	FOOD SAFETY LAB / MILK QUALITY IMPROVEMENT PROGRAM *Standard Operating Procedure*	

Title: *Salmonella* Invasion of HIEC-6 Cells			
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Salmonella **Invasion of HIEC-6 Cells**

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SECTION 1 INTRODUCTION

1.1 Purpose

The purpose of this document is to create a protocol for the maintenance and infection of human intestinal epithelial cell line cells (HIEC-6).

1.2 Scope

This SOP applies to the Food Safety Lab, including the Laboratory for Food Microbiology and Pathogenesis of Foodborne Diseases.

1.3 Definitions

HIEC-6: human intestinal epithelial cell line
FBS: fetal bovine serum
LB: Luria Bertani
PBS: phosphate buffered saline
Opti-MEM: optimal minimal essential medium
Split ratio: The split ratio defines the ratio with which cells are diluted when they are passaged into a new flask. For example, if ½ of the cells in one confluent T75 are seeded into a fresh T75 flask, then the cells were split at a 1:2 ratio (typically that would entail resuspending all cells from the confluent T75 in a given volume of media (e.g., 6 ml) and using half of the volume (e.g., 3 ml) to seed a new flask.

1.4 Safety

Salmonella enterica contains approximately 2600 serovars, nearly all of which are pathogenic to humans and some of which can cause serious illness. *S. enterica* is a BSL-2 level organism, and all necessary precautions (personal protective equipment such as lab coat, gloves, and when dealing with concentrated amounts, face shields/eye protection, should be worn; all laboratory BSL-2 regulations must be followed, etc., see http://sp.ehs.cornell.edu/lab-research-safety/bios/research-with-microbes-and-cell-lines/Documents/Checklist_BSL2.pdf .

Human cell lines are considered BSL-2 level materials. Prior to working with these cell lines all personnel are required to complete the EH&S training for handling bloodborne pathogens. Personal protective equipment including lab coats, gloves, and safety goggles must be warn at all times. All work should be conducted in a biosafety cabinet.

SECTION 2 MATERIALS

- **LB MOPS**: 5 g NaCl + 5 g yeast extract + 10 g Tryptone per liter supplemented with 100 mM MOPS at pH 6.7; autoclave for 15 min at 121ºC
- **PBS**
- **Pasteur pipettes**: sterilized, only opened in the biosafety cabinet
- **Tissue culture flasks**: (T25 [Fisher catalog # 29186-10] or T75 [Fisher catalog # 07-202-0000])
- **Water bath:** water needs to be changed every week; only ultrapure, autoclaved water may be added
- **Stripettes:** sterile, individually wrapped stripettes of 2 mL, 5 mL, 10 mL, and 25 mL volume
- **HIEC-6 media**: Opti-MEM medium (Gibco, catalog # 31985070) supplemented with 5 ng/mL Epidermal growth factor (Thermofisher catalog # PHG0315)
- 0.25% Trypsin EDTA (Thermofisher catalog # 25200072)
- **Incubator** with 5% CO_2 at 37°C
- **Triton X-100:** To prepare the lysis solution, add 0.5% v/v Triton X-100 to PBS; store at 4°C

SECTION 3 PROCEDURES

3.1. Thawing of HIEC-6 cells

Note: This SOP borrows heavily from the "Salmonella invasion of CaCo-2 cells" authored by Dr. Rodriguez-Rivera.

(1) Turn on the 37°C water bath. Let temperature equilibrate to 37°C (approx. 15 mins). Place the HIEC-6 media in the 37°C water bath. Allow the temperature to equilibrate to 37°C (approx. 15 mins). Turn on the UV light in the biosafety cabinet and allow the light to remain on for approx. 15 mins. After 15 mins, turn off UV light in the biosafety cabinet. Turn on the fluorescent light and blower. Open the biosafety cabinet and spray down the surface with ethanol. Transfer all materials to the hood (stripettes, PBS, Trypsin, Opti-MEM, new flasks). Label the flask with the name of the cell line, the passage number, date, and your initials.
(2) Remove a cryovial from liquid nitrogen and immediately place it into a small container of warm sterile water (a small beaker will work). Do not "float" the vial in the water bath but use enough water to cover the frozen cell pellet inside the cryovial.
(3) As soon as the cells have thawed, spray the vial liberally with ethanol and then wipe the cryovial before placing it in the biosafety cabinet. Use a 2 mL stripette to transfer the cells into the T25 (which already contains 5 mL of growth medium with 10% FBS). Gently rock the flask to distribute the cells, and then place the flask into a tissue culture incubator.

- Note: It is important to ensure that the liquid covers the entire bottom of the flask. The bottom of the flask is coated with a polymer to encourage attachment and is slightly hydrophobic; if a corner is uncovered (doesn't have any media covering it) it will remain that way.

(4) After 12-24 hours replace the media in the T25 flask (make sure to first follow the procedures outlined in above step 1 to pre-heat the media and disinfect the biosafety cabinet).
(5) After the cells have reached 90-100% confluency, trypsinize the cells as described in 3.2 below and seed all cells into a T75 flask (which represents a 1:3 passage).

3.2. Maintaining HIEC-6 cells in a T-75 flask

HIEC-6 cells should be maintained in HIEC-6 medium (Opti-MEM + 10 ng/µL EGF) in a T75 flask. When performing invasion assay experiments you need to maintain a continuous supply of HIEC-6 cells for invasion assays. HIEC-6 cells should be split every 3 to 4 days at either a 1:3 or 1:4 split ratio (1:3 split cells usually need to be split again after 3 days, after a 1:4 split cells usually need to be split again after 4 days).

While a new flask can typically be seeded using either a 1:3 or a 1:4 split ratio, many people have found it useful to split cells at a 1:3 ratio when seeding a new T75 flask while preparing a 24-well plate, followed by a 1:4 split three days later, which yields a T75 with a confluent HIEC-6 monolayer after another 4 days, which can be used to seed another 24-well plate. This protocol provides for a 7-day cycle, where a new 24-well plate for an invasion assay is seeded on the same day every week.

Guidelines for splitting ratios to be used based on tissue culture cell numbers

Cell average per Large Grid on Hemocytometer	Split Ratio	mL of Cell Suspension Used
20-60	1:3	3.0
60-110	1:4	2.2

Maintenance of HIEC-6 cells in T75 flasks:

(1) Check the cells daily under the inverted microscope for any sign of contamination and check the percent of confluence or growth rate of cells. Once the cells reach 80 -100% confluence they are ready to be passaged into a new T75 flask and/or seeded into 24-well plates. To seed a 24-well plate a T75 flask with cells at 100% confluency is needed.
(2) Prepare the biosafety cabinet (turn on the UV light for 15 mins., then spray liberally with 70% Ethanol), preheat all media (PBS, HIEC-6 cell media) at 37°C in the tissue culture water bath, and move stripettes and tissue culture flasks to the sanitized biosafety cabinet.
(3) Place the flask that is to be passaged in the biosafety cabinet.
(4) Remove the HIEC-6 media from the T75 flask with a Pasteur pipette, maintaining the flask at a ~45° angle. Avoid touching the walls of the flask.
(5) Use 5 mL of PBS to wash the bottom corners of the flask avoid touching the bottom of the flask. After washing, place the flask horizontally without swirling, shaking, or rocking it. Then remove the PBS with a Pasteur pipette.
(6) Add 1 mL of 0.25% Trypsin-EDTA solution and incubate for 5 min in tissue culture incubator. Use the inverted microscope to confirm the detachment. When you see that most of the cells have detached, place the flask in the biosafety cabinet. Do not over-trypsinize the cells (i.e. incubate with trypsin EDTA more than 7 minutes).
(7) Once cells are mostly detached add 9 mL of pre-warmed HIEC-6 media (using a 10 ml stripette) to the flask and pipette up and down against the corner of the flask vigorously to breakup any remaining clumps of cells.
(8) When splitting cells without setting up a 24-well plate for a new invasion assay transfer 2.2 or 3 mL of cell suspension (for a 1:4 or 1:3 split ratio, respectively), based on counts (presented above in Table 1), into a new T75 flask and add HIEC-6 media to a final volume of 10 mL.

(9) Label flask with: (i) split date, (ii) passage number, (iii) split ratio, (iv) your name, and (v) the medium added and the cell line name (HIEC-6).

3.3 Counting HIEC-6 cells using the hemocytometer

The concentration of cells in the cell suspension is determined using a hemocytometer (see **Fig. 1**). The hemocytometer and its weighted coverslip must be carefully cleaned (first, clean with 70% ethanol, distilled water and then dry with kimwipe) before the cell suspension is

applied for counting.

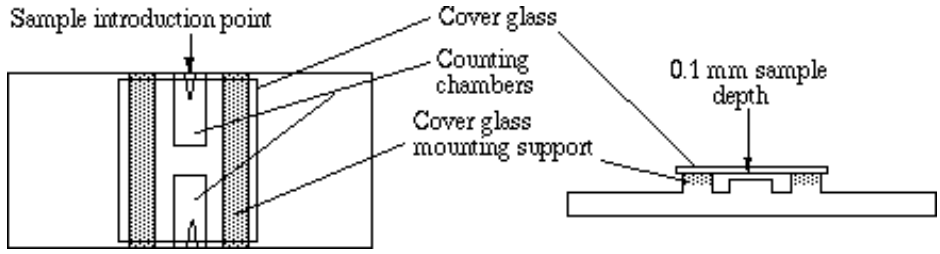


Figure 1. The hemocytometer

The specific counting procedure is as follows:
(1) Transfer 0.1-0.2 mL of a cell suspension to a 1.5 mL Eppendorf tube. Place the weighted coverslip evenly onto the middle section of the hemocytometer. It is very important the cells were recently agitated otherwise the count will be inaccurate. If the cells have been sitting in the hood for >1 minute use a sterile stripette to pipette the cells up and down several times.
(2) Transfer 10 µL out of the cell suspension onto one of the notched sides of the hemocytometer by placing the pipet tip to the notch and slowly allowing the area under the coverslip to fill by capillary action.
(3) Place the slide on the microscope platform, set from the lowest to the appropriate magnification (i.e. 10X objective) to focus on the grid pattern of the corner square (see Fig. 2).
(4) There are 16 small squares per corner square and those correspond to an equivalent volume of 1 mm^3or 10^{-4} mL. Count the number of cells in each corner square.
(5) Once you have the 4 cell counts add them up and continue with the next step.
(6) Calculate the # of cells/mL to obtain the volume of cell suspension needed to seed one 24-well plate:

(6a) Calculate the average cell number per square
Calculation: Add cell counts of 4 corner squares and divide this number by 4 = average # of cells per large square
Example: (189+168+134+162) / 4 = 163.3 cells

(6b) Calculate the number of HIEC-6 cells/mL
Calculation: Average # cells/square x 10^4 = cells/mL
Example: 163.3 x 10^4 = 1.63 x 10^6 cells/mL
(6c) Calculate the # of mL of this cell suspension that are needed to seed n # of wells:
mL of cell suspension = $\frac{(3 \times 10^5 \text{ cells/well}) * \#\text{ of wells}}{\text{conc. of cells in suspension}}$
Example: if you want to seed 20 wells with the cell suspension containing 1.63 x 10^6 cells/mL (Note: always add an extra well to the calculation)
$\frac{(3 \times 10^5 \text{ cells/well})}{1.63 \times 10^6 \text{ cells/mL}} * 21$ wells = 3.9 mL of cell suspension
(6d) To calculate the number of mL of media to add use the following equation:
mL of HIEC-6 media = # of wells - # of mL of cell suspension to add

Example: in our above example this would equate to: 21-3.9 mL = 17.1 mL of HIEC-6 media need to be added to 3.9 mL of cell suspension in order to seed 20 wells (with 1 extra well to account for pipetting errors).

The standard protocol for seeding a 24-well plate

(1) Transfer the volume of the HIEC-6 cell suspension needed (as calculated in 3.3.6c) into a new 50 mL Falcon tube. Then add the HIEC-6 cell medium to obtain the final volume (section 3.3.6d). The concentration (i.e. cells/mL) should be **3 x 10^5** cells.
(2) Seed the 24-well plate by adding 1 mL of the cell suspension to each well, place 24-well plate in the 37°C tissue culture incubator. Cells should be at 90-100% confluency ~ 20-24 hrs after seeding.
(3) The remaining cell suspension may be used to seed additional plates or T75 flasks.

3.4 Growth of *Salmonella* for invasion assays

3.4.1 *Salmonella* controls for invasion assays

Previous Acc. No.	FSL No.	Type of Control	Serotype	Provided by
CA-32 (ATCC 14028)	FSL R8-4084	Positive	Typhimurium	Craig Altier
CA-501	FSL R8-4083	Negative **Δ*hil*A**	Typhimurium	Craig Altier
CA-772	FSL R8-4082	Negative **ΔsirA**	Typhimurium	Craig Altier

***Controls can be found in -80 Freezer no. 2, in Tower 23**

(1) Transfer single colonies from recently cultured plates (ideally no longer than 1 week old) into 5 mL of LB with 100 mM MOPS pH 6.7 in red-capped tubes. Incubate the cultures for 16-18 h at 37°C. This media was selected because it represents the pH of the ileum (Ref. 1).
(2) After 16-18 h of incubation, pulse vortex the cultures, and transfer 100 µL of each culture to a clean 1.5 mL microcentrifuge tube containing 900 µL of PBS. Pulse vortex for 3-5 sec.
(3) You will use your 10^{-1} dilution. The goal is to have a multiplicity of infection (MOI) of 10 bacteria per cell (Ref. 1). Note: this assumes that the concentration of bacteria is ~1 x 10^9 cfu/mL in your overnight culture. This should be used as an approximation, but should be tested with your specific strain(s).

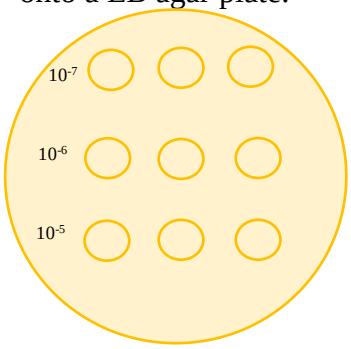
3.5 Invasion assay

(1) Transfer 20 µL **(from the 10^{-1} dilution tube)** into a single well and repeat this step for all the different strains or samples. **Pipet up and down** into the well. Avoid touching

the walls or the bottom of the well.

(2) After all the wells have been inoculated, return the 24-well plate to the 37°C incubator for 60 minutes in the "infected" tissue culture incubator.

(3) During the incubation time, plate the inoculum to enumerate the levels of bacteria added. This can be done by making serial dilutions in PBS (i.e. transfer 100 µL into 900 µL) to 10^{-7}. Spot 20 µL of the 10^{-5}, 10^{-6}, and 10^{-7} dilutions in triplicate (as shown below) onto a LB agar plate.



Allow the plate to dry completely for at least 1 h. Incubate plates at 30°C for 16-20 h.

Invasion continuation

(1) After 60 minutes of incubation, remove the 24-well plate from the tissue culture incubator and place it into the biosafety cabinet.
(2) Using a Pasteur pipette remove the medium from each well.
(3) Distribute 1 mL of PBS (pre-warmed to 37°C) into each well to wash the cells. Remove the PBS from all the wells with a Pasteur pipette. Perform this step three times.
(4) Add 1 mL of fresh HIEC-6 cell medium with 20 µg/mL to each well. Incubate the 24- well plate in the tissue culture incubator for 60 min.
(5) After 60 min, remove the medium with a Pasteur pipette and wash the cells 3 times by adding 1 mL of PBS, and then remove with a Pasteur pipette.
(6) Add 200 uL of Trypsin EDTA to each well, and incubate the plate for 3-5 min at 37°C in the "infected" tissue culture incubator.
(7) After the 3-5 min incubation, the cells should be noticeably detached. Add 800 µL of the cell lysis solution (ice cold 1% Triton X-100 buffer). Incubate on ice for 5 min to lyse the eukaryotic cells. Pulse vortex every minute.
(8) Perform serial dilutions (10^{-1} to 10^{-3}) in PBS and plate 100 µL of each dilution on a separate LB plate.
(9) Incubate the plates at 37°C overnight and then count the colonies to calculate the number of *Salmonella* that successfully invaded the HIEC-6 cells.
(10) After 24 h, count the number of colonies on each plate.

SECTION 4 TROUBLESHOOTING

SECTION 5 REFERENCES

(1) *Lawhon, S. D., R. Maurer, M. Suyemoto, and C. Altier. 2002. Intestinal short-chain fatty acids alter Salmonella typhimurium invasion gene expression and virulence through BarA/SirA. Mol. Microbiol. 46:1451–1464.*

SECTION 6 METHOD VERSION & CHANGES

VERSION	DATE	EDITOR	COMMENTS
Version 1	12/22/2020	ram524	Original SOP