FOOD SAFETY LABORATORY
CORNELL UNIVERSITY

Listeria monocytogenes Caco-2 cell invasion assay

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TABLE OF CONTENTS

1. INTRODUCTION 3
1.1 Purpose
1.2 Scope
1.3 Definitions
1.4 Safety

2. MATERIALS 4

3. PROCEDURE 6

4. REPORTING AND LABELING 8

5. TROUBLESHOOTING 10

6. REFERENCES 11

SECTION 1 INTRODUCTION

1.1 Purpose

The purpose of this document is to set forth **standard** guidelines for performing an assay for testing *Listeria monocytogenes* isolates and strains for their ability to invade Caco-2 human intestinal epithelial cells using 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 Lab, including the Laboratory for Food Microbiology and Pathogenesis of Foodborne Diseases.

1.3 Definitions

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

Caco-2 cells represent a human derived cell line and *L. monocytogenes* is a BSL-2 pathogen. Appropriate protective measures need to be taken when working with *L. monocytogenes*. All waste from these experiments needs to be treated as BSL-2 waste.

SECTION 2 MATERIALS

- **Caco-2 media (MEM Alpha 1X. 12561-056) with 20% FBS and NO pen-strep added**. This medium is commercially ordered from the Supply Center.
- **PBS.** Lab stock in media room pH 7.4
- **Sterile distilled water.**
- **0.1% Trypsin-EDTA.** This medium must be prepared as detailed in the SOP entitled "Preparation of 0.1% Trypsin-EDTA"
- **T75 tissue culture flasks**
- **Tissue culture 24-well plates**
- **Sterile 2, 5, and 10 ml disposable pipettes**
- **Hemocytometer**
- **Appropriate number of BHI plates for enumeration of inoculum and *L. monocytogenes* that invaded the Caco-2 cells**
- **BHI broth and 15 ml conical tubes or necessary media for growth of *L. monocytogenes* prior to infection**
- **Gentamicin (50 mg/ml)**

SECTION 3 PROCEDURES

3.1. 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. 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).

3.2. Maintaining Caco-2 cells in a T-75 flask

When performing invasion assay experiments you should maintain Caco-2 cells in a T75 flask to have a continuous supply of Caco-2 cells for invasion assays. For routine maintenance of Caco-2 cells they 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 grad 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

The standard procedure for maintaining Caco-2 cells is as follows:
(1) Check cells daily under the inverted microscope to check for indications of contamination and for growth rate of cells.
(2) When cells are 80 -100% confluent, prepare all necessary reagents and materials in the hood for splitting; cells need to be at 100% confluency when splitting to see a 24 well plate.
(3) Remove media from Caco-2 cells and wash 3x with 10 ml of sterile PBS.
(4) Add 2 ml of 5X trypsin/EDTA solution and incubate in tissue culture incubator for 5 min. or until cells begin to loosen from bottom flask (may require up to 10 min. if your flask is very confluent). Check cells for detachment and separation under inverted microscope. Do not rap flask to detach cells from flask (as per ATCC instructions: "to avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach"). If you see mostly clumps of cells and few individual cells return your flask to the incubator and trypsinize longer (usually 2 additional minutes is sufficient). Be careful to not over-trypsinize cells (i.e., do not trypsinize the cells for longer than 7 min. total.
(5) Once cells are clearly detached and mostly separated, add 7 ml of pre-warmed Caco-2 media with antibiotics (use 10 ml pipette). Pipette up and down against corner of flask vigorously to breakup any remaining clumps of cells.
(6) 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 Caco-2 media with antibiotics to a final volume of 20 ml.
(7) 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).

3.3. Splitting and harvesting Caco-2 cells to seed a 24 well plate for an invasion assay

Notes: (i) at 100% confluency a T75 contains approximately 2.8 x 10^6 to 8.4 x 10^6 Caco-2 cells.
(ii) A total of 1.25 X 10^6 Caco-2 cells are needed to seed 25 wells at a target cell density of **5.0 x 10^4** cells/well (while we only seed a 24 well plate calculating cells needed for 25 wells will provide 1 ml of extra cells)
(iii) 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.
(iv) The invasion assay described here has also been performed in 6 well plates containing three 12 mm coverslips per well. For comparison, while one well in a 24 well plate has a surface area of 200 mm^2; one well in 6 well plate has a surface area of 962 mm^2.
Infection experiments in 6 well plates have traditionally used an infectious dose of 1 x 10^8 *L. monocytogenes* cells per well. As of December 2005 the 6-well protocol is not in use in the FSL; if you think that you should use, for some reason, a 6-well format for a Caco-2 invasion assay, you must discuss this with Martin first.

Special note on Caco-2 cell densities used to seed 24 well plates
As of December 2005, two types of protocols were in circulation, one that called for a seeding density of **5.0 x 10^4** Caco-2 cells/well and another that called for a seeding density of **1.0 x 10^5** Caco-2 cells/well. ***The standard procedure for this assay involves using a seeding density of 5.0 x 10^4 Caco-2 cells/well.*** Anyone using a seeding density of **1.0 x 10^5** Caco-2 cells/well as of December 1, 2005 should complete on-going experiments (i.e., experiments that will be used for a given paper) continuing to use a seeding density of **1.0 x 10^5** Caco-2 cells/well. ***Anyone starting invasion assays for a new project must use a seeding density of 5.0 x 10^4 Caco-2 cells/well.***

In order to start the procedures for seeding a 24 well plate for an invasion assay one needs to have a T75 flask with 100% confluent Caco-2 cells. The standard procedure for seeding a 24-well plate is set up so that an aliquot of the Caco-2 cells harvested from a T75 flask is used to seed a 24-well plate, while another aliquot of the Caco-2 cells is used to seed a new T75 flask to maintain a Caco-2 cell stock. 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.

The standard protocol for seeding a 24 well plate and splitting Caco-2 cells into a new T75 flask is as follows:
(1) When cells are 100% confluent prepare all necessary reagents and materials in the hood.
(3) Remove media from Caco-2 cells and wash 3x with 10 ml of sterile PBS.
(4) Add 2 ml of 5X trypsin/EDTA solution and incubate in tissue culture incubator for 5 min. or until cells begin to loosen from bottom of flask (may require up to 10 min. if your flask is very confluent). Check cells for detachment and separation under inverted microscope. Do not rap flask to detach cells from flask (as per ATCC instructions: "to avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach"). If you see mostly clumps of cells and few individual cells return your flask to the incubator and trypsinize longer (usually 2 additional minutes is sufficient). Be careful to not over-trypsinize cells (i.e., do not trypsinize the cells for longer than 7 min. total.
(5) Once cells are clearly detached add 7 ml Caco-2 medium **without antibiotics**. Pipette up and down against corner of flask to break up clumps of cells. This protocol gives you a total of 9 ml of cell suspension. Check flask under inverted microscope to verify you have adequately separated cells.
(6) Remove 0.5 - 1 ml of the re-suspended Caco-2 cells and transfer into a 1.5 ml Eppendorf tube (use 5 or 10 ml pipette, DO NOT USE PIPETTMAN). This aliquot will be used to enumerate the Caco-2 cells by hemocytometer (as described under **3.4** below) to assure that the 24 well plate used in the invasion assay is seeded with a highly standardized amount of Caco-2 cells.
(7) Transfer 3 ml of the Caco-2 cell suspension into new T-75 flask and add Caco-2 media **with antibiotics** and adjust to a final volume of 20 ml; label the flask with split ratio (i.e., 1:3), date, cell type etc.
(8) Transfer the volume of the Caco-2 cell suspension calculated to contain a total of 1.25×10^6 Caco-2 cells (this usually would be around 2 ml of the cell suspension) and add Caco-2 media **without antibiotics** to final volume of 25 ml yielding a cell suspension at a concentration of **5.0×10^4** cells/ml to a sterile 50 ml conical tube and mix well by pipetting.
Note: A volume of 25 ml of a cell suspension at 5.0×10^4 cells/ml are required to seed a full 24 well plate for an invasion assay; of 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;
(9) Seed 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.

3.4. 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 (dry wet hemocytometer first, then clean with 70% ethanol, then dry with kimwipe) before the cell suspension is applied for counting.

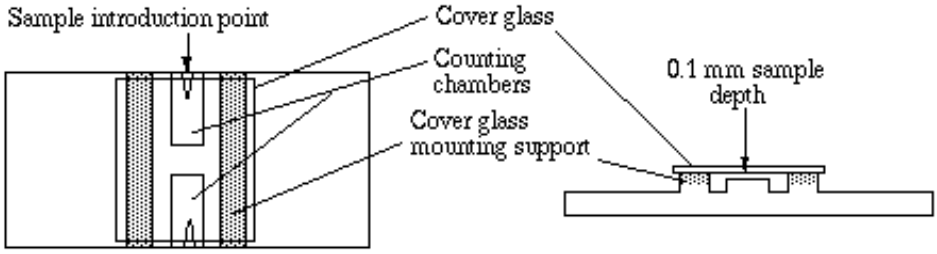

Fig. 1. The hemocytometer

The specific counting procedure includes:
(1) Place the weighted coverslip evenly on the middle section of the hemocytometer.
(2) Pull 20 µl 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 10µl per chamber--Do not overfill or underfill the chamber!!
(3) Allow the cells to settle briefly and place the slide onto the microscope stage, focusing on the grid pattern (see Fig. 2) using the 10X objective.
(4) Count the cells in the large corner squares. Each large c square contains 16 small squares and has a volume of $1mm^3$or 10^{-4} ml.

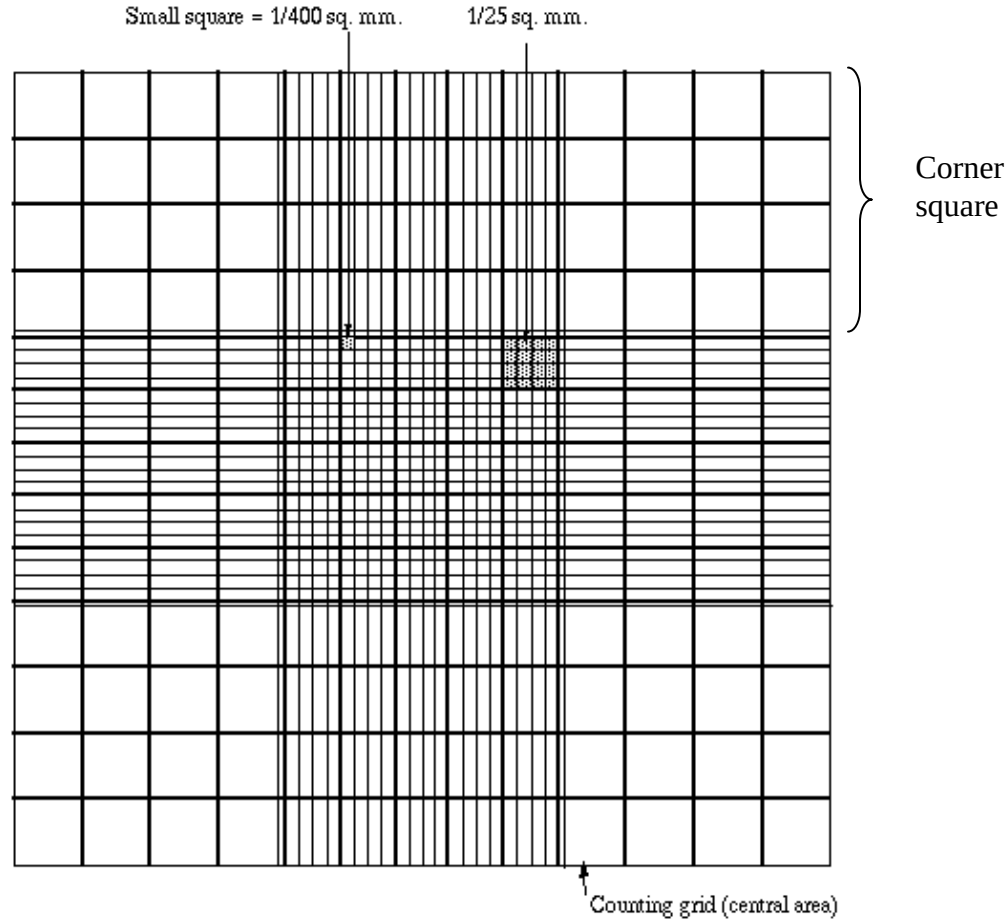

Fig. 2. The hemocytometer counting squares

(5) Calculate the # of cells/ml to obtain the volume of cell suspension needed to seed one 24-well plate:

(5a) 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

(5b) 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

(5c) Calculate the amount of the cell suspension that contains a total of 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

(5d) 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 without antibiotics need to be mixed together)

3.5 Growth of *L. monocytogenes* for invasion assays

Different growth protocols can be used to prepare *L. monocytogenes* for the invasion assay. The standard protocol that is used to screen wildtype *L. monocytogenes* isolates and null mutants in selected virulence genes involves growth of *L. monocytogenes* at 30ºC without shaking. This protocol has been used to characterize a number of *L. monocytogenes* isolates and mutants for their ability to invade Caco-2 cells (e.g., Nightingale et al., 2005). This protocol is outlined in detail below (3.5.1). This growth protocol is not used for any invasion assay experiments involving characterization of an *L. monocytogenes* Δ*sigB* null mutant, since we have found that the Δ*sigB* null mutant grows slower than the wildtype strain when grown at 30ºC without shaking. At this point, the standard protocol for any screening experiments characterizing large numbers of wildtype *L. monocytogenes* isolates involves growth at 30ºC without shaking as outlined in **3.5.1** below. For all assays that are designed to characterize *L. monocytogenes* mutants in regulatory systems (*sigB, ctsR, prfA*, etc.) the standard growth procedures for *L. monocytogenes* cells to be used in Caco-2 cell invasion assays involves growth at 37 C with shaking to stationary phase (as outlined in **3.5.2** below). If the effect of growth conditions, stress exposure, etc. on invasiveness is being tested, then strains will be grown at various other conditions prior to Caco-2 cell invasion assays. If you're unsure which growth conditions to use for experiments you must discuss your experiments with Martin.

3.5.1. Growth of *L. monocytogenes* at 30ºC without shaking

(1) Start bacterial culture 18 h before infection experiments by inoculating 2 ml BHI in a 15 ml conical tube with a well isolated *L. monocytogenes* colony from BHI plate with a fresh culture (no older than 2 weeks; cultures need to be re-streaked from stock cultures maintained at –80ºC if they are older than 2 weeks).

(2) Incubate conical tubes on side @ 30ºC without shaking for 18 h (the *L. monocytogenes* cell density after 18 h will be approximately 2.0 x 10^9 bacteria/ml).

(3) At 20 min before the invasion assay prepare bacterial inoculum using the following steps:

(3a) Transfer 1 ml of 18 h. culture into 1.5 ml microcentrifuge tube.

(3b) Centrifuge cultures @ 13,000 RPM for 5 min. to pellet cells.

(3c) Re-suspend cells in 1 ml of PBS.

3.5.2. Growth of *L. monocytogenes* at 37ºC without shaking

(1) Start o/n culture by inoculating a 5 ml BHI tube with a single well-isolated colony and incubate at 37°C with shaking for at least 12 but not more than 18 h.
(2) Transfer 1% of the o/n culture (50 ul) to a new 5 ml BHI tube and grow at 37°C with shaking until your cultures reach an OD_{600} of approximately 0.40. This should take about 3.5 hours. All isolates will not reach an OD_{600} of 0.40 at exactly the same time so you will need to use an adjustment ratio to synchronize your cultures.
(3) Use an adjustment ratio to determine appropriate volume with which to inoculate your next tube or side-arm flask. For example, if your culture has an OD_{600} of 0.45 and you are inoculating your second passage into a 5 ml BHI tube, a 1% adjusted inoculum volume would be 44.4 ul.
(4) Grow the second passage of your cultures to desired condition (e.g., early log or stationary phase).

3.6 Invasion assay

(1) Prepare all necessary reagents and equipment in the laminar flow hood. Specifically prepare Caco-2 media with 150 μg/ml gentamicin and warm this media in 37°C water bath; for each 24 well plate used, a total of 25 ml Caco-2 media with 150 μg/ml gentamicin needs to be prepared.
(2) At 30 min before infection carefully aspirate old Caco-2 media without antibiotics from the wells in the 24-well plate and gently add 1 ml of fresh pre-warmed Caco-2 media without antibiotics to each well.
(3) Add appropriate amount of *L. monocytogenes* (either in PBS or directly from culture, depending on growth protocol, see 3.5 above) to each well. Use at least one well as an uninoculated control. For *L. monocytogenes* cells grown at 30°C without shaking infect each well with 10.5 μl of undiluted culture (2.0×10^9 bacteria/ml). Push 24 well plate back and forth to distribute inoculum within wells.
This is time point **T=0**.
(4) Immediately after the infection bacteria present in the inoculum need to be quantified by spread plating (see 4a) or using the spiral plater (see 4b); this step can be eliminated when large scale invasion efficiency screenings are performed. While people completing on-going experiments should not switch the plating method used, enumeration using the spiral plater is the recommended procedure.
(4a) Spread plating protocol: Spread plate 100 μl of the 10^{-6} and the 10^{-7} dilutions of the o/n cultures on BHI in duplicate.
(4b) Spiral plating protocol: Spiral plate (select exponential/50 on the machine) the 10^{-5} dilution of the o/n culture on BHI in duplicate.
(5) At time point **T=30 min after infection** gently wash cells in each well 3 times with 1 ml sterile PBS. For each washing step remove the media or PBS from all 24 wells using a vacuum pump with an attached Pasteur pipette and then add PBS to all 24 wells. [**pump should be on low vacuum**; needle on gauge should barely exceed 0 psi (or mm Hg) without Pasteur pipette attached]. After the last PBS wash, gently add 1 ml pre-warmed Caco-2 media **without antibiotics**. Return to incubator for 15 min.

(6) At time point **T=45 min after infection** remove media from each well using a vacuum pump with an attached Pasteur pipette and then add 1 ml Caco-2 media with 150 ug/ml gentamicin. Put water on ice so that it will be cold for recovery step (#7, below).
(7) At time point **T=90 min after infection** remove old media, gently wash cells 3 times with 1 ml of sterile PBS, and add 500 ul of cold sterile DI water to all wells and then pipette up and down vigorously (be consistent with pipetting pattern between replicates, i.e., vigorously pipette water into different areas of the well in a consistent pattern for a total of at least 30 pipetting cycles) to lyse and detach infected cells.
(8) Transfer lysed tissue culture cell suspension from each well to a 1.5 ml microcentrifuge tube and vortex for approximately 30 s to complete lysis of Caco-2 cells.
(9) Enumeration of intracellular (invaded) bacteria can be performed by spread plating (see 10a) or using the spiral plater (see 10b). While people completing on-going experiments should not switch the plating method used, enumeration using the spiral plater is the recommended procedure.
(10a) Spread plating protocol: Spread plate 100 µl of the undiluted, 10^{-1}, 10^{-2} and 10^{-3}dilution of the lysed tissue culture cells on BHI plates in duplicate. If the invasion phenotype of specific mutants is well known the dilutions plated can usually be reduced and adjusted. It is often sufficient to plate 100 µl of the 1:1 and 1:10 dilutions for strains expected to be deficient in invasion and to plate 100 µl of the 1:100 and 1:1000 dilutions for wild type strains.
(10b) Spiral plating protocol: Spiral plate (select the exponential/50 option) appropriate dilutions on BHI in duplicate. Typically, plating the undiluted lysed cell suspension is sufficient for invasion attenuated strains and plating either the 10^{-1}or 10^{-2} dilution is sufficient for fully invasive strains.

Overview table of major steps in the invasion assay and their timing

Step #	**Timing** (*t=0h is time of LM addition to Caco-2 cells*)	**Protocol #**	**Brief Description**
1	***-48 hours***		Seed 24 well plate
2	***-18 hours*** (time variable depending on growth protocol used)		Start growth *L. monocytogenes* strains or isolates 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 *L. monocytogenes*, enumerate inoculum
6	***30 min***		Wash infected cells, add fresh medium
7	***45 min***		Remove media, add new media with gentamicin
8	***90 min***		Wash infected cells, lyse cells with cold dist. water, enumerate invaded (i.e., intracellular) *L. monocytogenes*

SECTION 4 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 subcultured
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 5 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. Also, during washes do not disrupt monolayer by pipetting fluid directly on top of monolayer. Instead, add media/ PBS to sides of well at a slow rate. This problem may also be caused is cells are healthy. Proper trypsinization of the cells is an easy way to permanently damage the cells. If cells appear unhealthy (i.e. Detachment, unhealthy appearance of monolayer, cell vacuolization: do not use cells for an invasion assay)

SECTION 6 REFERENCES

Martin B. M. 1994. Tissue Culture Techniques: An Introduction. Boston: Birkhauser.

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