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## Method Article

# A modified method for rapid quantification of *Chlamydia muridarum* using Fluorospot



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## ABSTRACT

Although manual enumeration of *Chlamydia* inclusion forming units is the most widely accepted means of quantification in the field, it is both time consuming and subject to inherent investigator bias. We report here a rapid, *i.e.*, minutes vs. hours, modified automated Fluorospot means of assessment that is linear (<1200 dots per well). Because the Fluorospot enumerated tissue culture plate/well can also be quantified using traditional manual counting, newly derived Fluorospot data can easily be compared to previously established manual enumeration data requiring no new reference norms.

- Concurrent enumeration of chlamydial IFU using automated and manual methods of counting on same tissue culture plate.
- Rapid method of counting chlamydial IFU reducing time from hours to minutes.

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## ARTICLE INFO

**Method name:** A modified method for rapid quantification of *Chlamydia muridarum* using Fluorospot

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## Specifications Table

Subject Area:	<i>Immunology and Microbiology</i>
More specific subject area:	Infectious Disease
Method name:	A Modified Method for Rapid Quantification of <i>Chlamydia muridarum</i> using Fluorospot
Name and reference of original method:	Wang, S., et al., A novel automated method for enumeration of <i>Chlamydia trachomatis</i> inclusion forming units. <i>J Immunol Methods</i> , 2007. 324(1-2): pp. 84-91.
Resource availability:	McCoy cells Black 96-well tissue culture plates with clear bottom (Corning, NY, USA). Phosphate buffered saline (PBS) 4% (v/v) Saponin (aqueous solution) Eagle's Minimum Essential Medium (EMEM) EMEM with 10% (v/v) FBS (EM-10) 0.05% (w/v) trypsin- Ethylenediaminetetraacetic acid (EDTA) in PBS Cycloheximide Gentamicin Primary (Rabbit anti- <i>C. muridarum</i> outer membrane complex) and secondary (Alexa Fluor (AF)488-conjugated Goat, anti-Rabbit) antibodies 4',6-diamidino-2-phenylindole (DAPI) nuclear stain

## Method details

*Chlamydia trachomatis* (*Ct*) is a Gram-negative obligate intracellular pathogen, and currently a leading cause of sexually transmitted infection (STI) worldwide. It is estimated that each day more than 1 million STIs are acquired, and each year some 130 million new *Chlamydia* infections arise worldwide. In 2014, over 1.4 million cases were reported in the United States taxing the American healthcare system in excess of \$500 million in direct medical costs [1]. Due to the asymptomatic nature of *Ct* infection, an exact number of infected individuals remains unknown [2]. Because *Ct* is an obligate intracellular bacterium, if left untreated in either symptomatic or asymptomatic patients, infection leads to development of ectopic pregnancy, infertility, and pelvic inflammatory disease (PID) [3,4]. The incidence of *Ct* has increased worldwide due to lack of organized healthcare infrastructure [5] in Third World developing countries, the unavailability of a vaccine [6,7], and asymptomatic nature of the infection [8,9].

Quantification of *Chlamydia* inclusion forming units (IFU) is a laborious process requiring growth of confluent monolayers of HeLa or McCoy cells in a 6, 24, 48 or 96 well tissue culture plate format [10–12]. Subsequently, the confluent monolayer must be infected with either genital swab or tissue homogenate material for 20–48 h depending upon serotype. Once established, cells are fixed, stained with serotype specific primary antibody, and subsequently labeled with a fluorophor conjugated secondary antibody. Following labeling, the well contents are typically enumerated manually using a fluorescence microscope, and 5–20 random fields per well. This is both labor intensive and time consuming especially in time course studies requiring multiple periodic sampling over several days. Furthermore, manual quantification may incur an inherent investigator bias toward a preconceived outcome.

Various methods, i.e., PCR, ELISA, and flow cytometry have been employed for quantification of chlamydial IFU [13–23]. While these techniques are available and in use, the inability of PCR to discriminate between active or residual infection [20]; the high detection threshold limit required for ELISA [16]; the inability of flow cytometry to provide accurate information regarding size and structure of chlamydial IFU or its location within a cell [17], and the labor intensive nature of immunochemical approaches [16] requiring highly trained technical personnel underscores the need for alternative approaches. We report here a modified, alternative, Fluorospot assay for visual inspection of infected host cells with the added capability of concurrent traditional enumeration allowing comparison of newly derived automated Fluorospot findings to previously well-established manually based *Chlamydia* norms.

## Description of equipment

### Scanning of stained cells

CTL Immunospot S6 Universal Analyzer and Immunospot Easy-Count software (Cellular Technology Limited, Cleveland, OH, USA) were used to enumerate positively stained cells. Manufacturer's recommended analyzer parameter settings: counting mask size (%), 100; normalize counts of mask 'off'; sensitivity, 200; minimum spot size, 0.0009 sq.mm; maximum spot size, 10.1012 sq.mm; oversized spots were estimated; spot separation, 0.00; diffuseness, small; objects, inverted detailed; over-developed area handling 'active'; background balance 'on'; background balance, 100; fill holes 'off'; hair removal 'on'; audit spots 'inactive'; edge effect compensation 'on', edge compensation level, 5.0; separation enhancement 'on'; aperture [pix], 50, and enhancement weight 50.

## Experimental procedures

### Bacteria

*C. muridarum* seed stocks were propagated in HeLa 229 cells. At 24 h *post* infection, HeLa cells were mechanically disrupted using glass beads. Following high-speed centrifugation (40,000 × *g* for 30 min at 5 °C), bacterial pellets were purified on a Renografin (E.R. Squibb and Sons, Inc., Princeton, NJ, USA) gradient as previously described [24]. Briefly, the cell monolayer was inoculated with stock *C. muridarum* ( $1 \times 10^7$  IFU), and rocked for 1 h at room temperature. Subsequently, the flask was incubated for 1 h at 37 °C in 5% CO<sub>2</sub>. Culture media was removed and replaced with fresh EM-10 (Eagle's Minimum Essential Media (EMEM), with 10% Fetal Bovine Serum, FBS) containing gentamicin (50 µg/mL), and cycloheximide (0.5 µg/mL). The monolayer was monitored for formation of inclusion bodies at ~20 h *post* inoculation. Once inclusions are visible microscopically, cells are harvested by scraping the monolayer into a 50 mL canonical tube while on ice. The contents were sonicated on ice for 30 s to release *C. muridarum*. The cell suspension was centrifuged at 300 × *g* for 10 min at 5 °C, and the resulting supernatant was centrifuged at 40,000 × *g* for 30 min. The supernatant was decanted, and pellet material was resuspended in 1 mL Sucrose-Phosphate-Glutamate (SPG) media, and stored at –80 °C until used. Throughout this study, the same *C. muridarum* seed stock was used.

### Mice

Four-six week old C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME), and allowed to acclimate for at least 1 week prior to experimentation. All experiments were conducted in compliance with guidelines issued by the Institutional Animal Care and Use Committee of The University of Texas at San Antonio (approved protocol MU012).

### Infection

- 1 One day prior to infection, cells were seeded at  $5 \times 10^4$  cells per well (black 96 well tissue culture plate with a clear bottom, Corning, NY, USA).
- 2 The tissue culture plate was incubated overnight at 37 °C in 5% CO<sub>2</sub>. Cell confluence was observed every few hours on the day of infection, and infection was carried out only if the monolayer was 70–90% confluent.
- 3 At confluency, culture media was removed. EMEM (200 µL) was added to each well, and incubated for 30 min at 37 °C in 5% CO<sub>2</sub>.
- 4 Dilution of samples:
  - a Elementary Body (EB) stock titration: A1:100 stock dilution was prepared by addition of 10 µL EB stock to 990 µL SPG. A 1:10,000 starting dilution was prepared by adding 10 µL 1:100 stock dilution to 990 µL SPG. A 3-fold serial dilution was set up for as many serial dilutions required.

- b Sample obtained by swabbing: The swab head was clipped and immersed aseptically in a sterile 1.5 mL tube containing 500  $\mu$ L sterile SPG, and was agitated thoroughly, *i.e.*, vortexed for 1 min. A 100  $\mu$ L aliquot was removed and transferred to 400  $\mu$ L sterile SPG (1:5), and mixed by vortexing. For *C. trachomatis* (all days) and *C. muridarum* from day 10 onward, a dilution series of 1:50 or 1:100 was used. For *C. muridarum* (days 1–9), a 1:100 or 1:150 dilution was used due to higher bacterial titers.
- 5 EMEM was removed by aspiration, and the monolayer was not washed. Aliquots (50  $\mu$ L) of each diluent were carefully transferred onto the side of each respective well, placed in a centrifuge plate holder, and centrifuged for 1 h at  $500 \times g$ .
- a Following centrifugation, EM-10 of media containing 2  $\mu$ g/mL cycloheximide and 10  $\mu$ g/mL gentamicin was added and infected plates were incubated for 20–24 h (*C. muridarum*) or 40–60 h (*C. trachomatis* serovars), *post* initial infection.

### Staining

Staining of *Chlamydia* IFUs was accomplished as previously described [10].

- 1 Each wash step consisted of aspiration of the contents of each well followed by addition of 200  $\mu$ L 1X Phosphate Buffered Saline (PBS), and immediate removal of the well contents by aspiration.
- 2 Cells were fixed with 150  $\mu$ L 4% (v/v) paraformaldehyde for 10 min at room temperature followed by aspiration of well contents.
- 3 Perforation of cells was achieved by addition of 150  $\mu$ L 4% saponin (v/v) for 30 min at room temperature. The contents of the well were removed by aspiration, and fixed cells washed 2X's with PBS.

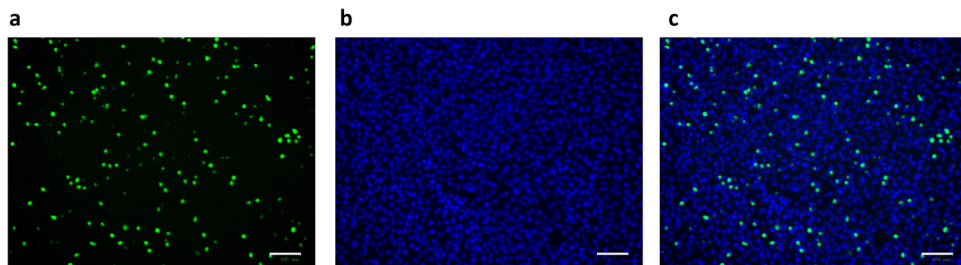
Note: 150  $\mu$ L 0.1% (v/v) Triton-X100 in PBS can be used, but greater repeatability was observed with 4% saponin.

- 4 Blocking was achieved by addition of 200  $\mu$ L EM-10 media for 30 min at room temperature overnight at 4 °C followed by aspiration.
- 5 100  $\mu$ L primary antibody (rabbit anti-*C. muridarum* outer membrane complex was kindly provided by Dr. Guangming Zhong, Department of Microbiology, Immunology, and Molecular Genetics, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78229) diluted 1:2000 in EM-10 media was added, incubated at 37 °C for 1 h, and washed 3Xs with PBS.
- 6 100  $\mu$ L secondary antibody (AF488-conjugated goat anti-rabbit, Sigma Chemical, St. Louis, MO) diluted 1:400 and 4',6-diamidino-2-phenylindole (DAPI, Sigma Chemical, St. Louis, MO) diluted 1:1000 in blocking EM-10 media was added to each well, incubated at 37 °C for 1 h, and washed 3 times.
- 7 Following addition of 50  $\mu$ L 1X PBS, IFUs were enumerated.

### Enumeration

#### Manual

- 1 Starting with the highest dilution at 10-X magnification, the number of inclusions in the center, upper right, lower right, lower left, and upper left sections of the well were counted.
- 2 If the cell monolayer does not cover the entire field or has swaths missing, the field was moved past the inadequate area until a full monolayer came into view.
- 3 If fewer than 5 inclusions were visible per field at the lowest magnification, inclusions present in three wells were counted. Conversely, if more than 30 inclusions were visible, the next highest magnification was used and inclusions were counted.
- 4 If the sample infection rate reached 100% (one inclusion per cell), these samples were not counted.



**Fig. 1. Fluorescence microscopy of *Chlamydia* infected HELA 229 cells.** Representative microscopic fields of *Chlamydia* infected host cells stained with (a) AF488-conjugated secondary antibody, (b) DAPI nuclear reagent, and (c) merged image. The white bar represents 100  $\mu\text{m}$ . Infection and fluorescence staining of *Chlamydia* and host cells was carried out as previously described under 'Experimental Procedures'.

- 5 Each dilution was evaluated at the lowest magnification increasing the magnification accordingly. Five fields for each well were counted. The dilution and magnification for each count was recorded.
- 6 Determination of IFU/mL in a given sample was based on the mean number of inclusions per view, the ratio of the view area to that of the total well, dilution factor, and inoculum volume as described below [13].

$$\text{IFU/mL} = (\text{IFU counted}/\text{Fields counted}) (\text{Dilution}) [\pi r^2 / \pi (A/2)^2] / \text{Inoculation vol (mL)}^{-1} \quad (1)$$

Where:  $r^2 = (\text{well radius})^2$

FOV = field of vision

A = FOV eye piece/objective magnification

$\pi(A/2)^2 = \text{Area of radius viewed}$

#### Automated

- 1 Quantification was achieved by scanning using the CTL Immunospot S6 Universal Analyzer.
- 2 Determination of IFU/mL was achieved using the Easy-Count Software as described below:

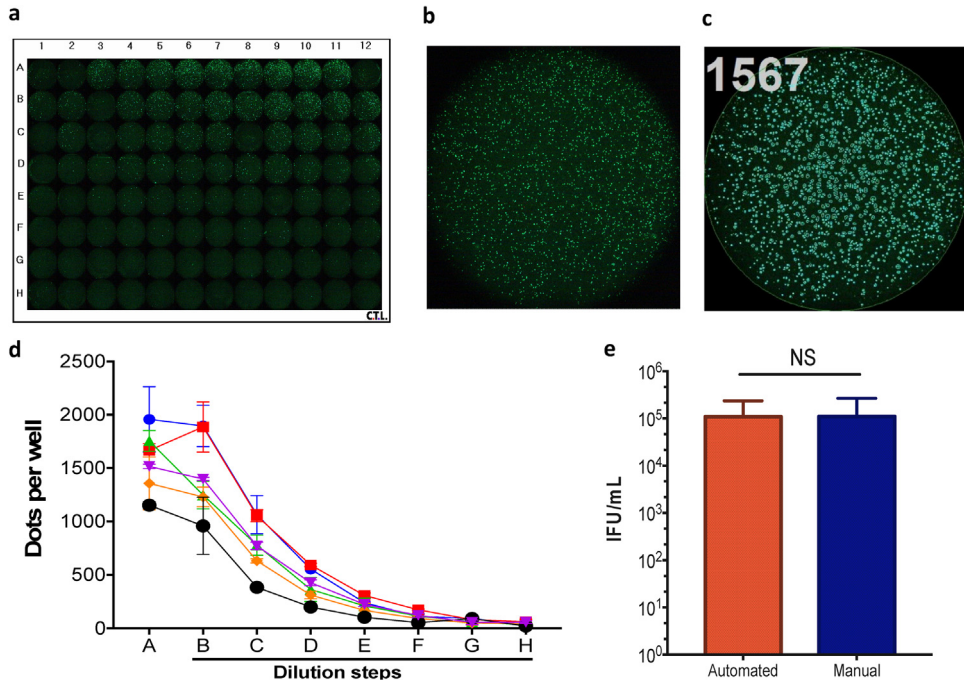
$$\text{IFU/mL} = (\text{Counts/well})(\text{Dilution}) / \text{Inoculation vol (mL)}^{-1} \quad (2)$$

#### Statistical analyses

Statistical analyses were performed using Prism 7 software (GraphPad, La Jolla, CA, USA). All data shown are representative of at least 2 experiments, and each experiment shown was analyzed independently. For multiple comparisons, 2-way ANOVA with Tukey's *post hoc* test (parametric distribution) and Kruskal-Wallis *post hoc* test (nonparametric distribution) were used. Differences were considered statistically significant if  $P < 0.05$ .

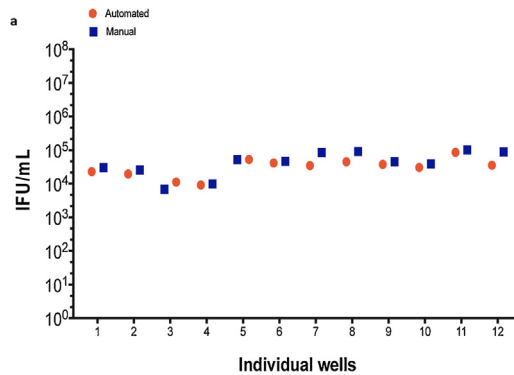
#### Results

Using a black 96-well plate with a clear bottom, McCoy fibroblast cells were seeded at  $5 \times 10^4$  cells per well, incubated overnight, and subsequently infected with  $2\text{--}8 \times 10^8$  *C. muridarum* per well. Fluorescence microscopy was used to evaluate IFUs present, and cell confluency (Figs. 1a and b, respectively). Shown in Fig. 1c is a merged image of Fig. 1a and b showing *Chlamydia* infected confluent cells. Shown in Fig. 2a is a CTL Immunospot S6 Universal Analyzer imaged 96 well plate. Replicates (1 and 2; 3 and 4; 5 and 6; 7 and 8; 9 and 10; 11 and 12) of *C. muridarum* infected cells (Row A-left to right: inocula containing  $1.19 \times 10^3$ ,  $4.71 \times 10^3$ ,  $1.05 \times 10^4$ ,  $1.85 \times 10^4$ ,  $2.86 \times 10^4$ , and  $4.09 \times 10^4$  IFUs), respectively, were serially diluted 2-fold, giving rise to rows B-H. Shown in Fig. 2b is a representative CTL Immunospot S6 Universal Analyzer imaged *Chlamydia* infected well compared to a representative



**Fig. 2. Enumeration of *Chlamydia*.** Cells were infected with *Chlamydia* at a MOI = 1, and serially diluted 2-fold (B–H). (a) Entire 96 well plate imaged with the CTL Immunospot S6 Universal Analyzer. Replicates (1 and 2; 3 and 4; 5 and 6; 7 and 8; 9 and 10; 11 and 12) of *C. muridarum* infected cells (Row A–left to right) contained  $1.19 \times 10^3$ ,  $4.71 \times 10^3$ ,  $1.05 \times 10^4$ ,  $1.85 \times 10^4$ ,  $2.86 \times 10^4$ , and  $4.09 \times 10^4$  IFUs, respectively, and were serially diluted 2-fold giving rise to rows B–H. (b) A representative CTL Immunospot S6 Universal Analyzer imaged infected well. (c) A representative Immunospot Easy-Count Software quantitated well derived from the CTL Immunospot S6 Universal Analyzer image. (d) Graphical analysis of 2-fold serial stock dilutions. Inocula (IFU  $\times 10^3$ ): Black closed circles, 1.19; Orange closed diamonds, 4.71; Purple inverted closed triangles, 10.5; Green closed triangles, 18.5; Red closed squares, 28.6; Blue closed circles, 40.9. Starting, undiluted sample = A. The bar on the 'x' axis represents the respective 2-fold dilution steps (B–H). (e) Comparison of manual and automated quantitation. Using equations 1 and 2 to calculate IFU/mL, the bar graphs represent the average of all values derived for all 96 wells. Infection of cells, fluorescence labeling (Fluorospot ELISA), and calculation of *Chlamydia* IFU values was carried out as previously described under 'Experimental Procedures'. NS = not significant.  $P < 0.05$  Two-way ANOVA with Sidak. Graphical analysis is representative of 2 independent experiments.

quantitated well derived image using the Immunospot Easy-Count Software, *i.e.*, 1567 *Chlamydia* IFU counted (Fig. 2c). Shown in Fig. 2d is a graphical analysis of serially diluted *Chlamydia* inocula. With exception of the starting samples containing  $1.19 \times 10^3$  IFUs (black circles), a nonlinear relationship between IFUs present and dots/well detected was observed for all starting samples as well as their respective diluents. Although the range of linearity for the  $1.19 \times 10^3$  containing sample is narrow, *i.e.*, approximately 1200 down to 200 dots/well, these data points do reflect a general linear relationship between IFU present and dots/well detected supporting the usefulness of this enumeration approach. Shown in Fig. 2e is comparison of IFU/mL values obtained manually to that obtained using the CTL Immunospot S6 Universal Analyzer. Based upon the linearity observed in Fig. 2d (Black dots), results shown in Fig. 2e were based upon enumeration values which fell well within the range of linearity, *i.e.*,  $< 1200$  IFUs prior to calculation of IFU/mL indicating no significant difference between manual and automated approaches. Consistent with *in vitro* *Chlamydia* infected McCoy fibroblast cells (Figs. 1 and 2), no quantitative difference was observed when comparing automated and manual IFU evaluation of *in vivo*, *i.e.*, intravaginally inoculated *Chlamydia* monitored by genital tract swabbing (Fig. 3). Results shown in Fig. 3 were based upon enumeration values (mean  $\pm$  SD =  $15.5 \pm 9.2$ ) which fell within the range of linearity prior to calculation of IFU/mL.



**Fig. 3. *In vivo* Chlamydia challenge-comparison of manual and automated quantitation.** Mice were challenged intravaginally with  $5 \times 10^4$  *Chlamydia* IFU. Respective genital tracts were swabbed 9 days *post* infection. Individual well comparison using manual (Blue closed squares) and automated (Orange closed circles) quantitation. Each data pair represents a different animal. IFU/mL were calculated according to Eqs. (1) and (2).

## Discussion

Considering the high incidence of chlamydial infections [8], manual counting of IFU by microscopy [10,24] is not a practical, high throughput, diagnostic approach nor is it adaptable for large scale screening required for development of vaccines and/or antibiotic candidates. Manual, microscopic counting is very labor/cost intensive requiring trained laboratory personnel, and is inherently prone to technician bias as it requires selection of microscopic fields for enumeration. To overcome these logistical concerns, our findings provide a fast, quantifiable alternate methodology. Values reported here were derived from experimental conditions resulting in confluent reporter cell monolayers. However, a reduced field of view, *i.e.*, 50% analogous to a nonconfluent monolayer resulted in proportionately reduced IFU values when compared to the full field view, *i.e.*, 100% (Data not shown). The method reported here is also amendable to further simplification, *i.e.*, single antibody based direct fluorescence enumeration using AF-488 conjugated antibody targeting specific surface antigens.

Although automation overcomes the pitfalls of manual counting, the gold standard for determining infectious *Chlamydia* sp., is that of manual assessment, *i.e.*, counting by technical personnel. The advantage of the approach reported here resides in further modification of the Fluorospot assay using a black 96-well plate with a clear bottom. Cost effective methodologies, *i.e.*, PCR and flow cytometry have been used for chlamydial detection. However, limitations/drawbacks of these techniques highlight the need for alternative assays. The Fluorospot system is not near as expensive as more sophisticated instrumentation, *i.e.*, whole animal *in vivo* imaging [25] and/or imaging flow cytometry systems [26], that could be employed for chlamydial burden detection in infected samples.

## Author contributions

JK, TF, JPC, RG, BPA conceptualized the study. JK carried out the experimentation. JK, TF, JPC, RG, and BPA analyzed the experimental findings. JK, RG, JPC, and BPA drafted, and edited the manuscript.

## Declaration of Competing Interest

Authors declare that they have no competing interests.

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