Title: Salmonella Caco-2 Cell Invasion Assay			
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Salmonella Caco-2 Cell Invasion Assay

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SECTION 1 INTRODUCTION

1.1 Purpose

The purpose of this document is to set forth **standard** guidelines for performing an assay for testing *Salmonella* spp. isolates and strains for their ability to invade Caco-2 human intestinal epithelial cells grown in 24-well plates.
The standard protocol described here must be used by everyone to perform the Caco- 2 cell invasion assay. Any and all modifications of this protocol need to be approved by Martin Wiedmann before being incorporated into this standard SOP. Training of new personnel in performance of the assay must use this standard SOP, modified versions of this SOP cannot be used for training.

1.2 Scope

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

1.3 Definitions

Confluence: Is the measure of the density of cells in the T75 flask or 24-well plate. At 100%, essentially all of the available surface should be covered by cells. Similarly, at 50% confluence, only half of the available surface should be covered by cells.

Split ratio: The split ratio defines the ratio with which cells are diluted when they are passaged into a new flask. For example, if half of the cells in one confluent T75 flask 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 spp. is a BSL-2 pathogen. Relevant information about the appropriate protective measures to work with BSL-2 pathogens can be found in the internal FSL webpage under the name of Laboratory Orientation Document or The Safety and Waste procedures SOP. All waste from these experiments needs to be treated as BSL-2 waste including Caco-2 cells.

SECTION 2 MATERIALS

Notes:

- The FSL internal webpage contains all the SOPs cited in this document.
- **Caco-2 cell medium**, **Dulbecco's Modified Eagle's Minimal (DMEM),** must be prepared as in the SOP entitled "Preparation of Caco-2 cell medium"
- **1X trypsin EDTA**

Materials:

- **Caco-2 cell medium (DMEM)** with 20% FBS, pre-warmed to 37°C.
- **Phosphate Buffered Saline (PBS)** pH 7.4. Located in the media room and should be sterilized by filtration if any problems occur; pre-warm to 37°C.
- **Sterile distilled water.**
- **1X trypsin EDTA.**
- **T75 tissue culture flasks**
- **Tissue culture 24-well plates**
- **Sterile disposable serological pipettes** (2, 5, and 10 mL)
- **Hemocytometer**
- **Luria Bertani (LB) plates** for enumeration of *Salmonella*
- **LB broth (5 mL)** in red-capped tubes.
- **Gentamicin (50 mg/mL)**
- **Tissue Culture incubator** at 37°C, 5% CO_2.

SECTION 3 PROCEDURES

3.1 Overview: Timeline for *Salmonella* spp. invasion assay

Step	**Timing** (*t=0 h is time of Salmonella inoculation to Caco-2 cells*)	**Brief Description**
1	**-48 hours**	Seed 24-well plate with the Caco-2 cells
2	**-18 hours**	Start growth *Salmonella* spp. strains or isolates in LB broth for assay
3	**- 30 min**	Change medium in 24-well plate to fresh medium
4	**- 20 min**	Prepare bacterial inoculum
5	**0 min**	Infect Caco-2 cells with *Salmonella*, make dilutions to enumerate inoculum
6	**1 hour**	Wash infected cells, add fresh medium
7	**1 h 15 min**	Remove media, add new media with gentamicin
8	**2 h 15 min**	Wash infected cells, lyse cells with cold distilled water, and enumerate bacteria that invaded the cells.

3.2 Thawing of Caco-2 cells

For detailed information on thawing of cells in order to start a tissue culture cell line not currently grown in the laboratory consult the protocol entitled "General Protocol for Freezing and Thawing Permanent Cell Lines". Briefly, the following steps should be performed:

(1) 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, but use enough water to cover the frozen cell pellet inside the cryovial.
(2) As soon as the cells have thawed, use ethanol to wipe the cryovial and then pipette the cells into the waiting T25 (which already contains 5.0 mL of growth medium with 20% FBS, pre-warmed to 37°C). Rock the flask to distribute the cells, and then place the flask into a tissue culture incubator.
(3) Wait about 4 hours, then remove the flask from the incubator and look at the cells inside with an inverted microscope. Most of the cells should be attached, but there will be probably a few "floaters". These may settle down, or they may be cells that did not survive being frozen. If most of the cells are still floating, return the flask to the incubator. Four hour is usually enough time for cells to reattach and if cells look attached at 4 h after thawing, aspirate the media (which contains DMSO, which may be toxic to the cells) and replace the media with 5.0 mL of fresh growth media. Return the flask to the incubator. If cells are not attached after 4 h, re-check them after 12 h (24 h at the very latest) and replace the medium then.
(4) Be sure to check the cells after 24 hours. The cells should be attached and growing. Remember that the shock of freezing will probably introduce a bit of a log in the growth cycle,

so adjust your time frame for splitting.
(5) After the cells have come to confluence, trypsinize the cells as described in 3.2 below and seed all cells into a T75 flask (which represents a 1:3 passage).

3.3 Maintaining Caco-2 cells in a T-75 flask

Caco-2 cells should be maintained in DMEM medium, in a T75 flask.
When performing invasion assay experiments you have to maintain a continuous supply of Caco-2 cells for invasion assays. Caco-2 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). If tissue culture cells are extremely dense (i.e., >110 average cell number per Corner Grid on the hemocytometer) cells may also be split 1:5 (there should rarely be a need for this, if you feel this step is needed consult a senior technician or graduate student with invasion assay expertise). In general, tissue culture cells used for seeding a 24-well plate for the invasion assay should have been spit 1:4, cells that were split 1:3 or 1:5 should, in general, be passaged once with a 1:4 spit before seeding a 24-well plate.

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 Caco-2 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	Split Ratio	Cell Suspension Used (mL)
20-60 cells	1:3	3.0
60-110 cells	1:4	2.2
>110 cells*	1:5	1.8

* there should rarely be a need for this, if you feel this step is needed consult a senior technician or grad student with invasion assay expertise

Maintenance of Caco-2 cells in T75 flasks:
**Note: Before starting, the DMEM and PBS should be pre-heated in a water bath to 37°C and the 1X Trypsin-EDTA should be at room temperature.*

(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.
(2) Once the cells reach 80 -100% confluence, all necessary reagents and materials must be prepared in the hood for splitting into a new T75 flask and seeding in 24-well plates. To seed a 24-well plate a T75 flask 100% confluent is needed.
Remove the DMEM from the T75 flask, maintaining the flask at ~45° angle. Avoid touching

the walls of the flask.

(1) Use 10mL of PBS to wash the bottom corners of the flask avoiding touching the bottom.

(2)After washing place the flask horizontally without swirling, shaking, or rocking it. Then remove the PBS. Perform 3 washes total.

(3) Add 2 mL of 1X trypsin EDTA solution and incubate for 5 min in tissue culture incubator. The cells should begin to detach from the bottom of the flask. Use the inverted microscope to confirm the detachment. When you see under the microscope if most of the content of the flask are clumps of cells or just a few detached cells, put the flask in the incubator to trypsinize longer (2 more minutes is enough). Do not over-trypsinize the cells (i.e. incubate with trypsin EDTA more than 5 minutes)

(4) Once cells are detached and mostly separated, (without removing the 2 mL of trypsin EDTA) add 7 mL of pre-warmed Caco-2 media using a 10 mL serological pipette.

Pipette up and down against corner of flask vigorously to breakup any remaining clumps of cells. The total 9 mL have to be transferred from the T75 flask to a 50 mL Falcon tube. The 9 mL should be sufficient to split the cells into a new T75 flask and seed a 24-well plate.

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), into new T75 flask and add the DMEM + 20% FBS to a final volume of 17 mL.

(3) 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 (Caco-2). See section 5 for more details about labeling.

3.4 Counting Caco-2 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 and then dry with kimwipe) before the cell suspension is applied for counting.

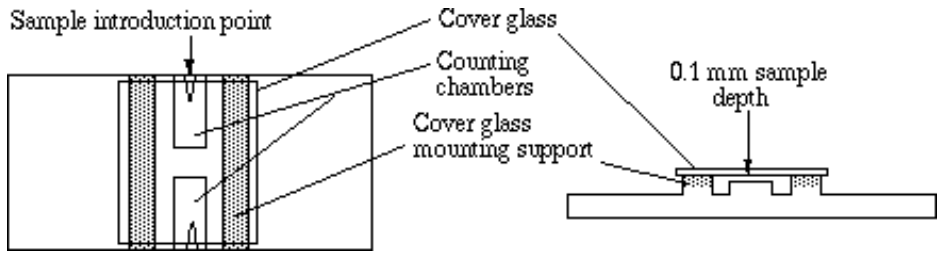

Figure 1. The hemocytometer

The specific counting procedure includes:

(1) Transfer 0.5-1mL from the Falcon tube containing 9mL (section 4.2, step 7) into an (1.5mL) Eppendorf tube. Place the weighted coverslip evenly onto the middle section of the hemocytometer.

(2) Pull 10 µL out of cell suspension into a pipet tip and apply to 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. Enough liquid should be introduced to just cover the mirrored surface. It will not take more than 10 µL per chamber--Do not overfill or underfill the chamber!

(3) Wait a couple of seconds until the cells settle, then place the slide onto the microscope, 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 corresponds to an equivalent volume of 1 mm^3or 10^{-4} mL. Count the amount of cells in each corner square.

(5) Once you have the 4 cell counts add them up and continue with the next step.

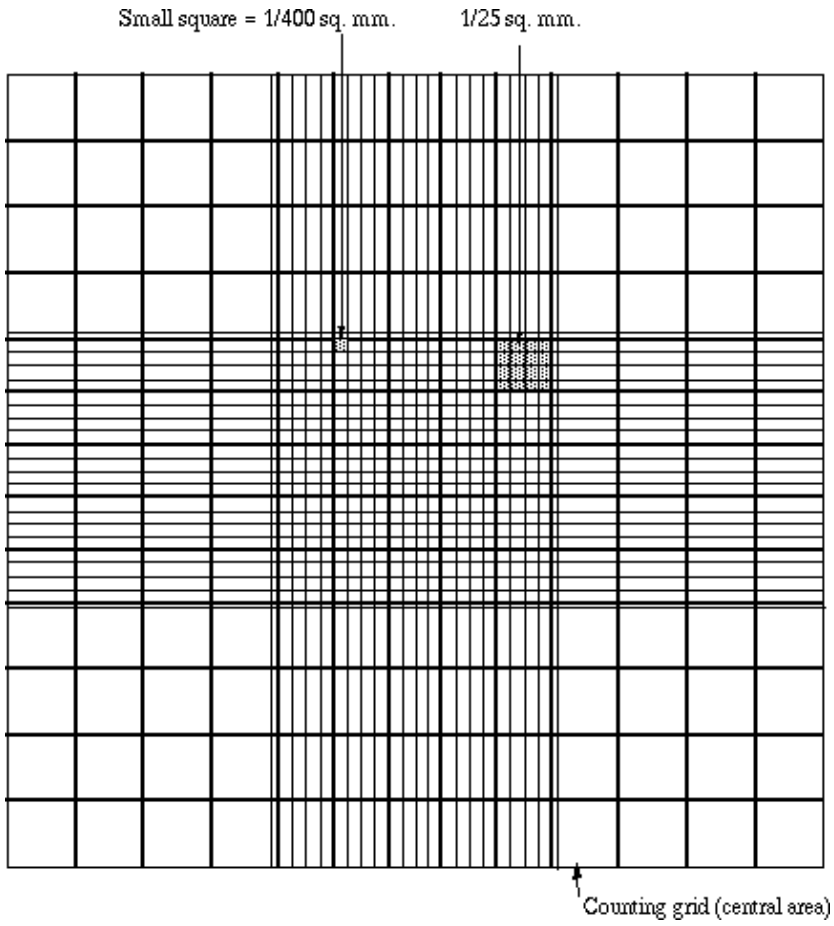

Figure 2. The hemocytometer counting squares

(1) 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 Caco-2 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 amount of the cell suspension that contains a total of 1.25 x 10^6 (because 25 wells X 5.0 x10^4= 1.25 x 10^6)

Caco-2 cells (which represents the total number of cells needed to seed a 24-well plate)
Calculation: 1.25 x 10^6 cells (needed to seed 25 wells) / (Cell number/mL) = mL of suspension that contains 1.25 x 10^6 Caco-2 cells
Example: 1.25 x 10^6 cells/1.63 x 10^6 cells/mL = 0.77 mL

(6d) Calculate the amount of media and the amount of cell suspension that need to be mixed to have 25 mL of media at 5.0 x 10^4 cells/well (which equals a total amount of 1.25 x 10^6 Caco-2 cells)
Calculation: 25 mL - mL of suspension that contains 1.25 x 10^6 Caco-2 cells = amount of media that needs to be added to the cell suspension volume that contains 1.25 x 10^6 Caco-2 cells
Example: 25 mL - 0.77 mL = 24.23 mL (therefore 0.77 mL of cell suspension and 24.23 mL of Caco-2 cell medium need to be mixed together)

3.5 Seeding a 24-well plate for an invasion assay

Notes:

- After you use the hemocytometer and calculate the volume needed to complete a volume of 25 mL, then you can continue with this section.
- At 100% confluency a T75 contains approximately 2.8 x 10^6 to 8.4 x 10^6 Caco-2 cells.
- To seed 25 wells at a target cell density of 5.0 x 10^4 Caco-2 cells/well (calculation of cells needed for 25 wells will provide 1 mL of extra cells), a total of 1.25 x 10^6 Caco-2 cells are needed (see step 5c, section 4.3).
- With a seeding density of 5.0 x 10^4 Caco-2 cells/well in a 24-well plate, wells should be about 80-90% confluent after 48 h. Monolayers will not be 100% confluent.

- Special note on Caco-2 cell densities used to seed 24-well plates: Anyone starting invasion assays for a new project must use a seeding density of 5.0 x 10^4 Caco-2 cells/well.

Reminder: In order to start the procedure for seeding a 24-well plate for an invasion assay a T75 flask with 100% confluent Caco-2 cells is needed. As mentioned above (section 4.2) the 9 mL harvested from the T75 flask are sufficient to use one aliquot from it to seed the 24-well plates and other aliquot for a new passage into a new T75 flask for further experiments.

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. This yields a T75 with a confluent Caco-2 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.

The standard protocol for seeding a 24-well plate

(1) Briefly, the total of 9 mL of cell suspension obtained (section 4.2) from harvesting the T75 flask can be used to split the cells into a new T75 flask. The cell concentration is calculated, and the cells are seeded in a 24-well plate.
(2) The cells are enumerated and the amount of cells needed to seed the 24-well plate is calculated. Transfer the volume of the Caco-2 cell suspension into a new 50 mL Falcon tube (should be around 2 mL). Then add the Caco-2 cell medium to reach a final volume of 25 mL (section 4.3). The concentration should be **5.0×10^4** cells/mL.
(3) Seed a 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. Incubate the plate for 48 hours and then check the confluency level.

Note: A volume of 25 mL of cell suspension at 5.0×10^4 cells/mL are required to seed a full 24-well plate for an invasion assay; for seeding 2 or 3 plates 50 mL and 75 mL, respectively, of a cell suspension at 5.0×10^4 cells/mL are required. When Caco-2 cells are maintained in a T75 as described, one T75 should yield enough cells to seed 2 – 3 24-well plates for invasion assays.

Seed a 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. The Caco-2 cells in these 24-well plates should be ready for an invasion assay after 48 hours.

3.6 Growth of *Salmonella* spp. for invasion assays

3.6.1 *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**

3.6.2 *Salmonella* growth instructions for invasion assays

(1) Streak out isolates from frozen glycerol stocks onto LB agar plates; be sure to streak sufficiently to achieve individual colonies. Incubate the plate(s) at 37°C overnight.
(2) Transfer one isolated colony into 5 mL LB broth red-capped tubes. Incubate the cultures 18h at 37°C. Tubes must be incubated horizontally.
(3) After 18 h of incubation culture tubes are vortexed and then 1 mL is removed from the tubes and distributed into each Eppendorf tube. The Eppendorf tubes are centrifuged at (13,000) 7,500 rpm for 5 min. The supernatant is decanted and 1 mL of PBS is added to the tubes to resuspend the pellets.
(4) Dilute overnight cultures to **10^5** and plate this dilution in duplicate. Use either the 10^0 or 10^{-1} dilution to inoculate the epithelial cells. The goal is to have a multiplicity of infection (MOI) of 10 bacteria per cell (Ref. 4). Then, continue to the invasion assay section. Steps 3 and 4 must be performed at least 20 minutes before the invasion assays.

3.7 Invasion assay

3.7.1 Invasion Assay Steps

Remember to perform step 3 and 4 from section 4.5 before the invasion assay start.

1. Remove the 24-well plate from the tissue culture incubator after 48 h of incubation, and place it into the fume hood.
2. Using a sterile Pasteur pipette remove the old medium in each well (Do not touch the bottom of the well).
3. Distribute 1 mL of new DMEM 20% FBS into each well.
4. Incubate the 24-well plate for 30 minutes in the tissue culture incubator.
5. After the incubation period place the 24-well plate into the fume hood.
6. Transfer 20 µL **(usually 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. Use a second well as duplicate if necessary.
7. After all the wells have been inoculated, incubate the 24-well plate for 60 minutes in the tissue culture incubator.
8. During the incubation time, dilutions can be made from the bacterial cultures to plate them and calculate the inocula concentration.

3.7.2 Plating to calculate the inocula concentration

9. Transfer 100 µL from the red-capped tubes into 1.5 mL Eppendorf tubes containing 900 µL PBS.

If you are performing regular spread plating or pour plating technique you must do a

1. serial dilution up to 10^{-6}, in some instances to 10^{-7}. Then plate all the dilutions in duplicate on LB plates.
2. If you are using the spiral plater remember to plate every isolate in duplicate.
    a. Turn on the spiral plater.
    b. press the "clean" button to clean the device.
    c. Vortex the *Salmonella* dilutions 3 seconds before using them.
    d. To push the tip of the pipette down, press the button with the little arrow that points down (inverted triangle).
    e. Insert the tip into the tube avoiding any contact with the Eppendorf tube walls. Do not immerse the tip deep into the tube. Press the "Fill-max" button.
    f. Place the plate onto the tray. Be sure that it has the right alignment. Press "plate" button.
    g. Remove the first plate from the tray and place a second plate (duplicate plate). Press "plate" button.
    h. After plating the duplicate, press "clean" and start over from step c to h, for each sample's dilution.
    i. Incubate the plates at 37°C overnight and then count the cells to calculate the initial inoculum used in each well.

3.7.3 Invasion continuation

3. After 60 minutes of incubation, remove the 24-well plate from the tissue culture incubator and place it in the biosafety cabinet.
4. Using a Pasteur pipette, remove the medium from each well.
5. Distribute 1 mL of pre-warmed PBS into each well to wash the cells. Remove the PBS from all the wells. Perform this step three times.
6. Add 1mL of new Caco-2 cell medium into each well. Incubate the 24- well plate in the tissue culture incubator for 15 minutes.
7. After the incubation period remove the Caco-2 cell medium without antibiotics from each well. Add 1 mL Caco-2 cell medium with 150 µg/mL gentamicin. Incubate the 24-well plate for 60 minutes in the tissue culture incubator. (This step is done to kill the bacteria that are attached to the cells, but have not invaded the cells).
8. After incubating the 24-well plate, wash 3 times with PBS as described in step 14.
9. Add 500 µL of chilled-water into a well. Pipet vigorously 25 times. This time the bottom of the well can be touched, pushing the tip of the pipette against it. Then transfer the 500 µL to a 1.5 Eppendorf tube. Repeat this step for each well. This step ensures that the Caco-2 cells are lysed.
10. Transfer the 500 µL to an Eppendorf tube and perform serial dilutions up to 10^{-2}**.** Plate the dilutions using the instructions for the spiral plater (step 11 section 4.6)
11. Incubate the plates at 37°C overnight and then count the colonies to calculate the amount of bacteria that successfully invaded the Caco-2 cells.

3.8 Reporting and Labeling

The following information should be recorded in your lab notebook for each split or split/seed:

For split:

1. Passage number of flask to be sub-cultured
2. Observations prior to split (Macroscopic and Microscopic)
3. Batch of media used for the split as it appears in the media log
4. Lot # of trypsin/EDTA used
5. Time of trypsinization
6. Pipette-aid used
7. Cell count observed in each quadrant of the hemocytometer and average count
8. Split ratio and subculture volume
9. Passage number of new flask

For split/seed:

1. All information above should be recorded for split/seed
2. Batch of media used for seeding, as it appears in the media log
3. Calculations for the seed

SECTION 4 TROUBLESHOOTING

Problems previously encountered with this assay include

(1) Monolayer of infected Caco-2 cells detaches when infected cells are washed. This problem often is caused by using a vacuum that is too strong to aspirate mediate or PBS, adjusting the vacuum intensity can help solve this problem. This problem may also be caused by over confluent Caco-2 cells; if you can see that the Caco-2 cell monolayer is over confluent before invasion. Make sure you check your counting procedures and make sure you use your 24-well plates at 48 post-seeding. This problem may also be caused by disrupting the monolayer during PBS washes and can be solved by gently adding PBS to the wall of the well rather than pipetting directly onto the monolayer.
(2) It is no longer advised to use antibiotics to maintain the Caco-2 cells. If you notice that your cell lines have become contaminated, you should discard any potentially contaminated media, clean the bio-safety cabinets, water baths, and incubators before thawing a new cell aliquot.
(3) It is recommended that you routinely test cells for *Mycoplasma* contamination. A protocol detailing the method for testing for *Mycoplasma* contamination can be found on the Wiki. It is generally recommended that you test cell lines for contamination before starting experiments (i.e. before conducting invasion assays).

SECTION 5 REFERENCES

(1) *Martin B. M. 1994. Tissue Culture Techniques: An Introduction. Boston: Birkhauser.*

(2) *Freshney, R. I. 2000. Culture of Animal Cells. 4th ed. Wiley-Liss New York.*

(3) *Nightingale K., Wiedmann M. 2005. Listeria Caco-2 cell Invasion Assay.*

(4) *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	11/30/2005	Lorraine Rodriguez-Rivera & Martin Wiedmann	Original SOP
Version 2	04/27/2020	Maureen Gunderson	Renumbered Section 3. Updated SOP to new standard lab template.
Version 3	05/29/2020	Rachel Cheng	- Removed the use of antibiotics and 1% amino acid supplement (this is no longer used for culturing Caco-2 cells) - Changed the 5X Trypsin solution to 1X Trypsin solution (we now purchase a 1X Trypsin solution so the SOP for preparing Trypsin using the 5X stock solution has been archived) - Added the temperatures that the media and PBS should be pre-heated to prior to starting experiments - For section 3.3 step 3: Removed the sentence about avoiding tapping cells to dislodge them from the bottom of the flask as "gentle tapping" is preferable to over-incubating cells in Trypsin to loosen them from the bottom of the flask.