



Published in final edited form as:

*Med Mycol.* 2016 May 1; 54(4): 361–369. doi:10.1093/mmy/myv104.

## Reproducibility of CSF Quantitative Culture Methods for Estimating Rate of Clearance in Cryptococcal Meningitis

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### Abstract

Quantitative cerebrospinal fluid (CSF) cultures provide a measure of disease severity in cryptococcal meningitis. The fungal clearance rate by quantitative cultures has become a primary endpoint for phase II clinical trials. This study determined the inter-assay accuracy of three different quantitative culture methodologies. Among 91 participants with meningitis symptoms in Kampala, Uganda during Aug-Nov 2013, 305 CSF samples were prospectively collected from patients at multiple time points during treatment. Samples were simultaneously cultured by three methods: 1) St. George's 100mL input volume of CSF with five 1:10 serial dilutions, 2) AIDS Clinical Trials Group (ACTG) method using 1000, 100, 10mL input volumes, and two 1:100 dilutions with 100 and 10 mL input volume per dilution on seven agar plates; and 3) 10mL calibrated loop of undiluted and 1:100 diluted CSF (loop). Quantitative culture values did not statistically differ between St. George-ACTG methods ( $P=0.09$ ) but did for St. George-10mL loop ( $P<0.001$ ). Repeated measures pairwise correlation between any of the methods was high ( $r = 0.88$ ). For detecting sterility, the ACTG-method had the highest negative predictive value of 97% (91% St. George, 60% loop), but the ACTG-method had occasional (~10%) difficulties in quantification due to colony clumping. For CSF clearance rate, St. George-ACTG methods did not differ overall (mean  $-0.05 \pm 0.07 \log_{10} \text{CFU/mL/day}$ ;  $P=0.14$ ) on a group level; however, individual-level clearance varied. The St. George and ACTG quantitative CSF culture methods produced comparable but not identical results. Quantitative cultures can inform treatment management strategies.

### Keywords

Cryptococcus; culture; methodology; HIV/AIDS; accuracy; meningitis

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**Conflicts of Interest:** none.

## Introduction

Cryptococcal meningitis is the most common cause of adult meningitis in sub-Saharan Africa, causing ~20% of AIDS-related deaths.<sup>1,2</sup> This substantial burden has made cryptococcal meningitis the target of renewed clinical research interest and created the need for standardized metrics to monitor disease progression. Quantitative cryptococcal culture provides a measure of disease severity in cryptococcal meningitis, has improved the understanding of rates of response to antifungal treatment, and is independently associated with clinical outcome.<sup>3,4</sup> Yet, although quantitative culture burden is a prognostic indicator, quantitative CSF cultures are not used in routine care.

The rate of cerebrospinal fluid (CSF) fungal clearance, as measured by serial quantitative cultures and termed early fungicidal activity, is a common endpoint used in phase II clinical trials that assesses the microbiologic activity of novel antifungal induction regimens for cryptococcal meningitis.<sup>5</sup> Various quantitative culturing methodologies are used to quantify CSF fungal burden; however, the reproducibility between techniques is unknown.

In this study, we directly compare three established methods of quantitative CSF cultures for *Cryptococcus* in order to compare their inter-assay performance against one another in terms of absolute colony counts per mL of CSF and the calculated early fungicidal activity of CSF yeast clearance. We also examined the correlation between quantitative cultures and automated cell counting.

## Methods

We prospectively screened 91 HIV-infected participants with suspected cryptococcal meningitis at Mulago Hospital in Kampala, Uganda from August 13 through November 30, 2013. CSF samples (n=305) were collected at diagnosis (n=86), and at days 3 (n=58), 7 (n=60), 10 (n=44) 14 (n=45), and after day 19 (n=12) of follow-up during the pilot phase of the ASTRO-CM trial (ClinicalTrials.gov: NCT01802385). The ASTRO-CM pilot was a dose finding phase II clinical study of adjunctive sertraline 100–400mg/day added to amphotericin 0.7–1.0 mg/kg/day with fluconazole 800mg/day. Participants provided written informed consent, and applicable institutional review boards approved the study.

Fresh CSF specimens were simultaneously cultured by three different quantitative culture techniques of varying complexity. The “St. George’s Method,” as developed by Robert Larsen, Thomas Harrison, and colleagues, was the reference standard used for comparison.<sup>5–11</sup> This method uses 100  $\mu$ L input volume of undiluted CSF with five additional 1:10 serial dilutions in sterile H<sub>2</sub>O. Each Sabouraud dextrose agar plate received a 100  $\mu$ L volume of CSF and was distributed using five 4.5mm sterile glass marbles (Figure 1A). The second method, as developed by Robert Larsen and used by the AIDS Clinical Trials Group (ACTG), employs multiple input volumes (1000, 100, 10  $\mu$ L) of undiluted CSF and two sequential 1:100 dilutions each with two 100  $\mu$ L and 10  $\mu$ L input volume per dilution, distributed using an L-shaped spreader (Figure 1B). This ACTG method uses seven culture plates in total. Finally, a third, and deliberately simple “loop” method of a calibrated plastic 10  $\mu$ L loop was used to plate undiluted and 1:100 diluted CSF onto two culture plates

(Figure 1C). Due to delays in receiving supplies, 10  $\mu$ L loop cultures began in October 2013, one month after the first CSF sample was collected. For each method, CSF vortexing was performed prior to dilutions and prior to plating. For quantification of fungal CSF burden, distinct colonies on agar plates were counted and colony-forming units (CFU) per mL CSF were enumerated on the 10<sup>th</sup> day of culturing.

We performed additional quantitative analyses of CSF fungal burden at diagnosis. In order to quantify cryptococcal antigen titers in CSF, we performed Cryptococcal antigen lateral flow assays (CrAg LFA) using a strategy that minimized the number of LFAs needed to determine titer values (**Supplemental Appendix**). In addition, yeast cells were quantified using automated cell counts performed on 10  $\mu$ L of undiluted CSF using the TC20 Automated Cell Counter (Bio-Rad, Hercules, CA), and subtracting the CSF white cell count (determined by manual counting).

### Statistical Analysis

All statistical analyses were performed using Stata/IC 13.1 (StataCorp LP, College Station, Texas) on log<sub>10</sub>-transformed culture values (log<sub>10</sub> CFU/mL). We assessed the relationship between culture methods via linear mixed model with a random intercept for each participant. Qualitative agreement was also assessed with percent concordance. To compare the difference in individual cultures, a paired sample Student's *t*-test was performed.

We compared the calculated early fungicidal activity of CSF yeast clearance between the St. George and ACTG culturing methodologies by establishing a clearance rate for each individual based on a within-subject linear regression. The 10  $\mu$ L loop method had insufficient number of cultures for a robust comparison. Specifically, we explored the intra-subject variability over time of 47 persons with culture-positive meningitis and at least two CSF cultures. The early fungicidal activity of the rate of clearance of *Cryptococcus* CFU/mL CSF per day is generally logarithmic (i.e., linear on log<sub>10</sub> CFU/mL scale), and thus we considered the rate of clearance as calculated by linear regression.

### Results

Among 91 participants with suspected meningitis, we performed 305 lumbar punctures and quantitative CSF cultures. Of the 91 persons, 66 (73%) participants had CSF CrAg detected compatible with cryptococcal meningitis. The remaining 25 CSF CrAg-negative, culture negative participants were excluded from comparative analyses. The demographics of the cohort were typical for cryptococcal meningitis with a median age of 37 years (interquartile range (IQR), 32-42) and 35% (n=32) women. The median CD4 count was 24 cells/ $\mu$ L (IQR, 9-81), with 49% (n=45) currently receiving ART and 4% (n=4) having previously received ART but defaulted from HIV care. Seven (8%) patients had a prior diagnosis of cryptococcal meningitis. The median initial CSF WBC count was <5 (IQR, <5-70) cells/ $\mu$ L, and median CSF protein was 59 (IQR, 34-116) mg/dL. Table 1 displays the three quantitative culture methodologies and an automated cell count as performed in parallel on longitudinally collected CSF specimens over two weeks of induction antifungal therapy. At diagnosis, the median CSF quantitative culture among patients with a positive culture was 4.26 log<sub>10</sub> CFU/mL by the St. George's reference method (IQR, 3.45 to 5.32 log<sub>10</sub> CFU/mL; mean

$\pm$ SD,  $4.34 \pm 1.27 \log_{10}$  CFU/mL), and the median CSF CRAG LFA titer was 1:2,000 (IQR, 1:300–1:4,000).

### Quantitative Culture Agreement

A paired comparison of the St. George's and ACTG methods indicated no significant difference between their results of the two assays ( $P=0.09$  by paired  $t$ -test). However, the ACTG quantitative culture results were generally higher than the standard St. George's method. For every 1-unit increase in  $\log_{10}$  ACTG quantitative cultures, there was a corresponding 0.88 increase in  $\log_{10}$  CFU/mL (95% CI, 0.84 to 0.93) using the St. George's quantitative cultures among 229 valid culture pairs from CSF CrAg-positive participants. For every 1-unit increase in the 10  $\mu$ L calibrated loop method results, there was a corresponding 0.89 increase in  $\log_{10}$  CFU/mL from the St. George's method (95% CI, 0.82 to 0.96) among 133 pairs from CSF CrAg-positive participants (Supplemental Figure 1). A comparison of the ACTG and calibrated loop indicated a mean increase in 0.94  $\log_{10}$  CFU/mL (95% CI, 0.85 to 1.03) in ACTG results for every 1-unit increase in the calibrated loop method among (n=127 pairs). Among patients who had additional lumbar punctures after 14 days of amphotericin B-based therapy, 34% (12/35) of participants' CSF remained culture positive with a mean culture growth of 750 CFU/mL, 870 CFU/mL and 30 CFU/mL for the St. George's, ACTG, and 10  $\mu$ L loop methods, respectively, among those with positive cultures by any method.

Figure 2 displays the relationship between the St. George's and ACTG methods, which had the strongest correlation (Pearson  $r^2$  for repeated measures = 0.92,  $P<0.001$ ). Although the 10  $\mu$ L loop method absolute counts differed significantly, the loop method's quantitative culture results did correlate well with the St. George's method ( $r^2$  for repeated measures = 0.89,  $P<0.001$ ) and with the ACTG method ( $r^2$  for repeated measures = 0.88,  $P<0.001$ ). Among paired cultures performed by St. George and ACTG methods, 62% (143/229) differed by less than  $\pm 0.5 \log_{10}$  CFU/mL and 81% (181/229) differed by less than  $\pm 1.0 \log_{10}$  CFU/mL, excluding sterile cultures by both methods.

### Intra-subject Variability in Early Fungicidal Activity

We explored the intra-subject variability in the calculated rate of CSF clearance over two weeks among 47 subjects with at least two CSF quantitative cultures to identify potential differences in assays. The mean early fungicidal activity for the St. George's method was  $-0.45 \log_{10}$ CFU/mL CSF/day (95%CI,  $-0.56, -0.33$ ). The mean early fungicidal activity for the ACTG method was  $-0.40 \log_{10}$ CFU/mL CSF/day (95%CI,  $-0.51, -0.29$ ). The mean difference in the calculated rate of clearance for the ACTG method was  $-0.05 \log_{10}$ CFU/mL/day (95%CI,  $-0.11$  to 0.02,  $P=0.14$ ) in comparison with the St. George's method (Figure 4). Thus, the overall group mean and median estimates were similar by quantitative culture method when using the linear method. However, there was substantial variation on the individual participant level; the 10<sup>th</sup> and 90<sup>th</sup> percentiles for differences in the individual participant level calculated early fungicidal activity were  $-0.47$  and  $+0.12 \log_{10}$  CFU/mL CSF/day. These differences generally increased when fewer quantitative culture results were available. When only two serial quantitative cultures were available, the median difference in estimated early fungicidal activity was  $-0.16$  (IQR,  $-0.32, 0.05$ , n=14)

whereas when 3 cultures were available (with at least two with growth) the median difference was  $<-0.01$  (IQR:  $-0.05, 0.07$ ,  $n=33$ ) between the St. George and ACTG methods. Thus, in real world use, the early fungicidal activity estimate did not differ for the overall cohort with respect to the particular quantitative culture methodology utilized, yet there were individual patient level differences when calculating the intra-subject early fungicidal activity.

### Prediction of CSF Sterility, Limit of Detection

Based on the differences in volumes of CSF cultured with each technique, the limit of detection for the St. George quantitative culture is 10 CFU/mL, for the ACTG culture 1 CFU/mL, and for the loop culture 100 CFU/mL of CSF. For each method, we determined the negative predictive value where a true sterile culture was a negative culture by all methods. Among all negative CSF cultures, the ACTG method had the highest negative predictive for predicting CSF sterility of 97% (60/62), representing when the ACTG culture was sterile, all culture methodologies were sterile. The negative predictive value was also high at 91% (105/115) for the St. George method using 100 $\mu$ L. Conversely, the 10 $\mu$ L loop method was relatively insensitive at low fungal burdens having only a 60% (46/77) negative predictive value for a negative 10  $\mu$ L loop culture to represent a true sterile culture. In 4.3% ( $n=10$ ) of 234 paired cultures the ACTG method detected low level fungal growth not identified by the other methods, consistent with using the largest culture volume. Only 1 of the 10 was on an initial diagnostic culture, with the other 9 obtained from follow-up cultures collected at time of therapeutic LPs, and all 10 patients were CSF cryptococcal antigen positive. The median quantitative culture result for these 10 samples was 16 CFU/mL (Range 2–79). The false negative diagnostic culture had only 2 CFU/mL by the ACTG method with a CSF CRAG LFA titer of 1:200.

### Automated Cell Counter Quantification Method

The automated cell counter did not perform well, even when subtracting the CSF WBC cell count ( $R^2=0.46$ , difference on Day 1 =  $+1.52 \log_{10}$  CFU/mL, 95% CI, 1.01 to 2.03;  $P<0.001$ ) as compared with St. George culture methodology. Supplemental Figure 2 provides an image from the instrument. Automated cell counts did not decrease over 14 days of amphotericin B, counting yeasts both dead and alive.

### Discussion

Overall, the ACTG and St. George's methods of quantitative CSF cultures produced highly comparable results on a population level but not identical results on an individual level. The 10  $\mu$ L loop method, although quite simple, had a limited dynamic range with false negative cultures at low fungal burden and undercounting at high fungal burden. Despite these differences, the correlation between all three methods remained high, with repeated measures  $r^2$  values  $>0.88$ . These results suggest strong reproducibility of quantitative culture results between St. George's and ACTG methods.

We observed a small yet statistically significant negative correlation between mean and difference between the St. George and ACTG methods, suggesting that as the CFU count

increased, the ACTG method tended to count slightly higher than the St. George method. This same trend was seen between ACTG and loop methods as well, suggesting that the ACTG method overall yielded slightly higher colony counts at the extremes of low and high CFUs. This is an expected difference as the ACTG used one additional culture plate both at the low and high end.

The ACTG method was best for predicting true CSF sterility. This is likely explained by the ACTG plating of 1 mL of undiluted CSF, with better ability to detect growth <50 CFU/mL. In this setting, 10 individuals were found to have actively growing *Cryptococcus* by the ACTG method and no other. Nine of these were on follow up specimens collected from later therapeutic LPs, which would have minimal clinical implication but theoretically could result in a slightly more accurate calculation of the early fungicidal activity rate. A single discordant diagnostic specimen with positive growth by the ACTG method only at 2 CFU/mL was from a diagnostic lumbar puncture collected prior to antifungal treatment. In the setting of using CSF CrAg LFA for diagnosis, which is more sensitive than culture,<sup>12</sup> the diagnostic implications are minimal. However if using India ink, low burden infections will be missed as the sensitivity of India ink is ~40% among persons with CSF cultures having <1,000 CFU/mL.<sup>12</sup> These results suggest that a 1mL plate of undiluted CSF should be a consideration in a research setting when seeking to maximize culture detection.

Those with sterile CSF cultures by 14 days of antifungal therapy have historically had better survival, less immune reconstitution inflammatory syndrome, and less cryptococcal relapse compared with those with positive CSF cultures at 14 days when using fluconazole 400mg/day consolidation therapy.<sup>13,14</sup> Whether the absolute number of organisms which remain culture positive in the CSF at 14 days, or if there is a threshold level which further influences clinical outcomes, is unknown.

CRAG LFA titers are a strong method of estimating initial fungal burden, but CRAG titer correlation worsens over time and cannot determine the response to antifungal therapy.<sup>12,15</sup> Automated cell counts did not estimate fungal burden well, possibly as counting could not distinguish between living and dead *Cryptococcus* or cellular debris; however, even at baseline, automated cell counts correlated unexpectedly poorly with culture CFU counts. The automated cell counter reported could count cells 6–50 µm in diameter with a dynamic range between 50,000 and 10 million cells/mL (4.7–7 log<sub>10</sub>/mL), which should have been adequate but was not. Although most CSF samples registered on the cell counter, most often the automated counts far exceeded the CFU's on culture, even when correcting for CSF WBCs. This difference could be due to poor automated counting or clumping of organisms on culture. In either scenario, automated cell counting using this particular instrument did not perform well.

Despite attempts to standardize the three culturing methods, problems in protocol application were identified that may affect feasibility in resource-limited settings. Common problems with quantitative cultures included: poor sample plating and spreading supplies, which lead to colony clumping and the inability to provide an accurate quantitative count. This was very common with the 10 µL loop method and occasionally occurred (~10%) with the ACTG method. With the ACTG method, the low sample volumes (e.g. 10 µL) on some

plates (e.g. plate 2, 4, and 6) may allow the sample to dry before adequate even spreading occurs, resulting in clumping. Marbles generally worked better than spreaders for even distribution. Lastly is a consideration of the resources necessary to conduct quantitative cultures, during this three month project, approximately 1,500 agar plates were used for the St. George method (n=5 per CSF specimen) and 1,700 plates for the ACTG method (n=7 per CSF specimen). Thus, the resources to conduct quantitative cultures in a busy hospital microbiology lab may be sizeable. Although on sequential samples, it may be possible to limit the number of plates used to the relevant number based upon the prior results or initial CRAG titer. For blood and CSF CRAG-negative persons, we routinely now use ~1mL CSF on one culture plate only.

Identification of cryptococcal disease and meningitis has now become rapidly and readily available with screening employing the CRAG LFA assay. Either 40  $\mu$ L of blood or CSF can determine if cryptococcal disease is present within 10 minutes.<sup>16</sup> It is now feasible for quantitative CSF cultures at baseline to become the mainstay of assessing severity of disease so that appropriate treatment decisions can follow. Baseline CSF quantitative cultures can be readily assessed at 4–7 days after incubation. Follow up cultures collected while receiving amphotericin-based induction therapy, in contrast, generally require longer incubation times with ~15% of samples requiring >14 days of incubation before becoming culture positive (Larsen, personal communication). Intensive antifungal therapy with amphotericin B (with or without fluconazole), offers the most effective antifungal therapy but is not broadly available. However, those with mild disease can likely be rapidly switched to fluconazole monotherapy if the initial fungal burden is low and the predicted response would lead to negative cultures by day 14. In those with higher baseline fungal burdens, when the predicted response to amphotericin B indicates that CSF culture would not be sterile by 14 days for example, it may be prudent to evaluate the fungal burden at 14 days and consider continuing the amphotericin B based regimen or higher dose fluconazole (800–1200mg/day) until it is predicted the CSF cultures will be sterile. Future studies focused on 1) determining the optimal cutoffs of initial CSF fungal burden used for making antifungal duration decisions, and 2) evaluating the feasibility of a customized approach to antifungal therapy, are needed. However, shorter durations of amphotericin therapy could provide substantial cost savings to the healthcare system and limit toxicity in individuals with milder disease.<sup>9,17</sup> This approach can only be enabled by performing quantitative CSF cultures, and ongoing assessment of the optimal method for quantitation that balances accuracy with practicality across various settings remains a priority.

Overall, the St. George's and ACTG quantitative culture methods for *Cryptococcus* yielded highly reproducible but not identical results in a real world setting. We provide an example laboratory standard operating procedure template (**Supplemental Appendix**), based on the lessons of this project. Ultimately, the choice of quantitative culture method should be based upon local laboratory-specific capacity. In high-resource areas, quantitative CSF cultures should be routinely performed for CRAG-positive specimens.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

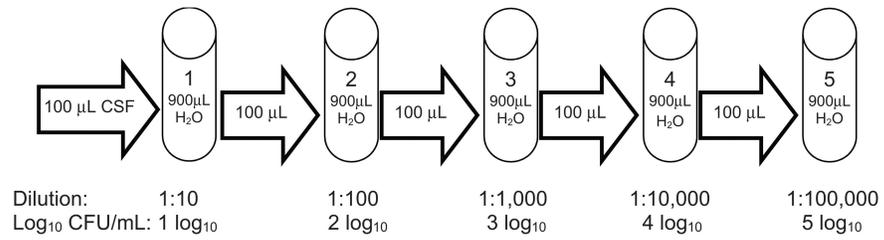
This research was made possible through support from the Fogarty International Center and National Institute of Neurologic Diseases and Stroke (R01NS086312, R25TW009345) and National Institute of Allergy and Infectious Diseases (U01AI089244, T32AI055433). This work was supported in part by the Doris Duke Charitable Foundation through a grant supporting the Doris Duke International Clinical Research Fellows Program at the University of Minnesota. Jonathan Dyal is a Doris Duke International Clinical Research Fellow. Tihana Bicanic was funded by a Wellcome Trust Intermediate Fellowship (WT 089966). We thank Tonny Luggya for laboratory work; Jane Francis Ndyetukira, Cynthia Ahimbisibwe, Florence Kugonza, and Alisat Sadiq for patient care.

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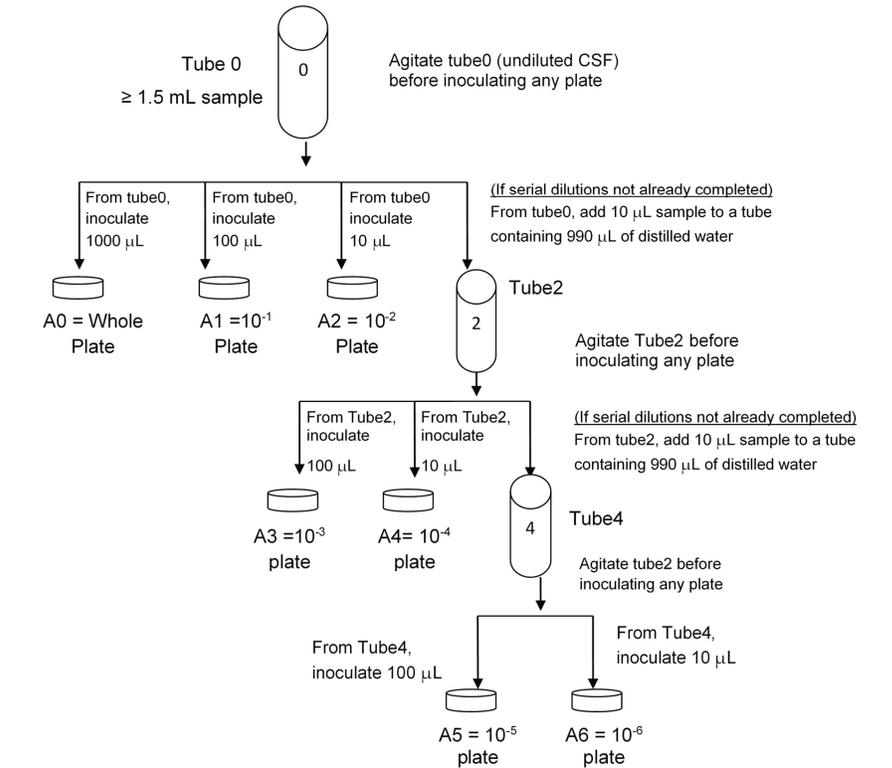
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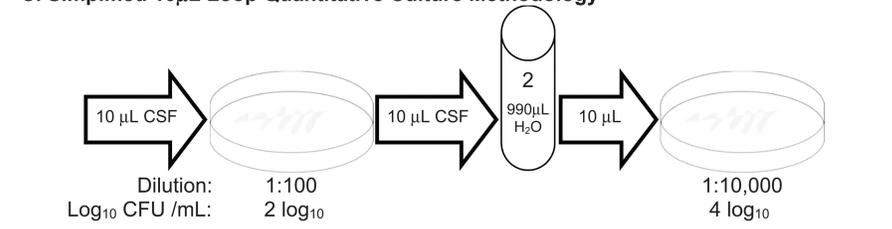
**A. St George's Quantitative Culture Reference Method**



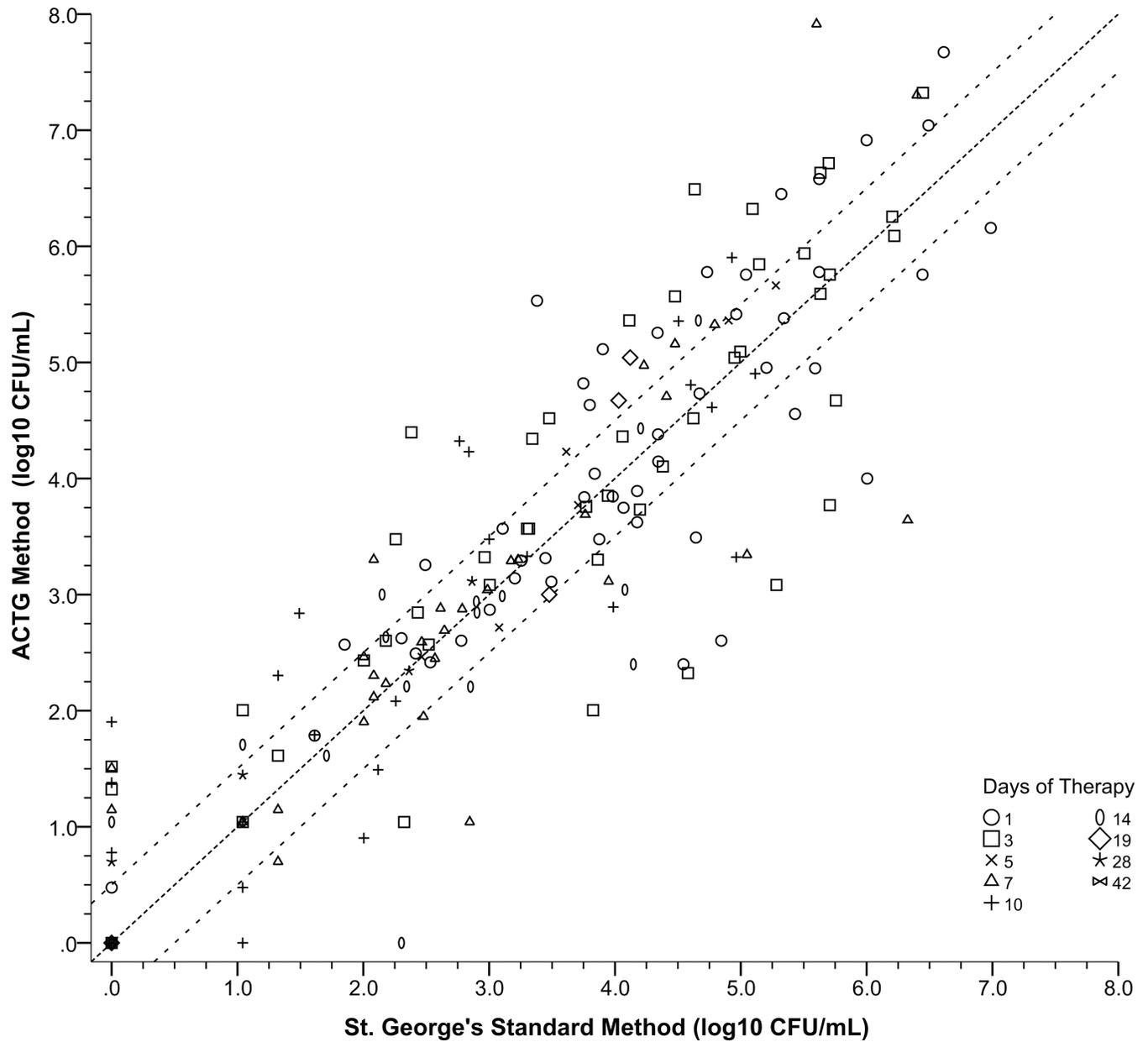
**B. ACTG A5225/HiFLAC Quantitative Culture Schema**



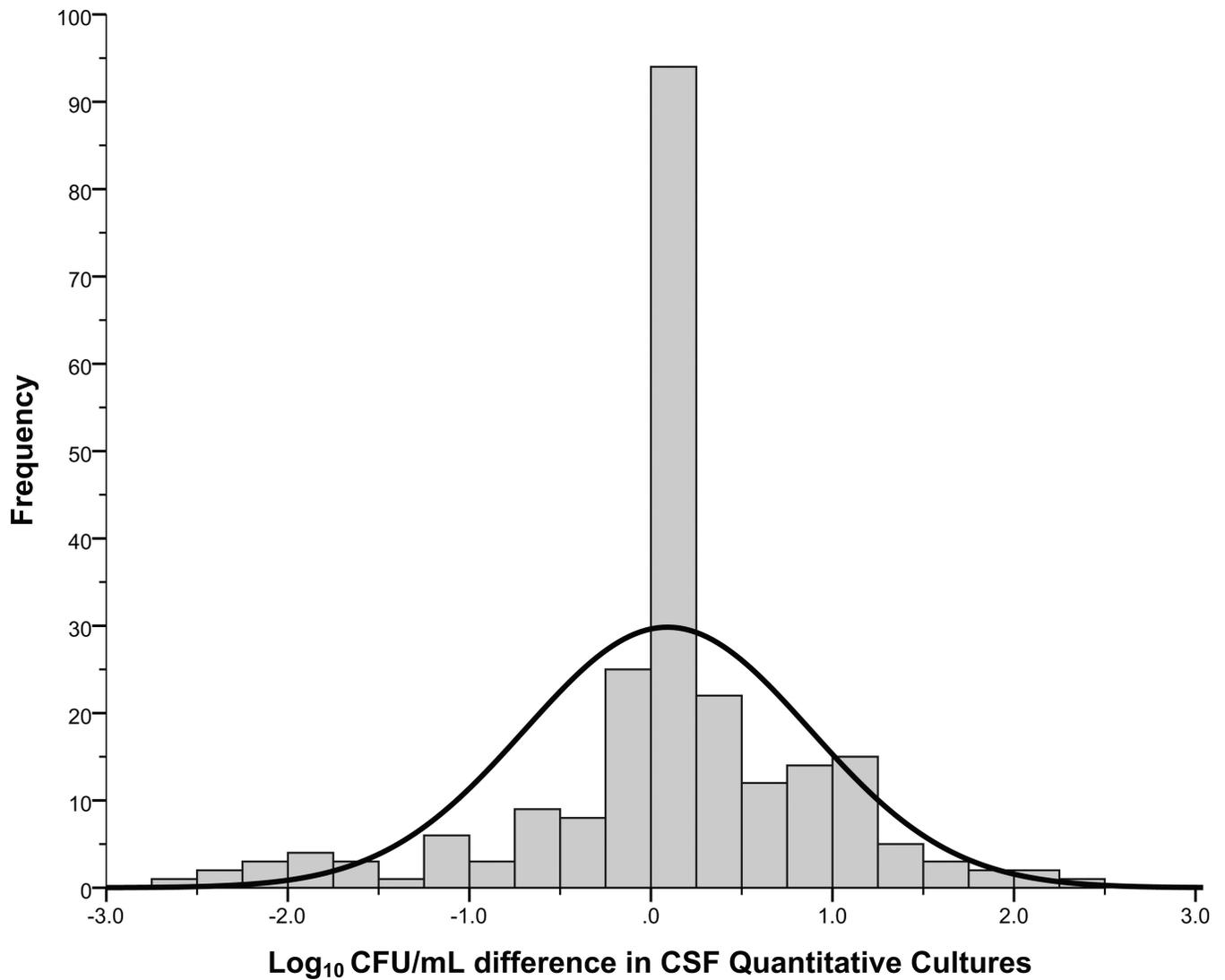
**C. Simplified 10µL Loop Quantitative Culture Methodology**



**Figure 1. Three Quantitative CSF Culture Methods**

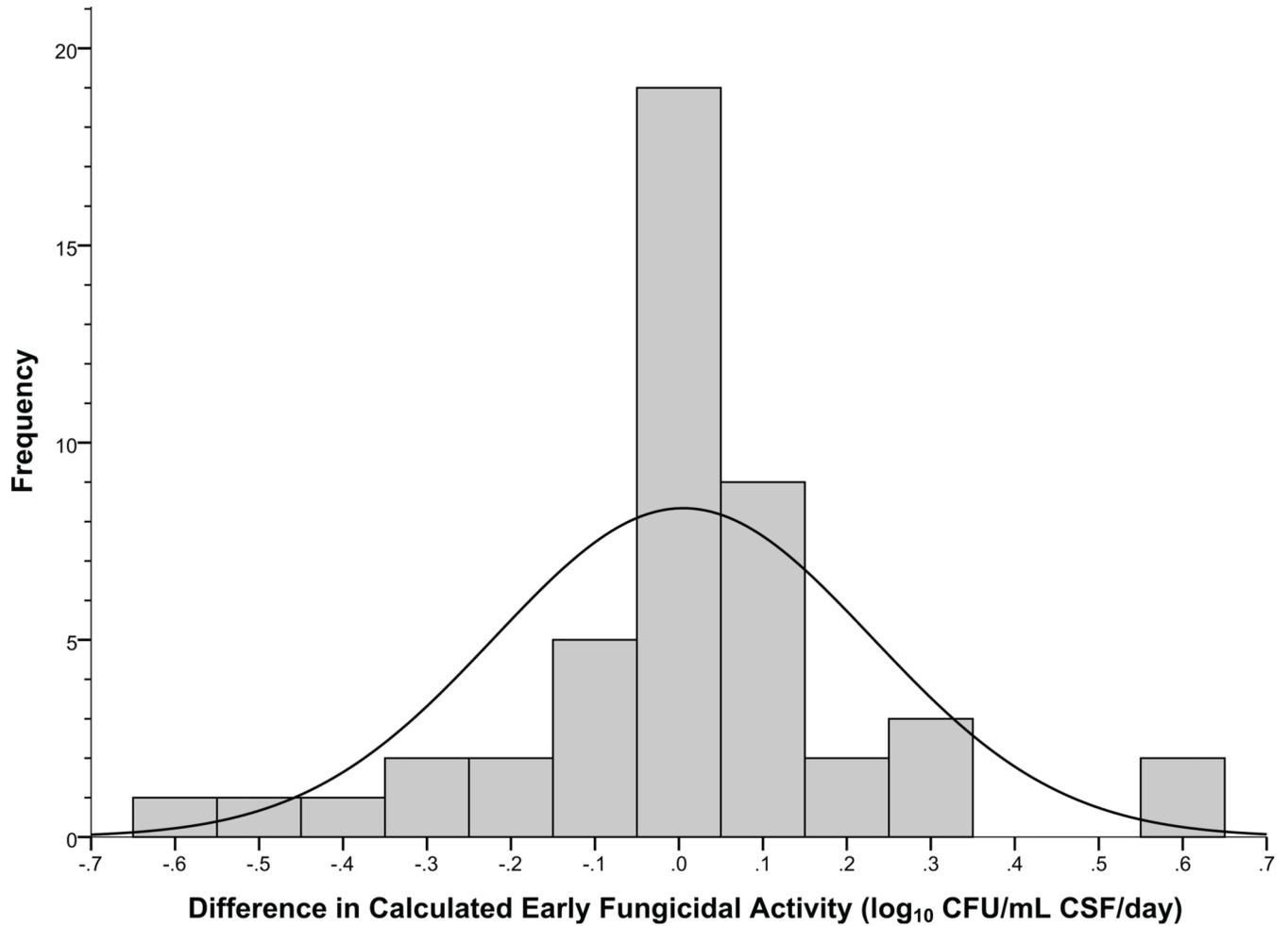


**Figure 2. Correlation between Quantitative Cultures by St. George Method and ACTG Method**  
 Center reference line displays 1:1 correlation with boundaries displaying  $\pm 0.5 \log_{10}$   
 CFU/mL. Paired specimens (n=229) were collected on day 1 (n=52), day 3 (n=49), day 7  
 (n=50), day 10 (n=34), day 14 (n=36), and after day 18 (n=8).



**Figure 3. Individual CSF Quantitative Culture differences between ACTG and St. George method**

The two methods correlated well ( $\beta=0.88$ , 95% CI, 0.84 to 0.93) with the ACTG method having slightly higher overall average growth (+0.09 log<sub>10</sub> CFU/mL). This was most apparent at the extremes of high and low culture values whereby the ACTG method tended to count slightly higher than the St. George method.



**Figure 4.** Differences in calculated early fungicidal activity of the rate of CSF clearance for ACTG quantitative culture method in comparison with St. George method as reference. Among 47 participants with culture positive cryptococcal meningitis and 2 CSF cultures, the mean difference in rate of clearance was  $-0.05 \log_{10} \text{CFU/mL/day}$  (95%CI,  $-0.11$  to  $0.02$ ,  $P=0.14$ ).

**Table 1**

Differences in Quantitative CSF Cultures by varied Techniques by Day of Treatment among persons with cryptococcal meningitis.

	St. George Culture Median (IQR)	ACTG Culture Median (IQR)	10mL Loop Median (IQR)	Auto Cell Counter Median (IQR)
<b>Overall N</b>	278	229	133	183
<b>Day 1</b>	4.0 (3.0, 5.1)	3.8 (2.6, 5.2)	3.5 (2.0, 3.9)	5.5 (5.0, 5.9)
<b>Day 3</b>	3.8 (2.3, 5.1)	3.7 (2.4, 5.4)	2.7 (0.0, 5.3)	5.7 (5.2, 6.3)
<b>Day 7</b>	2.1 (0.0, 3.5)	2.5 (1.0, 3.6)	0.0 (0.0, 2.5)	5.4 (5.0, 5.9)
<b>Day 10</b>	1.0 (0.0, 2.3)	1.2 (0.0, 3.3)	0.0 (0.0, 1.0)	5.2 (4.7, 5.7)
<b>Day 14</b>	0.0 (0.0, 2.3)	0.0 (0.0, 2.5)	0.0 (0.0, 0.0)	5.3 (4.8, 5.7)
<b>&gt;Day 14</b>	0.5 (0.0, 2.8)	1.9 (0.3, 3.1)	0.0 (0.0, 2.5)	5.2 (0.0, 5.5)

Data are median (IQR) log<sub>10</sub> colony forming units (CFU)/mL CSF collected among all persons with initial CSF cryptococcal antigen positivity, including sterile cultures. Twenty-five participants with negative CSF cryptococcal antigen and sterile cultures are not included.