APPLICATION NOTE

Reliable quantification of *Candida albicans* by cellometer X2 cell counter: correlation study with qPCR and ddPCR

Authors

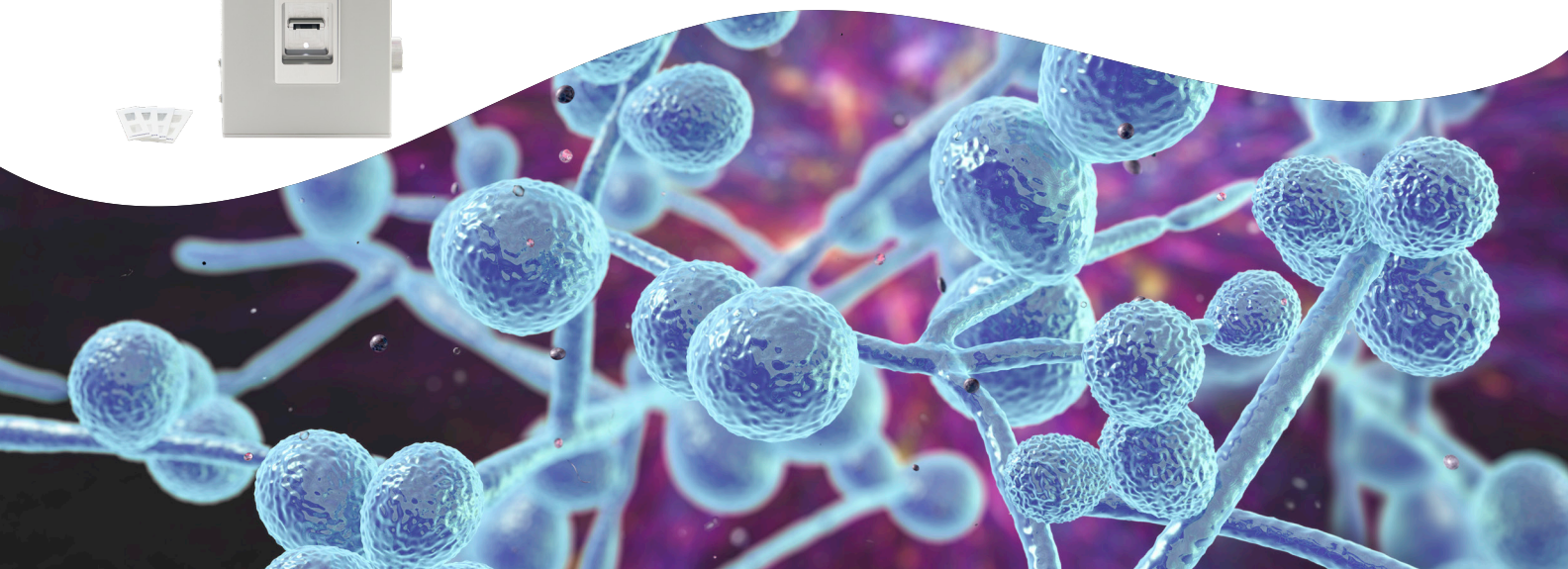
Macy Veling
Peishan Xie
Leo Li-Ying Chan
Mackenzie Pierce
Bo Lin
Yanhong Tong
Revvity, Inc.

Introduction

Candidiasis is a fungal infection that can be invasive and lead to serious illness if not properly treated. It is commonly caused by *Candida albicans (C. albicans)*, which is a top-ranking pathogen that falls within the critical priority group according to WHO in 2022 [1][2]. To facilitate the development of treatment methods, it is crucial to acquire reference *C. albicans* material with reliable quantification information for analytical studies such as limit-of-detection and nucleic acid-based detection.

Many yeast cell quantification methods were established decades ago and it is essential to select a fit-for-purpose method to generate reliable cell counting results to address specific biological questions. Traditional methods such as optical density can provide total cell estimation using standard curves, but they cannot differentiate live and dead cells for proper viability measurement or may generate false-positive signals from air bubbles. Manual counting with a hemacytometer under a standard microscope can determine live and dead cells with appropriate staining, but also can be time-consuming for multiple samples. Finally, colony forming unit (CFU) can measure viable clonogenic cell numbers, however, may require days for colony outgrowth and tedious manual counting procedures [3]. In addition, CFU is unable to measure dead or dying cells that may still retain genomic material necessary for analytical studies using PCR-based technologies.

For research use only. Not for use in diagnostic procedures.



Previously, several studies have demonstrated that CFU typically provides fewer viable cell counts compared to the actual stock due to plating efficiency and variability [4]. Nevertheless, this type of method generally requires larger sample sizes and is labor-intensive and time-consuming [5]. In the recent decade, the Cellometer X2 automated cell counter in combination with fluorescent stains has demonstrated to be a reliable image-based cell counting and viability method that can reduce hands-on time, making it more suitable for routine use [6].

Alternatively, nucleic acid detection-based methods have provided higher sensitivity and lower variability in quantitative measurement. For example, quantitative PCR (qPCR) can generate absolute quantification but it requires external reference standards. In contrast, newer technology such as droplet digital PCR (ddPCR), provides absolute quantification and no dependency on external reference. It is important to note that both methods require effective lysis to achieve accurate results when the whole intact organism is used [7], specifically for organisms like *C. albicans* which has relatively thick cell wall/biofilm structures making it difficult to be fully lysed.

Currently, there is no available reference count for *C. albicans* that can be used to understand the reliability and correlation between the Cellometer X2 direct cell counting method and nucleic acid-based qPCR or ddPCR methods. Here, we present a quantification correlation study between the Cellometer X2 automated cell counter (Revvity, Cat. # CMT-X2-S150) and the two nucleic acid detection methods (qPCR and ddPCR) using *C. albicans* and an in-house developed short lysis procedure.

Materials and Methods

C. albicans fresh culture preparation

C. albicans frozen stock was received from BEI (Cat. #NR-29445) and seeded on Candida BCG agar plate (Edge Biologicals) for two days at 30°C. After the formation of visible colonies, they were collected and resuspended in 1X PBS buffer. The resuspended fresh culture stock concentration was defined as 100X and stored at 4°C for up to 14 days before testing.

Cell staining and quantification using Cellometer X2 cell counter

The resuspended fresh culture stock (100X) was diluted with the yeast dilution buffer (Revvity, Cat. #CSK-0102-2mL) at a 1:1 or 1:4 ratio. 20 µL of each diluted sample was mixed with 20 µL of the yeast acridine orange/propidium iodide (AO/PI) dye (Revvity, Cat. # CSK-0102-2mL), incubated for 1 min, and subsequently loaded into the disposable counting chambers (Revvity, Cat. # CHT4-SD100-002). The cell counting slide was then inserted into the Cellometer X2 and the cells were allowed to settle prior to image acquisition and analysis. The samples were measured in triplicate per testing condition. The Cellometer X2 utilized one bright-field and dual-fluorescent imaging modes to automatically generate stock concentration, viability, and fluorescent intensity of target cells [6].

qPCR quantification

A 10-fold serial dilution was performed by using the resuspended fresh culture stock (100X) and sequentially diluted into an in-house developed lysis buffer (Revvity, Cat. # CMV-ELU-384) to generate concentrations at 10, 1, 0.1, 0.01, and 0.001X. Six replicates per concentration were prepared individually. 50 µL of each concentration was heat-treated at 95°C for 20 min. 10 µL of the heat-treated sample was then loaded to setup a final 15 µL TaqMan-based PCR reaction, with reagent A and reagent C from the *Candida auris* Real-Time PCR Kit (Revvity, Cat. #DXMDX-RGT-1001). Primers and probe specific to a *C. albicans* target gene, which presented as a single copy in the whole genome [8], were used for detection. A ddPCR quantified synthetic *C. albicans* plasmid with 5-fold serial dilution was used as the quantification standard. qPCR was performed on a QuantStudio™ Dx Real-Time PCR Instrument with 96-well fast block (Thermo Fisher). Standard curve calculation and analysis were calculated by the Quantstudio™ Test Development Software (version 1.0.3). Real-time PCR cycles were run as 37°C/2min, 94°C/10min, 40 cycles (94°C/10s, 62°C/15s, 65°C/45s).

ddPCR quantification

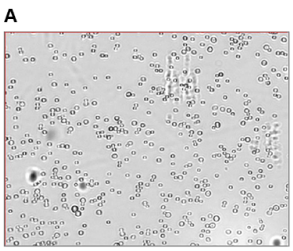
The resuspended fresh culture stock (100X) was diluted in an in-house developed lysis buffer (Revvity, Cat. # CMV-ELU-384) to generate a final concentration of 1X. Triplicates of 50 µL samples were heat-treated at 95°C for 20 min. After heat lysis, each sample was further diluted in 1X TE buffer to final concentrations of 0.1 and 0.01X. 5 µL of each testing concentration was mixed with the ddPCR

Supermix for Probes (Bio-Rad, Cat. #1863024), primers and probe specific to *C. albicans* [8], and 5U of KpnI-HF restriction enzyme (New England Biolabs, Cat. #R3142S). The reaction mix was incubated at 37°C for 30 min. The manufacturer recommended ddPCR experimental workflow was followed. 20 µL of reaction mix and 70 µL of Droplet Generation Oil for Probes (Bio-Rad, Cat. #1863005) were loaded onto DG8 Cartridges. Droplets were generated by the QX200 Droplet Generator (Bio-Rad) and all droplet contents were then transferred to a 96-well PCR plate. After PCR amplification on a C1000 Touch Thermal Cycler (Bio-Rad), the samples were analyzed on the QX200 Droplet Reader (Bio-Rad, version 1.7.4).

Performance Data

Automated cell counting of fresh culture stock

The Cellometer X2 system acquired brightfield and two-fluorescent channels sequentially to provide total, live, and dead cell counts (less than 30 sec per sample) (Figure 1A, 1B). The system software auto-calculated the original stock concentration, viability, and cell mean size based on the dilution factor parameter (Figure 1C). The average concentration of the tested stock concentration in this study was 6.32E8 cells/mL.



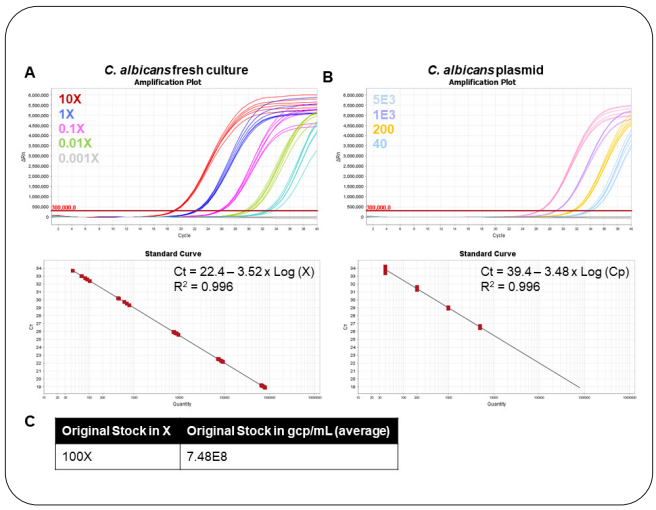
C

Cell Count		Concentration (cells/mL)	Mean Size (µm)
Total	7346	6.38E8	3.7
Live	5386	4.67E8	3.7
Dead	1960	1.70E8	3.7
Viability	73.3%		

Figure 1: *C. albicans* fresh culture stock concentration confirmation in cell numbers by Cellometer X2 cell counter. (A) Example of Brightfield image of *C. albicans*. (B) Example of fluorescent staining image of *C. albicans*. Live yeast is shown in green and dead in red. Scale bar not included in the image. (C) Summary of the culture stock concentration in cells/mL and viability automatically generated by the Cellometer software from the example.

qPCR quantification of fresh culture stock with standard curve

The fresh culture of *C. albicans* treated with an in-house lysis procedure followed by qPCR (total < 2 hours) provides efficient lysis and the average concentration of the original stock is 7.48E8 genome copies per milliliter (gcp/mL), which is approximately 1.2-fold higher than the result provided by cell counting method (Figure 2). Quantified synthetic plasmid was used to generate a standard curve for quantification analysis.



C

Original Stock in X	Original Stock in gcp/mL (average)
100X	7.48E8

Figure 2: *C. albicans* fresh culture stock concentration establishment by qPCR. (A) Amplification curve (top) of the *C. albicans* fresh culture with 10-fold serial dilution to 10X, 1X, 0.1X, 0.01X, and 0.001X concentration. The original stock is defined as 100X. Standard curve (bottom) of the fresh culture demonstrating the efficiency of the reaction (n=6). (B) Amplification curve (top) of the ddPCR quantified synthetic *C. albicans* plasmid at 5E3, 1E3, 200, and 40 copies per rxn (cp/rxn) with standard curve shown below (n=6), suggesting high efficiency of the *C. albicans* primers/probe in the reaction. (C) Average of the original stock concentration by qPCR.

ddPCR quantification of fresh culture stock

The fresh culture of *C. albicans* treated with the in-house lysis procedure followed by ddPCR (total ~4.5 hours) provides similar efficient lysis and the average concentration of the original stock is 3.60E8 gcp/mL, which is approximately 1.7-fold lower than the result provided by cell counting method, and 2-fold lower than the qPCR method (Figure 3). No external reference material is required for ddPCR.

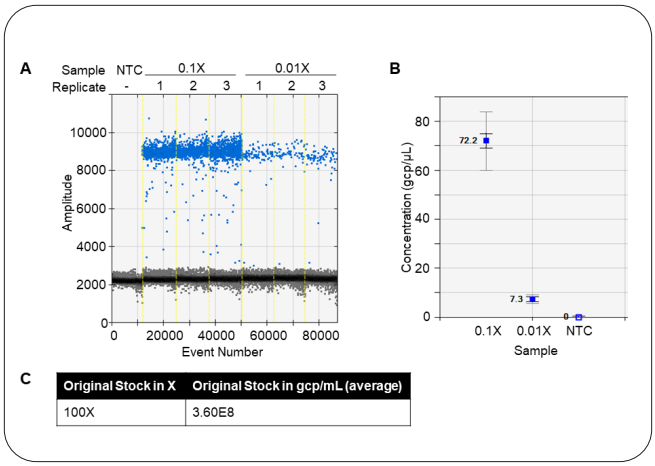


Figure 3: *C. albicans* fresh culture stock concentration establishment by ddPCR. (A) ddPCR amplitude plot of the fresh culture sample at 0.1X or 0.01X concentration (n=3). The original stock is defined as 100X. NTC: no template control. (B) concentration plot of fresh culture at individual tested concentration. Average is shown in the graph. gcp/µL: genome copies per microliter. Grey error bar: total error. Black error bar: Poisson error. (C) Average of the original stock concentration by ddPCR.

Conclusion

The study has demonstrated that the fresh culture stock quantification correlation of *C. albicans* is within 3-fold differences between the automated fluorescence cell counter system and the nucleic acid-based detection methods (qPCR and ddPCR) with the in-house developed lysis procedure. It shows that the Cellometer X2 cell counting system can be used as a routine, rapid, and reliable quantification method for fungi, such as certificate-of-analysis (CoA) of fungi stock, sample stability study, and analytical study sample unit determination.

Reference

1. CDC – Candidiasis: https://www.cdc.gov/fungal/diseases/candidiasis/index.html

2. WHO fungal priority pathogens list to guide research, development and public health action. Geneva: World Health Organization; 2022. Licence: CC BY-NC-SA 3.0 IGO.

3. Kavitha Sankaranarayanan, Raja Prabhu Ramachandran, Raji Sundararajan, Chapter 6 - Electrically-enhanced proliferation control of cancer-stem-cells-like adult human mesenchymal stem cells – a novel modality of treatment, Electroporation-Based Therapies for Cancer, Woodhead Publishing, 2014, Pages 127-159. ISBN 9781907568152. https://doi.org/10.1533/9781908818294.127.

4. Bhatti MA, Hjertstedt J, Hahn BL, Sohnle PG. Inefficient delivery of yeast cells as an explanation for reduced plating efficiency of Candida albicans. Med Mycol. 2002 Oct;40(5):465-9. doi: 10.1080/mmy.40.5.465.469. PMID: 12462525.

5. Wang R, Lorantfy B, Fusco S, Olsson L, Franzén CJ. Analysis of methods for quantifying yeast cell concentration in complex lignocellulosic fermentation processes. Sci Rep. 2021 May 28;11(1):11293. doi: 10.1038/s41598-021-90703-8. PMID: 34050249; PMCID: PMC8163860.

6. Berkes CA, Chan LL, Wilkinson A, Paradis B. Rapid quantification of pathogenic fungi by Cellometer image-based cytometry. J Microbiol Methods. 2012 Dec;91(3):468-76. doi: 10.1016/j.mimet.2012.09.008. Epub 2012 Sep 15. PMID: 22985717.

7. Vaitilingom M, Gendre F, Brignon P. Direct detection of viable bacteria, molds, and yeasts by reverse transcriptase PCR in contaminated milk samples after heat treatment. Appl Environ Microbiol. 1998 Mar;64(3):1157-60. doi: 10.1128/AEM.64.3.1157-1160.1998. PMID: 9501455; PMCID: PMC106384.

8. Bui HTV, Bui HT, Chu SV, Nguyen HT, Nguyen ATV, Truong PT, Dang TTH, Nguyen ATV. Simultaneous real-time PCR detection of nine prevalent sexually transmitted infections using a predesigned double-quenched TaqMan probe panel. PLoS One. 2023 Mar 6;18(3):e0282439. doi: 10.1371/journal.pone.0282439. PMID: 36877694; PMCID: PMC9987813.

Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA
www.revvity.com

For a complete listing of our global offices, visit www.revvity.com