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Automated image analysis with ImageJ of yeast colony forming units from cannabis flowers

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ABSTRACT

Currently, in the state of Colorado and all other states within the United States of America with legalized marijuana programs, testing is required for bacteria, yeast, and mold on marijuana products. The Code of Colorado Regulations, 1 CCR 212–1, considers a passing result when a 1 g sample contains $< 10^4$ colony forming units (CFU) for the total yeast and mold count (TYMC). These measurements are usually obtained by manually counting colonies on petri-dishes or 3 M™ Petrifilms™, which is a time consuming and user subjective process. Therefore, an automated counting method utilizing ImageJ has been developed for CFU analysis of TYMC on Petrifilms. The performance of this colony counting method was demonstrated by comparing manual and automated counts from marijuana flower samples containing spikes of *Candida albicans* as well as samples that tested positive for the presence of yeast and mold. Fifteen images of Petrifilms showing various concentrations of colonies were studied by fifteen users at two institutions using both the automated and manual counting methods. All counts from the automated ImageJ procedure were within 12% of those obtained manually. In twelve out of fifteen Petrifilms, the average count of the automated method was statistically similar to the manual counts. The statistical differences of the other three samples were observed to be random and caused by user errors. The automated counting method could be used to quickly count numbers that are as high as 400 CFUs, reducing time of analysis with improved documentation because the images and the electronic colony counts can be saved on a computer or cloud for long term storage and data access.

1. Introduction

Quality control testing in the food and marijuana industries is a requirement that involves cell enumeration of bacteria and fungi to comply with state and federal regulations. In US states where marijuana has been legalized, these regulations include the determination of total yeast and mold count (TYMC) in all consumer facing products to prevent health problems (Cescon et al., 2008; Verweij et al., 2000).

There are several methods used for cell enumeration in microbiological testing labs, including plate counting (Wilson et al., 2017), polymerase chain reaction (PCR) (Knight et al., 2018), and using a hemocytometer (Salm et al., 2010). Plate counting measures viable cells as colony forming units (CFUs) in dilute liquid culture samples by spreading the sample on agar plates so that a single cell results in a colony that can be counted by the naked eye. This process requires little instrumentation and few user skills (Wilson et al., 2017). PCR is a quick, automated method but requires expertise to perform and interpret, along with costly equipment and supplies, such as a thermal cycler and PCR reagents (Knight et al., 2018). With a hemocytometer, cells can be counted manually in a homogenous liquid sample. But this method requires a microscope, more user training, and does not distinguish between live and dead cells (Salm et al., 2010). Many laboratories still rely on manual plate counting, even though this technique is tedious, time-consuming, and subjective. Automation has the potential to simplify this process, reducing variability between analysts and increasing lab productivity (Cadena-Herrera et al., 2015). While several efficient software suites have been developed to automatically count cells, some of them are restricted to only certain type of cells and cannot be applied to coalescing yeast and mold colonies when concentrations are high. CellProfiler, for example, is a free of cost application that is able to count both mammalian and non-mammalian cells and has the ability for high throughput analysis. However, non-mammalian cells are limited to only round yeast cells. CellC is also a free of cost, modifiable software that is able to only count fluorescently labeled or tagged cells.

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The software is capable of distinguishing cell clusters of various species into single cells by a double staining process, hybridizing bacteria with species-specific probes and staining with 4′,6-diamino-2-phenylindole (DAPI) for the non-hybridized bacteria (Selinummi et al., 2005). Other cell counting software applications include CellCounter (Ho, 2019) and OpenCFU (Geissmann, 2013), which are designed for specific cell types and may require staining.

ImageJ has been utilized for counting colonies as an open-source image analysis program for Macintosh, Windows, and Linux operating systems (Wilson et al., 2017). Additionally, plugins and macros can be downloaded or created and applied to ImageJ for quicker and easier analysis. For example, Image-based Tool for Counting Nuclei (ITCN) is a plugin that works well for counting nuclei (Kuo and Byun, 2019), but has not been utilized for counting yeast and molds, probably due to their morphological diversity at higher concentrations. Automatic mammalian cell counting with ImageJ was previously reported but required the use of two consecutive macros using images that were obtained by a hemocytometer (Grishagin, 2015).

The automated ImageJ counting method of yeasts and molds reported here allows for the counting and discrimination of overlapping colonies of various morphologies even at concentrations considered too numerous to count (TNTC) by the US Food and Drug Administration (FDA) (Maturin and Peeler, 2001). Furthermore, the method only requires a simple photograph taken by a smartphone without requiring fluorescent staining, a microscope, or a hemocytometer. 3 M™ Petrifilm™ Rapid Yeast and Mold Count Plates were used in this method because they contain a color indicator to visualize yeast and mold growth (Beuchat et al., 1990; Vlaemynck, 1994).

3 M™ Petrifilm™ Rapid Yeast and Mold Count Plates are sample-ready-culture medium systems that contain nutrients, antibiotics, and a water-soluble gelling agent. Yeast and mold colonies on these plates can be distinguished by color, where yeasts appear as blue-green colonies with defined edges and no foci while molds are variably-colored colonies with foci and distorted edges (Bird et al., 2015; Chandrapati and Nordby, 2014). The color change on the Petrifilm is due to the enzymatic activity of phosphatases (Chandrapati and Nordby, 2014). A 3 M™ Petrifilm™ Plate Reader is commercially available for automated quantification of aerobic coliform and E. coli/coliform colonies. However, this reader is not yet compatible with the yeasts and molds Petrifilm due to the variability in colony morphologies. Thus, this automated counting method utilizing ImageJ for the analysis of yeast and mold CFUs on Petrifilms is advantageous especially for high CFU concentrations and was observed to be as effective as the manual method but outperformed the manual method at high concentrations.

2. Methods

2.1. Preparation of control and marijuana flower matrix spike of Candida albicans 3 M™ Petrifilms™

All sampling and Petrifilm preparation were performed at AgriScience Labs (Denver, CO). As a positive control and to assure the Petrifilms were working properly, only one yeast species, *Candida albicans* (*C. albicans*) (ATCC 10231*)* cells using KWIK-STIK™, were streaked on standard agar plates and incubated at 25 °C ± 2 °C for 24–48 h. After the incubation period, one isolated colony was transferred to 9.0 mL of buffered peptone water (BPW1, 1 g/L). Ten-fold serial dilutions (10^{-1} to 10^{-7}) were prepared with fresh BPW1 as the diluent. Each dilution was incubated for 24 h at 25 °C ± 2 °C, plated on Petrifilms (3 M™ Petrifilms™ Rapid Yeast and Mold Count Plate) and incubated at 25 °C ± 2 °C for 60–72 h. Following incubation, colonies were counted to determine the optimal concentration that allows the count to be quantifiable. For the marijuana flower spiked sample, 0.5 mL of the *C. albicans* culture that showed the best quantifiable concentration was added to a sterile, two-sided Whirl-Pak® bag with a finely perforated, polyethylene filter for separation (Nasco). A 1.0 g sample of autoclaved marijuana flower was added and diluted with 8.5 mL of BPW20 (20 g/L). For the *C. albicans* control sample (without marijuana flowers), 0.5 mL of the *C. albicans* culture was added to a sterile Whirl-Pak® containing 9.5 mL of BPW20. Three consecutive ten-fold dilutions (1:100, 1:1000, and 1:10000) of the control and flower spiked samples were made with BPW1 and plated on 3 M™ Petrifilms™.

2.2. Preparation of marijuana flower samples on 3 M™ Petrifilms™

A minimum of 2.0 g of marijuana flower sample was aseptically weighed and placed into one side of a sterile Whirl-Pak® bag. Samples were diluted ten-fold in the bag with BPW20 tempered to 36–38 °C by adding a volume of nine times the mass of the sample. The bags were then masticated by hand for 30 to 60 s to homogenize the sample. From the opposite side of the filter to which the flower sample was added, 1 mL of the resulting liquid was diluted into three consecutive ten-fold dilutions with BPW1 (1:100, 1:1000, and 1:10000). One mL of each dilution was plated onto separate 3 M™ Petrifilms™ and incubated for 60–72 h at 25 ± 2 °C.

2.3. ImageJ automated cell analysis

Images were taken with an iPhone 7, containing a 12 M-pixel (f/1.8) camera. Fifteen Petrifilm images of the samples were analyzed with ImageJ by fifteen users at Doane University (Crete, NE) and AgriScience Labs. For automated cell analysis, the image of the Petrifilm was opened in ImageJ. The area of the smallest visible colony on the film was measured in pixels squared by first using the oval region of interest (ROI) tool to outline the colony. The “Measure” tool under the “Analyze” tab opened a “Results” box with the determined area of the drawn circle. Then, a circle was drawn around the edge of the Petrifilm with the oval ROI tool (Fig. 1A). Under the “Edit” tab, “Clear Outside” was used to remove the area outside of the oval from the analysis area (Fig. 1B). Under “Image” and “Type”, the image was converted to 8-bit (Fig. 1C). The threshold, located in the “Image” tab under “Adjust”, was set by the user to make sure colonies were highlighted as black spots with a background white (Fig. 1D). It was important not to hit “Apply” after setting the threshold, but to just close out of the window. Under “Process” and “Binary”, the image was converted to a mask (“Convert to Mask”) before using the “Watershed” tool to split merging colonies with a 1-pixel wide line as shown in Fig. 1E. Lastly, a circle was drawn again around the edge of the Petrifilm eliminating any partial CFUs on the edge that were not to be included in the final CFU count. Under “Analyze”, “Analyze Particles” was selected. The area of the smallest colony previously acquired was used to set the parameter for the particle size range, beginning with a value lower than the area determined prior. For example, if the area was observed to be 577 pixels squared, the range was set as “377-Infinity”. The circularity parameter was set as “0.20–1.00” and an overlay was used to highlight and number the colonies counted. Making sure to select “display results”, “Clear results”, “Summarize”, and “Exclude on edges”, the results displayed the number of particles that fit the parameters within the image and was used as the total CFU count (Fig. 1F). In addition to the written standard operating procedure (SOP) generated, computer screen overlay videos were created to assist new users with the automated and manual counting methods (Table S1) .

2.4. ImageJ manual cell analysis

A “Cell Counter” plugin was downloaded for ImageJ from the National Institutes of Health (De Vos, 2019). The image of the Petrifilm was opened in ImageJ. Within the “Plugins” tab, the “Cell Counter” tool was opened. Manual cell counting was carried out by using the multi-point tool in which each countable colony was marked by clicking over it and a running count of the total colonies was recorded. In both methods, the total CFUs was determined by visible colonies including

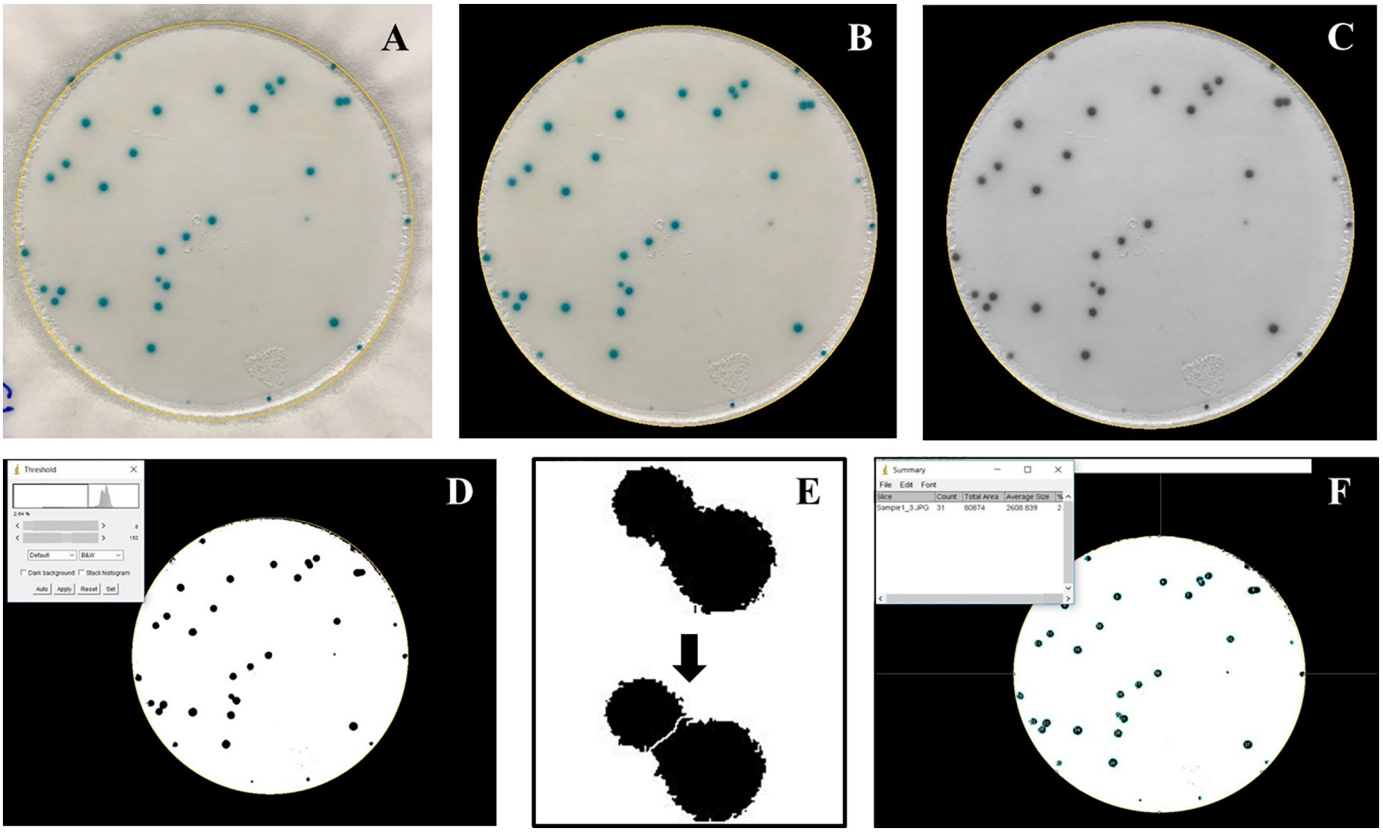

Fig. 1. ImageJ automated cell analysis of a Petrifilm image. A) Petrifilm colony forming area outlined with the oval ROI tool. B) Outside of the ROI cleared. C) Image converted to 8-bit. D) Threshold set to highlight colonies as black particles. E) Merging colonies split by a single pixel line via the "Watershed" tool. F) Particles included in the total count, highlighted and numbered in an overlay on the image.

Fig. 2. Two images that were used to instruct users how to test the automated and manual colony counting method. Users were instructed not to include fine plant particles (left image) or colonies on the edge of the Petrifilm (right image) in the count. However, faint colonies (right image) were counted.

faint colonies but excluded colonies on the outer edge of the Petrifilm and visible plant particles (Fig. 2).

3. Results and discussion

3.1. Petrifilm image analysis

Table 1 highlights the major advantages and disadvantages of the manual and automated cell counting methods. Both methods have the potential for variability from user to user or within one user, which are the major causes of error in the reported results. Users may insert a bias within both methods when selecting what constitutes a colony. Even though specific instructions were provided, some users may include faint colonies or count certain colonies on/close to the edges, while others do not. In some samples such as the AgriScience client marijuana flowers, small blue particles are visible and considered debris and should not be included in the total CFUs but may be misinterpreted as colonies. To address this issue, the automated method was designed to limit the number of particles in the results by restricting the analysis particle size to a specific diameter and only include particles in the count that were defined by their circular dimensions. The manual method also had greater potential for issues when colonies were

Table 1
Advantages and disadvantages of manual and automated CFU counting methods.

Manual		Automated	
Advantages	Disadvantages	Advantages	Disadvantages
• Inexpense • Low tech • Little training required • Quick	• Subjective to what counts as a colony and how many colonies are merged in a single spot • Not consistent between counts and users • No electronic record	• Inexpensive • More objective • Optimization available • Watershed feature available • Quick but could be quicker than manual in high count samples • Electronic record for data storage/review	• Some training • Computer and software required • Subjective due to thresholding and estimating particle size

crowded and users may easily lose track of which colonies have already been counted, even when the embedded grid in the Petrifilm was used. In the automated method, the "Watershed" tool was used to estimate the individual colonies within a crowded section so that every single colony was included within the count even when colonies overlap. The automated method also included factors that could potentially influence variability including the ROI drawn, the threshold set, and the estimate of the smallest colony particle size, which are all set and controlled by the user.

Fifteen Petrifilm images (Fig. 3) were obtained and analyzed by fifteen different individuals using ImageJ to determine CFU count, performing both the manual and automated methods described (Table S2). The results of the CFU counts from both methods were compared to determine if the automated method is an efficient alternative to the manual counting method. The fifteen images that were selected were actual client samples providing a realistic scenario of controls and flower samples in a daily analytical lab operation. Furthermore, the images show that the samples contain various colony morphologies and coverage, which is also a typical lab situation in official marijuana testing facilities. The spiked flower sample, the *C. albicans* control, and Flower Sample 1 (Fig. 3) were all officially reported to be TNTC when manually counted by AgriScience, with the number of colonies exceeding 250 per plate (Table S2). However, when ImageJ was used in the manual method to track the colonies and when those counts were compared with the ImageJ automated analysis, it became evident that the automated method was able to efficiently analyze all three samples with similar counts.

3.2. Statistical comparison of manual vs automated counting methods

The manual and automated CFU counts were analyzed with a series of statistical analyses. Average and standard deviation for both methods were calculated after each data set was tested for an outlier by Q and T_n tests at a 95% confidence level (Fig. 4; Table S2 and S3) (Mandel, 1978; Raschka, 2014). The percent difference was determined by comparing the automated method average to the average of the manual method to determine how closely the two methods functioned (Table S3). The highest percent difference was 12.3% for flower sample 1 (dilution 1:100), which had the most clustered colony growth, making it a challenge to count accurately with either method. All other images had percent differences of ≤ 10%. Calculated *p* values were used to determine if the averages from the two methods were statistically the same. Three of the fifteen images (flower 1 dilution 1:100 and 1:10000 and flower sample 3 dilution 1:1000) had p values < 0.05 (0.002, 0.01, 0.04, respectively), indicating that the counts were statistically different between the manual and automated method, which was confirmed by the F value. The F values (13.81, 8.82, 5.13, respectively) were greater than the F crit values (4.60, 4.67, 4.60), which also

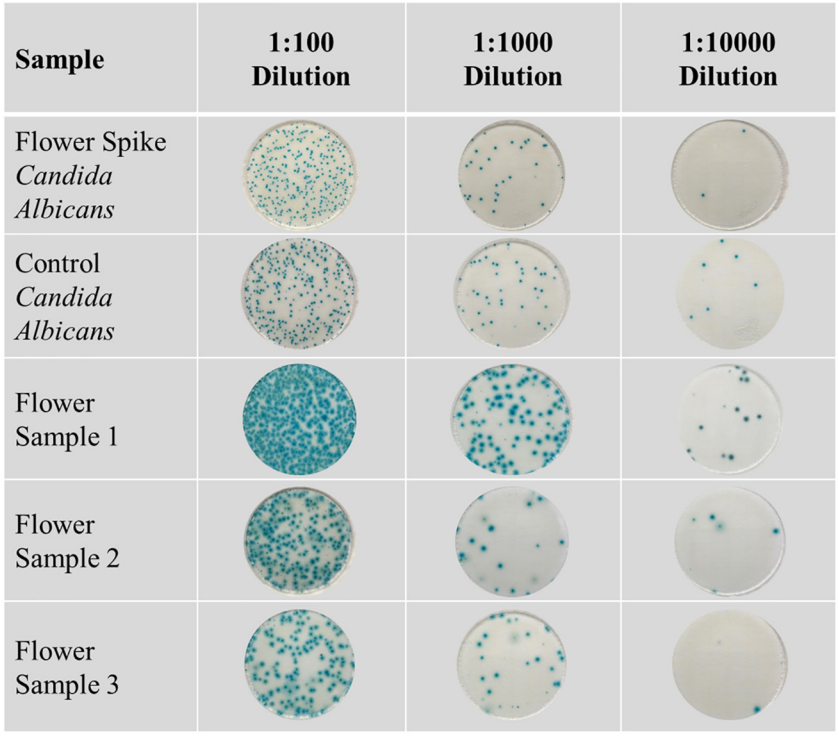

Fig. 3. Five different samples were plated on 3 M™ Petrifilms™ at various dilutions for analysis of yeast and mold CFU counts using both manual and automated methods. The matrix spike contains a mixture of multiple flower strains and was spiked with *C. albicans*. The control was prepared with only the *C. albicans* culture. Flower samples 1, 2, and 3 are all different marijuana flowers. Images of samples at dilution factors of 1:100, 1:1000, and 1:10000 were chosen for analysis.

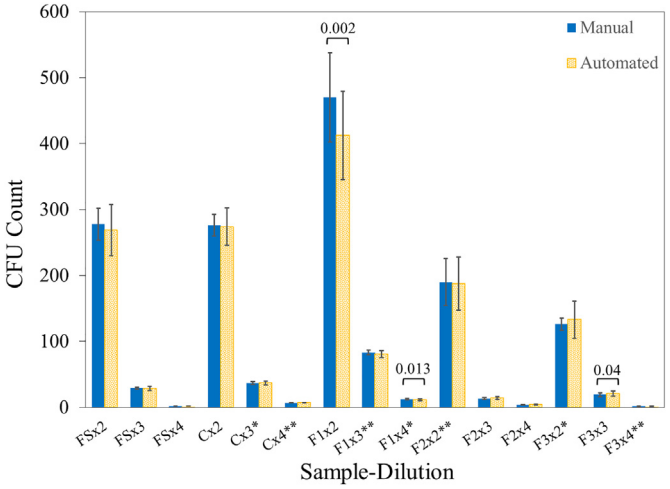

Fig. 4. Average CFU count from the image analysis of 5 samples (FS – Flower Spike *C. albicans,* C - Control *C. albicans*, F1 - Flower sample 1, F2 - Flower sample 2, and F3 - Flower sample 3) at three dilutions [1:100 (x2), 1:1000 (x3), and 1:10000 (x4)] using manual (solid blue bar) and automated (dotted orange bar) analysis. Three samples are considered to have CFU counts from the manual and automated method statically different and are marked with the calculated *p*-values. Error bars represent the standard deviation of user counts excluding any outliers determined. n = 15, *average and standard deviation of one method exclude an outlier where n = 14, **averages and standard deviations of both methods exclude an outlier where n = 14. Numerical values presented in Table S3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

confirmed the statistical difference in CFU counts for these three images. This statistical difference cannot be explained by either dilution factors or sample identity. If dilution was a factor to the performance of both methods, then the same dilution for every sample would have led to a similar statistical difference. If the sample identity was the reason for the statistical difference, then all dilutions of the same sample would have shown this difference between the counting methods as well. However, as seen in flower sample 1, the high and low dilutions (1:100 and 1:10000) were different but the middle dilution was not (1:1000). This clearly demonstrates there is no obvious explanation for the statistical difference and therefore, could be caused by user bias.

Table S4 also shows that the variations between the two methods are not associated with dilution factors or difficulties in counting colonies due to overcrowded growth. The average and standard deviation of the percent difference for each sample at the same dilution were fairly consistent throughout the three dilutions analyzed, with the 1:100 dilution showing the greatest percent (4.6 ± 4.7%). This consistency suggests that the manual and automated method performed similarly. The average standard deviation showed that the variability of both methods decreased as samples were more dilute, which was an expected outcome as the colonies become more spread out and easier to count. However, the manual method had a lower average standard deviation for each dilution as compared to the automated method, demonstrating that user variability was a greater factor in the automated method. Finally, the average *p*-values for all three dilutions comparing the two methods were 0.41, 0.34, and 0.32, respectively with a standard deviation leading to the conclusion that both methods are similar in determining CFUs. Overall, the manual counting method was easy and sufficient when the CFU count was low, but the automated count was a quicker alternative when the CFU count was large and colonies were crowded.

4. Conclusion

The automated counting method for yeast and mold colonies from marijuana flowers using ImageJ allows for counting of colonies even at concentrations considered too numerous to count by the FDA. Based on manual and automated cell counts of fifteen users, three of the fifteen sample images were observed to have a significant difference between the automated and manual counts, but this difference is believed to be due to user error and not a method error. Overall, the automated method was easily learned by the users when they were provided with a written procedure and a quick instructional video. This method could serve as an additional or alternative counting tool in state regulated cannabis testing labs where clients and consumers depend on accurate results before cannabinoid containing products are sold or consumed. The electronic record of the images and CFU counts provides the clients and lab facilities long term documentation, easy electronic storage, and data review. This is particularly important when a sample fails at $> 10^4$ CFUs per gram at which point the entire batch of marijuana biomass from where the sample originated would have to be destroyed. Therefore, the electronic evidence of the failed sample is important for the business, the testing lab, and the consumer.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.105681.

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