APPLICATION NOTE

A quick and direct Candida albicans lysis efficiency comparison using the Cellometer X2 fluorescent cell viability counter.

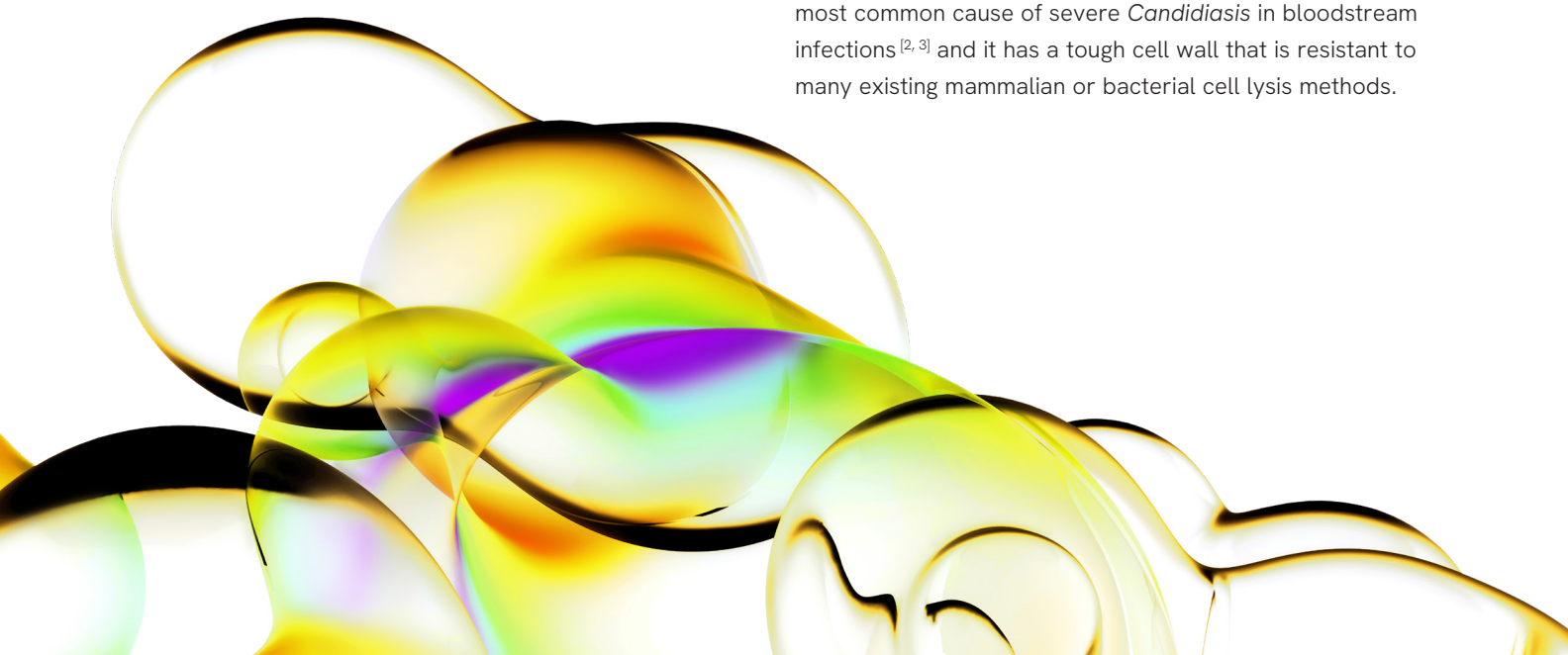
Authors

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

Revvity Inc.

Introduction

A fit-for-purpose lysis method with easy visualization/detection is crucial for laboratories to perform any research activities involving nucleic acid and protein detection or cell membrane integrity measurement. Attractive lysis methods that have been broadly used including thermal lysis, mechanical force, chemicals, biologicals, or sonication (a combination of chemical and physical methods). Existing outcomes for efficiency measurement can be categorized into two major characteristics: phenotype or genotype, each with its own strengths and weaknesses. For instance, nucleic acid-based detection has been well established for lysis efficiency studies but is sometimes not feasible for direct measurement due to inhibition caused by chemicals. As such, sample purification is often needed prior to detection, which could introduce process variation. Alternatively, direct lysis efficiency can be measured by image-based technology. Traditional brightfield microscopy is cost-effective but has limited resolution with only one staining dye option. Multi-color fluorescent microscopy, such as confocal, can provide more biophysiological information at cellular and sub-cellular levels for its price but is not necessarily needed for a simple lysis efficiency study. Here, we utilized the Cellometer™ X2 fluorescent cell viability counter, a simple dual-fluorescent-based cell counting instrument generally used for measuring stock concentration, viability, and cell size/diameter [1], to support direct lysis efficiency studies. Lysis methods from thermal lysis, chemical-based buffers, and enzymatic reagents of choice are demonstrated in the study. *Candida albicans (C. albicans)* is selected as the yeast model microorganism because it is the most common cause of severe *Candidiasis* in bloodstream infections [2, 3] and it has a tough cell wall that is resistant to many existing mammalian or bacterial cell lysis methods.



Materials and methods

Sample preparation for pH-based chemical and thermal combined lysis study

Freshly grown C. *albicans* culture on an agar plate was collected and resuspended in 1X PBS buffer. The starting culture stock concentration is defined as 100X. The resuspended 100X culture stock was diluted to 10X in lysis buffer differing by pH (8.0, 9.5, or 12). Heat treatment was applied at 95°C for 5, 10, or 20 minutes (min) to each sample. Controls at 0 min without heat treatment for each lysis buffer type were included for comparison. After incubation, 15 µL of each 10X sample was mixed with 15 µL of the yeast acridine orange/propidium iodide (AO/PI) dye from the ViaStain™ Yeast Kit (Revvity, Cat. #CSK-0102-2mL). 20 µL of the dye-mixed sample was then loaded into a disposable counting chamber (Revvity, Cat. # CHT4-SD100-002) and subsequently inserted into the Cellometer X2 fluorescent cell viability counter (Revvity, Cat. # CMT-X2-S150). The cells were allowed to settle inside the chamber for 5 mins incubation prior to image acquisition and analysis. All images were captured at a final 5X concentration.

For the qPCR experiment, a serial dilution of the 100X culture stock was performed to reach final concentrations at 20, 2, 0.2, and 0.02X. 50 µL of each sample was incubated either at 95°C or room temperature (RT) for 5 mins. 5 µL of each sample was loaded to a final 20 µL PCR reaction (Promega, Cat. # M7405). A set of primers/probe specific to a C. *albicans* target gene, which is present as a single copy in the genome, was applied in the PCR reaction [4]. Three replicates per testing condition were included. QuantStudio™ Dx 96 (ThermoFisher) was used with real-time cycles run at 94°C/2 mins, 40 cycles of 94°C/10 sec, 62°C/15s, and *65°C/45s (*: signal record).

Sample preparation for detergent-based chemical lysis study

A 100X fresh culture in PBS buffer was first diluted to 50X in the same PBS buffer, followed by a second round of dilution to 25X in control buffer (pH 8~8.5), buffer with 1% SDS, or buffer with 1% SDS and Proteinase K (18 mg/mL). The number of total cells per reaction is approximately 1.5E7 cells/rxn. The samples were incubated at RT throughout the whole process. At each time point (0, 15, 30, 60, and 90 min), 3 µL of sample from each tube was taken out and mixed with 12 µL of Yeast dilution buffer and 15 µL of AO/PI dye from the ViaStain Yeast Kit. The same loading and image procedure described above was applied.

Sample preparation for enzymatic-based lysis study

A 100X fresh culture in PBS buffer was first diluted to 50X in the same PBS buffer, followed by a second round of dilution to 25X in control TE buffer (pH 8.0) or TE buffer that contains a total of 5U Zymolyase (Zymo Research, Cat. # E1004). The number of total cells per reaction is approximately 4.3E7 cells/rxn. The samples were incubated at RT throughout the whole process. At each time point (0, 30, 60, 90, and 150 min), 3 µL of sample from each tube was taken out and mixed with 12 µL of Yeast dilution buffer and 15 µL of AO/PI dye from the ViaStain Yeast Kit. The same loading and image procedure described above was applied.

For lysis efficiency comparison between Zymolyase and Proteinase K, a 3-fold serial dilution of the 100X fresh culture in liquid amies (BD, Cat. # 220245) was performed to reach final concentrations at 1, 1/3, 1/9, and 1/27X. The lysis buffer and Proteinase K from the chemagic™ Pathogen NA Kit H96 (Revvity, Cat. # CMG-1033-G) were used in manufacturer-recommended amounts as the control condition. The same lysis buffer with Zymolyase (1U/rxn) spike-in was set up as the test condition. 320 µL of each prediluted sample was spiked into both buffer types and incubated at RT for 30 mins, followed by an automated extraction procedure on the chemagic 360 system (Revvity). Six replicates per testing condition were included. 10 µL of the extracted DNA was loaded to set up a final 15 µL PCR reaction. The Reagent A (PCR buffer) and Reagent C (enzyme mix) from the *Candida auris* Detection Real-time PCR Reagents (Revvity, Cat. # DXMDX-GRT-1001) with the same *customized C. albicans* primers/probe set were used to set up the reaction. CFX 96 (Bio-Rad) was used with real-time cycles run at 37°C/2 mins, 94°C/10 mins, 42 cycles of 94°C/10 sec, 62°C/15s, and *65°C/45s (*: signal record).

Results

pH-based chemical and thermal combined lysis efficiency comparison

Three detergent-free-buffers, ranging from mild to high pH (pH 8.0, 9.5, or 12), were used in combination of heat treatment (HT) at 95°C for 0, 5, 10, or 20 mins for *C. albicans* lysis efficiency comparison. The viability result, measured by the Cellometer X2 fluorescent cell viability countersoftware, showed that the proportion of propidium iodide (PI) positive cells of *C. albicans* (in red) increased from 8.37% to nearly 50% in the mild pH 8.0 buffer as the duration of heat treatment extended from 0 to 10 mins (Figure 1-2). Interestingly, the PI positive cells (49.57% - 49.87%) were also acridine orange (AO) positive (50% - 50.43%) in a 1:1 ratio between the 5- and 10-min heat-treat conditions. This indicated that the entire *C. albicans* population was close to 100% damaged, yet they were not completely lysed or destroyed. In contrast, the viability in both the medium and high pH buffers, with a 5-min heat treatment, showed 0% AO marker, indicating 100% cell death. In fact, the PI marker was almost lost in the high pH 12 buffer even at 5-min heat-treat condition when compared to the medium pH 9.5 buffer, suggesting higher pH further enhances cell destruction to completion. An additional control in the strong pH 12 buffer at 150 mins without heat treatment confirmed that only approximately 20% viability was reduced from 0 min (data not shown), suggesting that a buffer with high pH property alone is insufficient to destroy *C. albicans*, even with 3.5 hours of incubation.

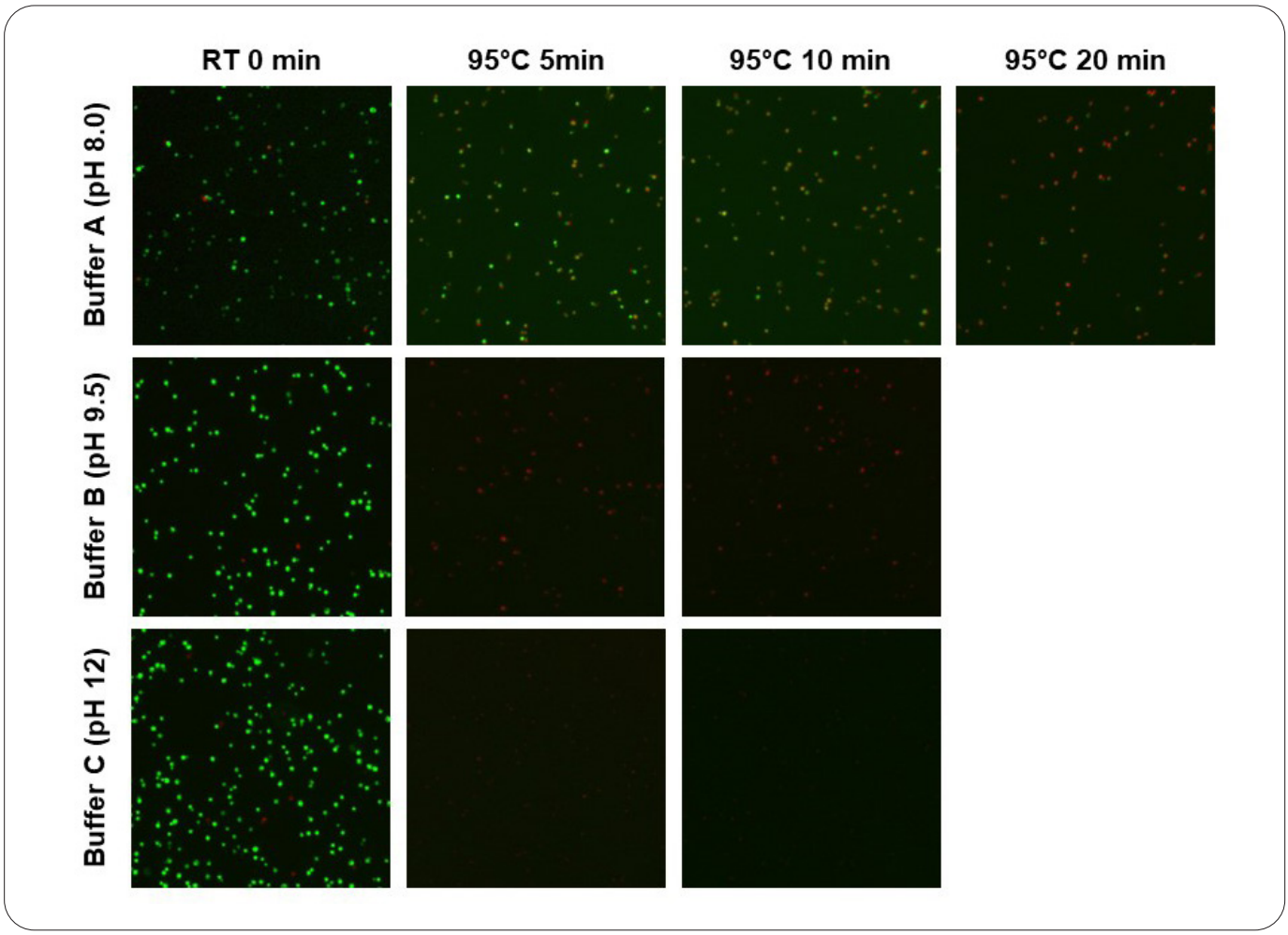


Figure 1: Fluorescent images of *C. albicans* AO/PI staining in buffers with different pH at various heat-treat conditions. Green: AO, live cell marker. Red: PI, dead cell marker. Yellow: merge of AO/PI, damaged cell marker with cell structure retained. RT: room temperature. Scale bar not shown.

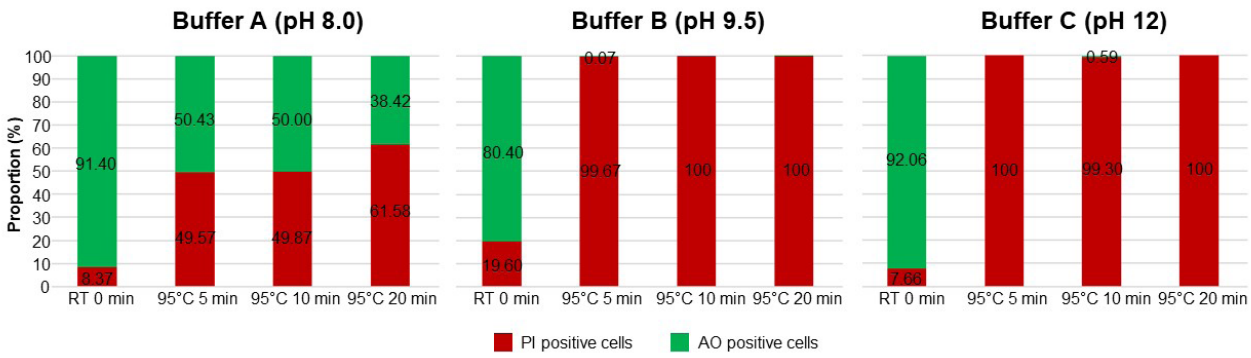


Figure 2: Proportion in percentage of AO or PI positive cells in different lysis buffers at various treatment time points.

To confirm the phenotypic observation that a combination of higher pH and HT enhances lysis efficiency more effective than either one, a genotypic confirmation using qPCR was applied. Given the same absolute input condition, qPCR results consistently showed that the higher pH buffer produces a smaller Ct value than the milder pH buffer, in both room temperature (RT) and HT cases (Figure 3). This confirms the same observation as measured by phenotype. It also revealed that the detection sensitivity is improved under high pH buffer conditions (Figure 3B). Anyhow, the combination of higher pH and HT proved to be the most effective of all. Surprisingly, HT did not seem to improve the efficiency of the pH 8.0 buffer at RT, possibly due to insufficient HT time or the tested concentration ranges being too low to see the differences.

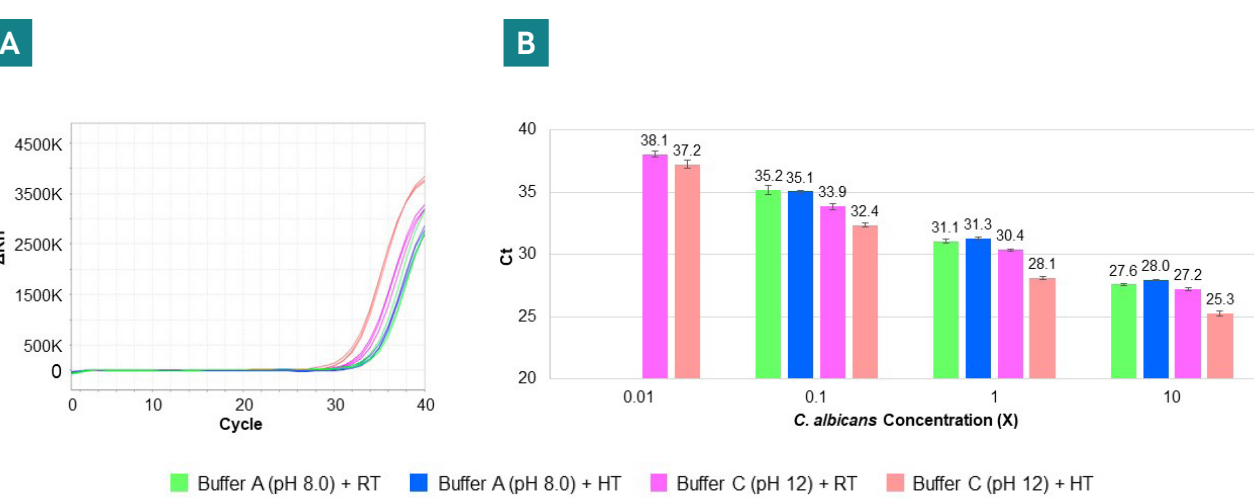


Figure 3: *C. albicans* lysis efficiency comparison between buffer A and C under RT or heat treatment (HT) followed by extraction and qPCR method (n=3 each). (A) Amplification curve shown at 0.1X concentration as an example. (B) Mean Ct value of specific treatment cases at various concentrations (n=3). *C. albicans* were not detected in Buffer A at the 0.01X concentration in both RT and HT cases.

Taken together, both phenotypic and genotypic methods illustrate that the lysis efficiency of the higher pH condition is enhanced by high-temperature treatment.

Detergent-based chemical lysis efficiency comparison

Although buffers with high pH under high-temperature treatment can effectively destroy the viability of *C. albicans* in 5 minutes, it would be more convenient if an RT solution is available to replace the high-temperature step. To explore an RT solution for thick yeast cell wall destruction, a mild basic buffer (pH 8~8.5) with strong detergent SDS was examined over a 90-min time course study. Proteinase K was also included in the study as it is commonly combined with SDS in many bacterial and mammalian cell lysis buffer recipes. The total cell (brightfield), AO positive, and PI positive cell numbers measured at each time point were normalized to the pre-treatment condition for comparison over time. In the control, the cell numbers of total (brightfield), AO positive, and PI positive remained relatively similar from 0 to 90 mins (Figure 4 and 5A). When SDS was added, it led to a 4-fold increase in PI positive cells compared to the control at 15-min incubation. These PI positive cells from 15- to 90-min are also nearly all AO positive at which their ratio was close to 1:1 (Figure 5B). As the total cell numbers remain consistent over the study period, these observations suggested that the buffer with SDS at RT is sufficient to permeabilize the *C. albicans* cell wall at 15 mins but incapable of fully destroying the cell structure even after 90 mins incubation. Strikingly, adding Proteinase K to the SDS buffer didn't seem to improve cell destruction at RT, at least not by phenotypic observation at a fixed time point (Figure 5). Extended incubation up to 150 mins also didn't change phenotypic observation, but effective lytic activity using the same amount of Proteinase K and total cells has been observed by qPCR (data not shown). A time-lapse study of live cells at single-cell resolution will be needed for in-depth phenotypic observation. Nevertheless, this study demonstrates that a buffer with SDS, in the presence or absence of Proteinase K, can effectively damage the cell wall of *C. albicans* at RT, but it is insufficient to fully lyse the cell structure even over a 90 mins incubation. This study also demonstrates the potential of using the Cellometer X2 fluorescent cell viability counterfor direct lysis efficiency study with a strong detergent, which is generally not feasible with qPCR due to detergent inhibition.

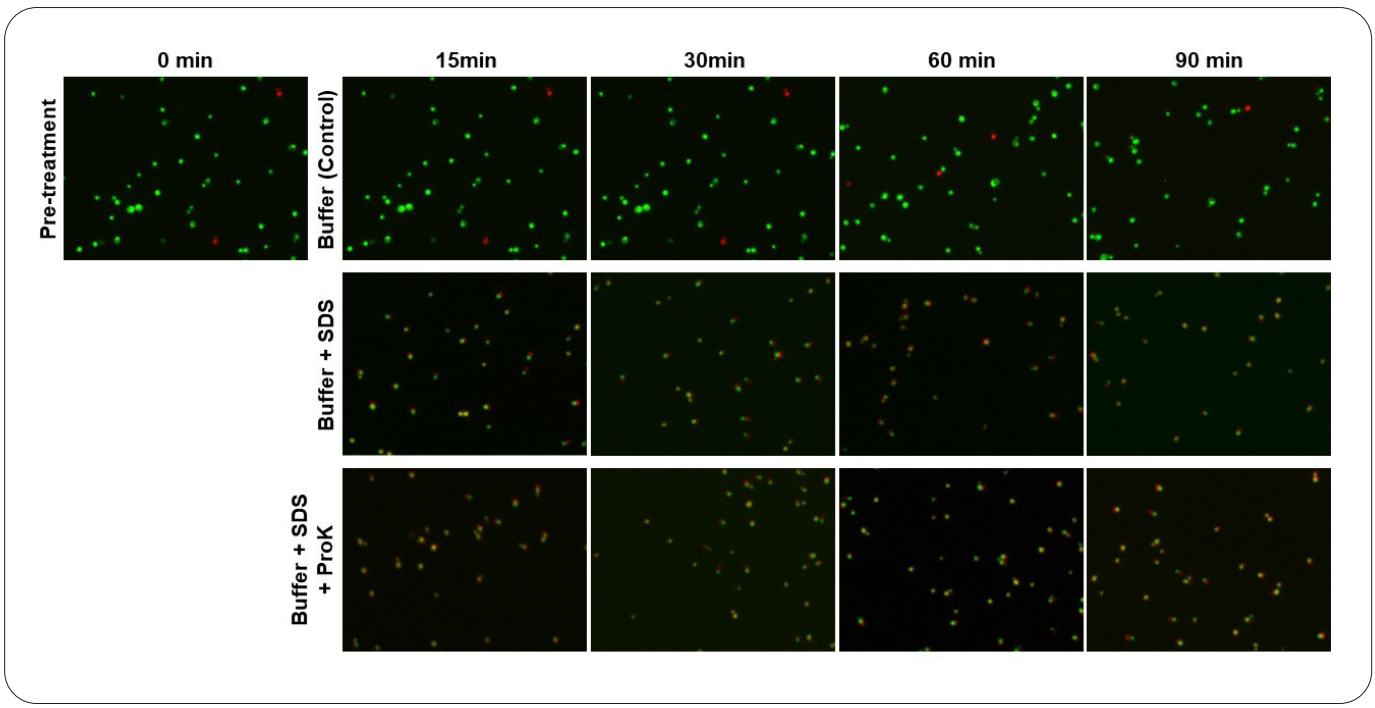


Figure 4: Fluorescent images of *C. albicans* AO/PI staining in control buffer, and SDS buffer with or without Proteinase K (ProK) over a 90 mins RT incubation. Green: AO, live cell marker. Red: PI, dead cell marker. Yellow: merge of AO/PI, damaged cell marker with cell structure retained. Scale bar not shown.

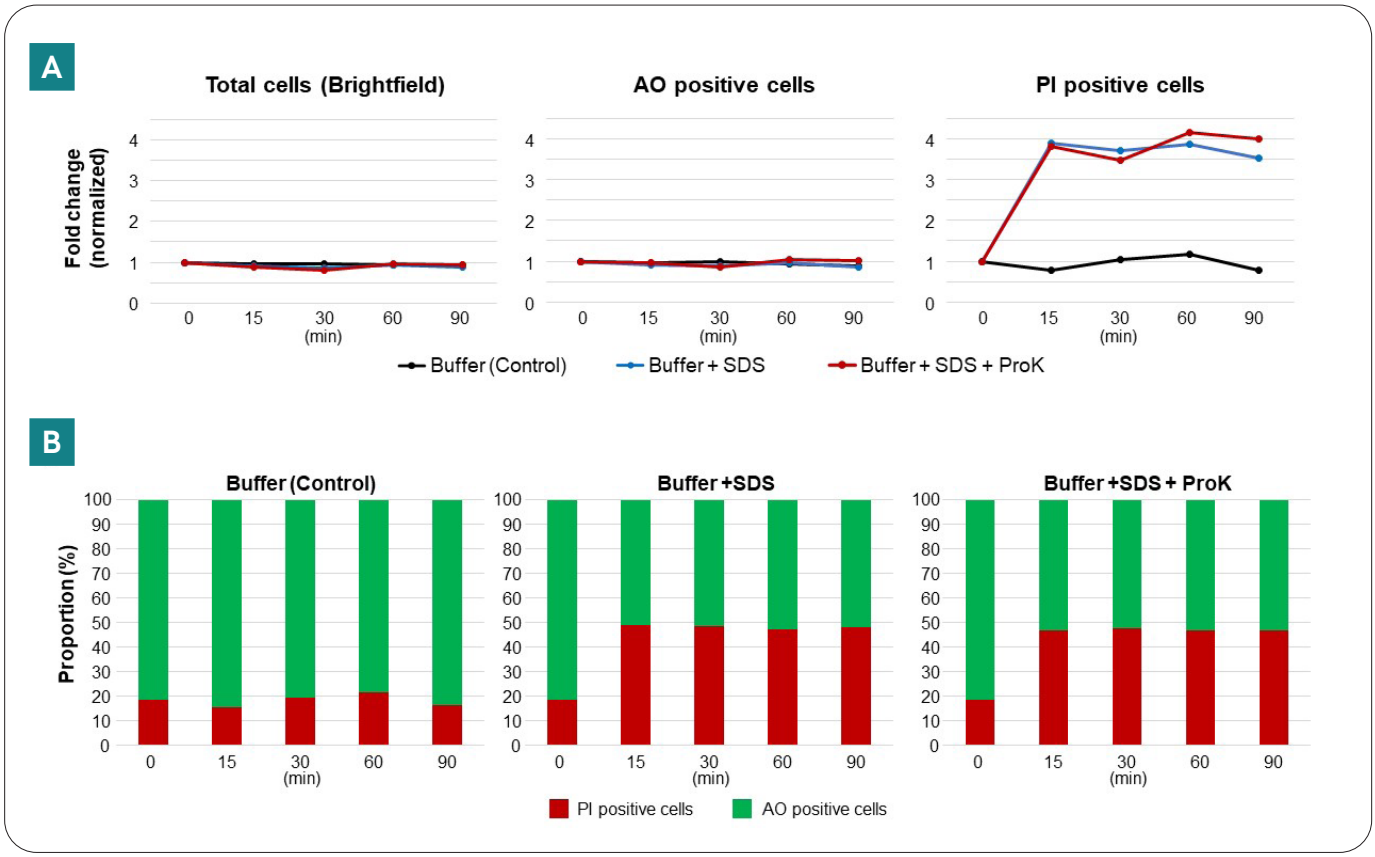


Figure 5: Lysis efficiency comparison of control buffer, SDS buffer, and SDS buffer with ProK over a 90 mins time course at RT. (A) Fold change of total, AO positive, and PI positive cells under each buffer type over time. (B) Proportion in percentage of AO or PI positive cells in individual buffers over time.

Yeast-specific enzymatic lysis efficiency comparison

Given that a buffer with strong detergent, in the presence or absence of Proteinase K, is insufficient to fully lyse *C. albicans* at RT, an alternative enzymatic method [5] using Zymolyase was evaluated over a 150-min time course study. A pre-treatment control and the follow-up timelapse in conditions with or without the Zymolyase were performed for comparison (Figure 6A). The total cell (brightfield) and the AO positive cell numbers measured at each time point were normalized to the pre-treatment condition for comparison over time. As a result, the proportion of cell numbers by total cells and AO positive cells showed a similar degree of cell reduction pattern (Figure 6B). This enzymatic reaction was able to reduce total and AO positive cell numbers down to half in 30 mins and further down to ~5% by 150 mins. In contrast, the control showed that ≥ 70% of cells remained AO positive even at 150 mins. These results suggested that Zymolyase was able to destroy *C. albicans* effectively at RT. Interestingly, the PI marker failed to provide quantitative information. This is because clusters of red signals, which likely were the leftover indigestible genomic material, were observed. Hence, it is not quantitative for total damaged or dead cell count.

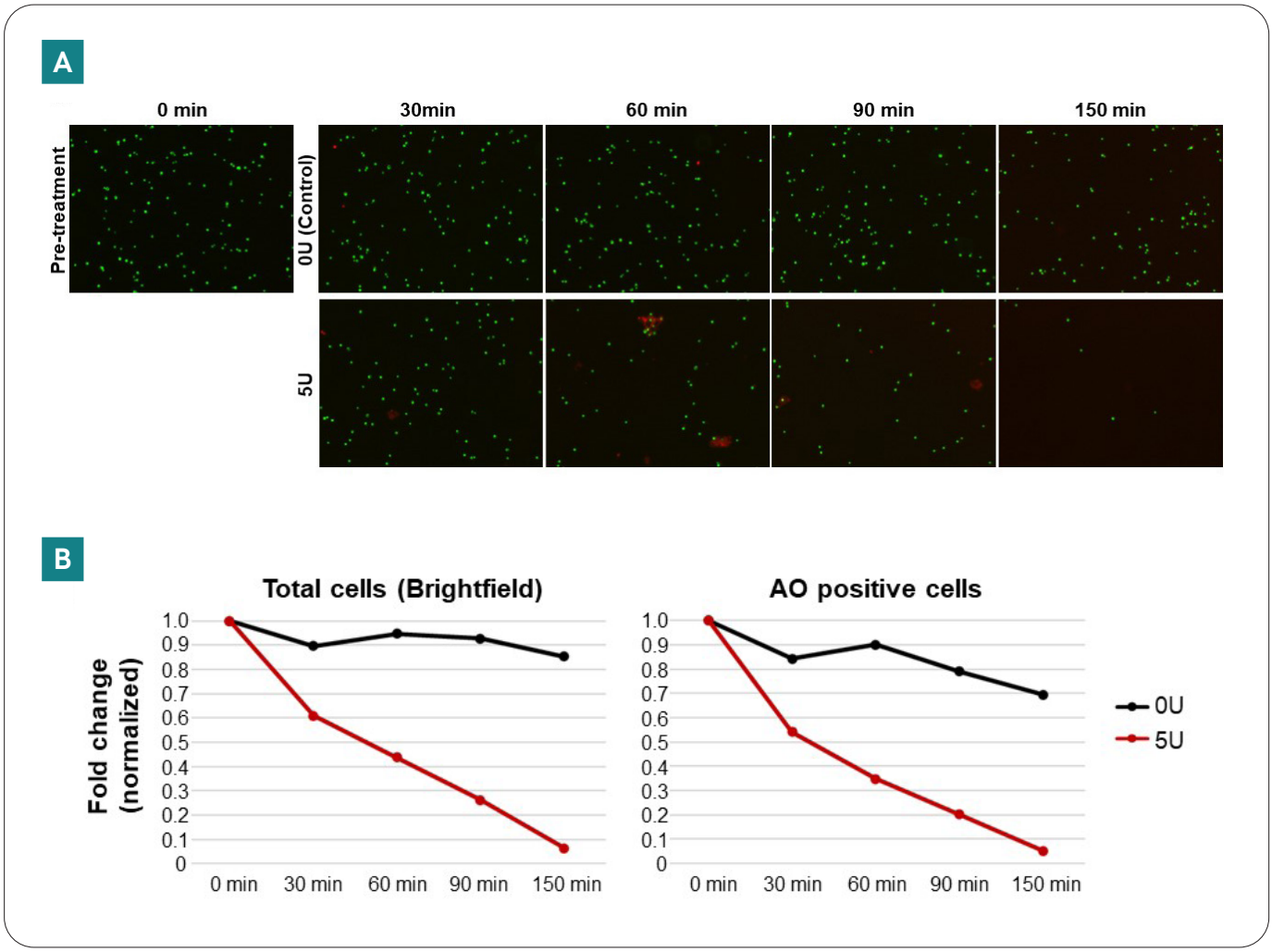
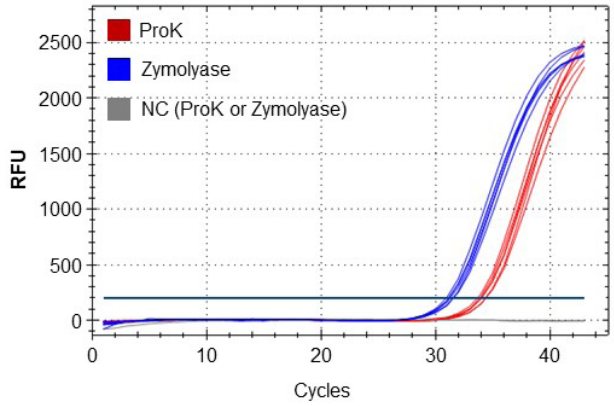


Figure 6: Lysis efficiency with or without 5U Zymolyase over time at RT. (A) Fluorescent images of *C. albicans* AO/PI staining shown at different time points in both control (0U) and Zymolyase treatment (5U). Green: AO, live cell marker. Red: PI, dead cell marker. Scale bar not shown. (B) Fold change of total and AO positive cells over time at each condition for comparison.

To confirm whether Zymolyase is more effective for lysing *C. albicans* than Proteinase K, an RT incubation of either enzyme in SDS buffer followed by extraction and qPCR study was performed. The result showed that the lowest detectable concentration of *C. albicans* is 9 folds more sensitive with Zymolyase than Proteinase K (Figure 7), confirming that Zymolyase indeed has better lysis efficiency than Proteinase K for treating *C. albicans.*

A



B

Input	Conc. (X)	ProK				Zymolyase			
		Ct Mean	Std Dev	N (Hit)	N (Hit) %	Ct Mean	Std Dev	N (Hit)	N (Hit) %
C. albicans	1	34.06	0.29	6/6	100%	31.28	0.24	6/6	100%
	1/3	**36.38**	**1.16**	**6/6**	**100%**	32.71	0.11	6/6	100%
	1/9	37.88	0.68	4/6	66.7%	34.61	0.63	6/6	100%
	1/27	38.84	0.59	2/6	33.3%	**36.70**	**0.73**	**6/6**	**100%**
NC	0	ND		0/1	0%	ND		0/1	0%

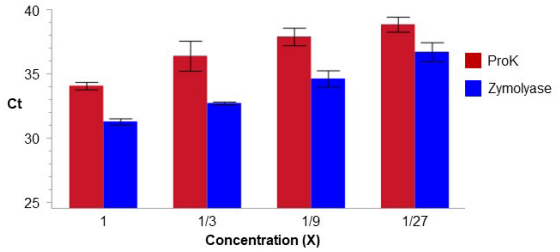


Figure 7: Lysis efficiency comparison of buffer with ProK or Zymolyase at RT by qPCR. (A) Example of qPCR amplification curve of buffer with ProK or Zymolyase at 1X concentration. NC: negative controls with either ProK or Zymolyase. (B) Table and bar chart showed the Ct mean value and detection rate of *C. albicans* at different concentrations between the two buffer conditions.

Overall, both phenotypic and genotypic studies demonstrate that the enzymatic method using Zymolyase provides effective RT lysis on destroying *C. albicans* as it also elevates the sensitivity detection of the species in qPCR assay.

Discussion

A successful RT solution for destroying *Candida* species is important as common lysis reagents designed for killing bacteria and mammalian cells may not be the most effective option, or additional treatment steps to facilitate lysis efficiency may often be required. A simple, affordable platform like the Cellometer X2 fluorescent cell viability counter, together with the ViaStain Yeast Kit, can accelerate such studies by providing quick phenotypic observation in a timely manner. Using the Cellometer X2 fluorescent cell viability counter and qPCR, this study has demonstrated that high pH-based chemicals, even at pH 12, are insufficient to destroy *C. albicans* unless supplemented by heat treatment (Figure 1–3). Moreover, it revealed that a buffer with SDS at RT, in the presence or absence of Proteinase K, effectively permeabilize *C. albicans* cell wall but is unable to destroy the whole cell structure (Figure 4–5). Lastly, enzymatic reagent such as Zymolyase, on the other hand, provides an effective RT solution as it can fully destroy the cellular structure of *C. albicans* without supplemental heat, chemical, or physical treatment (Figure 6). As a result, SDS and Zymolyase enhance lysis efficiency 9 folds better than SDS with Proteinase K at RT, suggesting a target-specific enzyme for destroying yeast cell wall is necessary not only to improve the complete destruction of the species but also for detection sensitivity (Figure 7).

An interesting phenomenon in which nearly all *C. albicans* cells remain both AO and PI positive for a long period of time in the pH- and detergent-based studies was not observed in the Zymolyase experiment (Figure 1, 4, 6). Perhaps, the *C. albicans* cell structure is being destroyed quickly, and the biophysiological transition from healthy live cell status to permeabilized to loss of complete structure is not captured. A time-lapse study of live cells will be needed to address such possibility. It is worth mentioning that some lysis buffers may not be compatible with direct mixing to the AO/PI dyes, and further process optimization (e.g., dilution or removal of lysis buffer) would be necessary to minimize potential chemical interference between the buffer and the dyes. It is also important to select appropriate marker(s) for quantification. For example, PI marker cannot be used for dead cell counting in the Zymolyase study as cell structure was destroyed and those PI positive clusters were likely the sticky genomic material left in the sample matrix. In summary, the Cellometer X2 fluorescent cell viability counter is feasible for performing quick and direct lysis efficiency studies.

Reference

1. 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.

2. Cravener MV, Mitchell AP. Candida albicans Culture, Cell Harvesting, and Total RNA Extraction. Bio Protoc. 2020 Nov 5;10(21):e3803. doi: 10.21769/BioProtoc.3803. PMID: 33659457; PMCID: PMC7854013.

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

4. 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.

5. Calvert CM, Sanders D. Inositol trisphosphate-dependent and -independent Ca2+ mobilization pathways at the vacuolar membrane of Candida albicans. J Biol Chem. 1995 Mar 31;270(13):7272-80. doi: 10.1074/jbc.270.13.7272. PMID: 7706267.