count 2 (x 10^6 Cells/ mL)	Viability 2 (%) (≥ 60%)
	☐ Pass ☐ Fail		☐ Pass ☐ Fail

Inoculation

Approximate Volume of Cells (mL)	Volume of 293TT TM (mL)	Flask Type / # Prepared

Cell Vial Label Information / Comments: ☐ Freezer Inventory Updated

Performed by/date:	
Reviewed by/date:	

Verify current version prior to use. Use of a superseded or obsolete document is prohibited.

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Attachment 6: 20001-03: 293TT Cell Culture Maintenance Form

Frederick National Laboratory for Cancer Research sponsored by the National Cancer Institute		Vaccine, Immunity and Cancer Directorate Standard Operating Procedure Form	
Form Title: 293TT Cell Culture Maintenance Form			
Document ID: 20001-03		Version:	6.0
Associated SOP: 20001		Effective Date:	13Aug21
Supersedes Version:	5.1	**Page 1 of 2**	

Cell Culture Maintenance

Working Passage #: _____ to Generated Passage #: _____ Lot #:_____

Equipment

Equipment Name	Equipment ID	Calibration Due Date
BSC	☐ HSL_007 ☐ HSL_008 ☐ HSL_076 ☐ Other:	
Inverted Microscope	☐ HSL_020 ☐ HPV_037 ☐ Other:	
Cellometer Auto 2000	☐ HSL_019 ☐ HPV_005 ☐ Other:	☐ N/A
$C0_2$ Incubator	☐ HSL_026 ☐ HSL_027 ☐ HSL_023 ☐ HSL_024 ☐ Other:	
Pipette: µL	PIP_	
☐ N/A Pipette: µL	PIP_	

Reagents

Reagent	Lot Number	Expiration Date
DPBS		
Trypsin-EDTA		
Maintenance Media (293TT MM)		
Vita Stain AOPI Staining Solution		

Cell Flask Confluency: _____%

Cell Count

Count Number	Cell Concentration (x10^6 Cells/mL)	Viability (%) (≥80%)
1		☐ Pass ☐ Fail
2		☐ Pass ☐ Fail
3 ☐ N/A Row		☐ Pass ☐ Fail
4 ☐ N/A Row		☐ Pass ☐ Fail
Average Cell Count (x10^6 Cells/mL)		
Percent Difference (%) (≤ 25%)		
Secondary Average Cell Count (x10^6 Cells/mL) ☐ N/A Row		

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Frederick National Laboratory for Cancer Research sponsored by the National Cancer Institute		Vaccine, Immunity and Cancer Directorate Standard Operating Procedure Form	
Form Title: 293TT Cell Culture Maintenance Form			
Document ID: 20001-03		Version:	6.0
Associated SOP: 20001		Effective Date:	13Aug21
Supersedes Version:	5.1	**Page 2 of 2**	

Flask Inoculation

Seeding Conc. of Flask ($x10^6$ Cells / Flask)	Total Volume Required (mL / Flask)	Volume of Cells (mL / Flask)	Volume of 293TT MM (mL / Flask)	Flask Type / # Prepared
☐ N/A Row				
☐ N/A Row				
☐ N/A Row				
☐ N/A Row				

Comments:

☐ N/A

Performed by/date:	
Reviewed by/date:	

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Attachment 7: 20001-04: 293TT Cell Culture Freezing Form

Frederick National Laboratory for Cancer Research sponsored by the National Cancer Institute		Vaccine, Immunity and Cancer Directorate Standard Operating Procedure Form	
Form Title: 293TT Cell Culture Freezing Form			
Document ID: 20001-04		Version:	6.0
Associated SOP: 20001		Effective Date:	13Aug21
Supersedes Version:	5.1	**Page 1 of 2**	

Equipment

Equipment Name	Equipment ID	Calibration Due Date
BSC	☐ HSL_007 ☐ HSL_008 ☐ HSL_076 ☐ Other:	
Centrifuge	☐ HSL_033 ☐ HSL_008 ☐ Other:	
Pipette: µL	PIP_	
☐ N/A Pipette: µL	PIP_	
CoolCell device	☐ HSL_041 ☐ HSL_042 ☐ Other:	☐ N/A
☐ N/A 2-8°C Refrigerator	☐ HSL_029	☐ N/A
☐ N/A -80°C Freezer	☐ HSL_105 ☐ HSL_022 ☐ Other:	
LN_2 Tank	☐ HSL_028 ☐ Other:	
	☐ LN_2 Freezer Inventory Updated	
	Rack #:	**Position:**

Reagents

Reagent Name	Lot Number	Expiration Date
Freezing Media (293TT FM)		

Calculations and Cell Reference

Average Cell Concentration ($x10^6$ Cells/mL)	Total Volume (mL)	Total # of Cells ($x10^6$ Cells/mL)	Volume of Conditioned Medium (mL) need for 10 x 10^6 (cells/ml)
Lot Number	**# of Aliquots Prepared**	**Passage # Used**	**Sample of Final Aliquot Label**

Cell Suspension

Cell concentration (x 10^6 Cells/mL)	Volume of 293TT FM (mL)	Volume of Cells (mL)

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Form Title: 293TT Cell Culture Freezing Form			
Document ID: 20001-04		Version:	6.0
Associated SOP: 20001		Effective Date:	13Aug21
Supersedes Version:	5.1	**Page 2 of 2**	

Comments: ☐ N/A	
Performed by/date:	
Reviewed by/date:	

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