How many replicates should be used in an ELISPOT assay and what is the cut-off for a positive response?

Marcus Dittrich, Paul V. Lehmann, and Alexey Y. Karulin

Cellular Technology Limited • R&D Department • 20521 Chagrin Boulevard • Shaker Heights, OH USA

INTRODUCTION: At present the answers to the above questions are largely based on empirical or mixed criteria. To come up with scientifically-validated answers, parametric statistical analysis has to be used, which in turn requires knowledge about the distributional properties of ELISPOT counts in replicate wells. The lack of such knowledge might be compensated by the use of non-parametric statistics like Bootstrap, Wilcoxon tests, DFR or the Chebychev inequality, however, these methods have lower power then parametric statistics (Student t-test, ANOVA). Establishing the distributional properties of ELISPOT counts requires large numbers of replicate wells. Since typical ELISPOT assays involve triplicate, or at most quadruplicate wells, presently there is a paucity of data that would permit defining reliable rules for the numbers of replicates and cut-off criteria for a positive response.

METHODS: PBMC of individual donors were plated with antigen (HCMV pp65 or HCMV pp6) in the specified number of replicate wells and in different cell numbers. IFN-γ was measured using the ImmunoSpot® Test Kit, and to assure low background, CTL-Test™ Medium was used. The spots were counted using an ImmunoSpot® S6 Core reader. The distributional properties of the spot counts in the replicate wells were analyzed using diagnostic plots (QQ plots) and Shapiro-Wilk statistical normality tests.

RESULTS: The results show that spot counts in a range between 6 per well and up to 350 per well follow Normal distribution (Figure 3). The Coefficient of Variation (CV, also called Relative Experimental Error) of ELISPOT data was shown to decrease from about 20% at ~30 spots per well to 5% for spot counts 350 per well (Figure 2). The ratio of spot counts for positive wells vs. negative controls increased with cell numbers plated, and with the use of signal-enhancing CTL-Test Plus™ Medium (Figure 4).

CONCLUSIONS: The Normal distribution of ELISPOT counts permits one to make precise predictions regarding the numbers of replicate wells needed and cut-off values. When statistical analysis is at the limits of confidence, experimental procedures can be implemented to reach confidence. Testing PBMC at higher cell numbers will help to establish positivity since spot counts and cell numbers are linear in the 25,000 – 1,000,000 PBMC/well range (Figure 1), both the signal-to-background ratio (Figure 4), and the CV (Figure 2) improves with the numbers of cells plated. Similarly, the use of signal-enhancing CTL-Test Plus™ Medium is suited to increase the signal-to-noise ratio (Figure 4). Thus, instead of testing three replicate wells at 300,000 PBMC per well as is commonly done, which requires 1.8 million PBMC for measuring the medium control and antigen-induced response, a single well each at 600,000 PBMC/well will provide more reliable data with 2/3rd of the cell material.

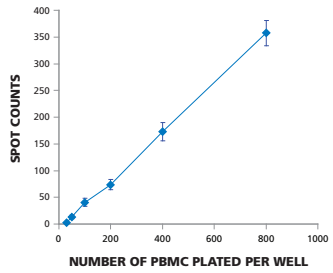

Figure 1: **ELISPOT counts and PBMC numbers plated are linear in the range between 25,000 and 1,000,000 PBMC per well.** PBMC of a donor were plated in 120 replicates for each of the numbers specified on the X axis per well. The mean number of IFN-γ ELISPOTs elicited by HCMV pp65 is shown on the Y axis. Error bars represent ±SD.

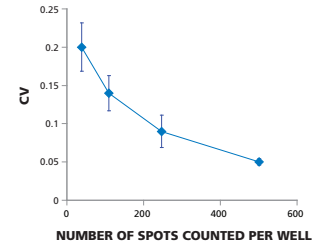

Figure 2: **Relative experimental error (coefficient of variation, CV) decreases with the number of spot counts per well.** PBMC were seeded at 400,000, 200,000, 100,000, and 50,000 cells per well, in 12 replicate wells, and the HCMVpp65-induced IFN-γ response was measured. The mean CV for each count category is shown with error bars showing the SD between 8 experiments.

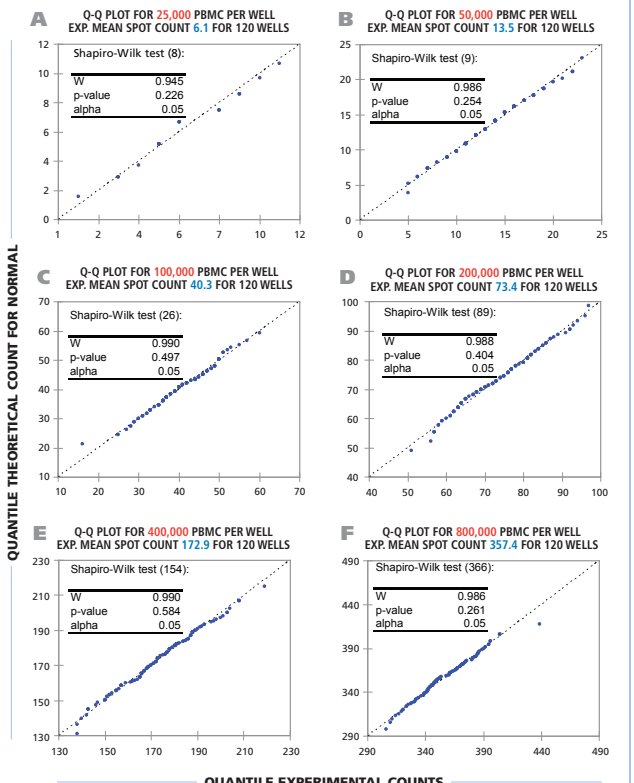

Figure 3: **Spot counts between 6 and 350 per well follow the Normal distribution function.** Therefore, parametric statistical tests (t-Test, Annova) can be used to discriminate between negative and positive responses in the full range of ELISPOT assay result span. The data shown in Figure1 were subject here to statistical analysis of the distributional properties of the spot counts. For each condition, represented in the panels A-F, the distributional properties of the spot counts were analyzed in the 120 replicate wells using diagnostic plots (QQ plots) and Shapiro-Wilk statistical normality tests with the significance level of α = 0.05 (shown in picture inserts). X axis labels the Quantile experimental counts, Y axis the Quantile theoretical counts for Normal distribution.

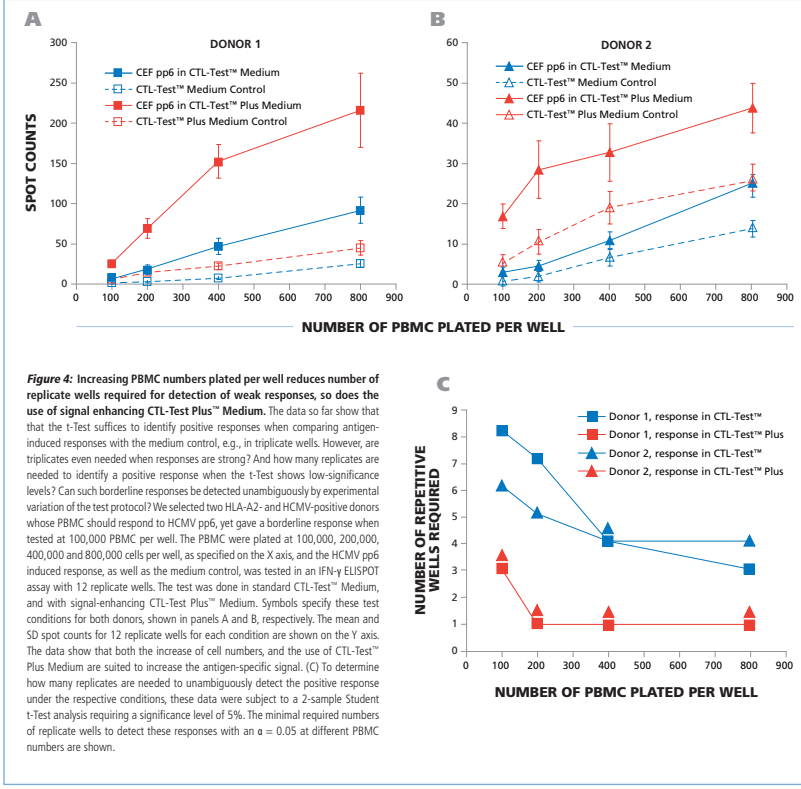

Figure 4: **Increasing PBMC numbers plated per well reduces number of replicate wells required for detection of weak responses, so does the use of signal enhancing CTL-Test Plus™ Medium.** The data so far show that that the t-Test suffices to identify positive responses when comparing antigen-induced responses with the medium control, e.g., in triplicate wells. However, are triplicates even needed when responses are strong? And how many replicates are needed to identify a positive response when the t-Test shows low-significance levels? Can such borderline responses be detected unambiguously by experimental variation of the test protocol? We selected two HLA-A2- and HCMV-positive donors whose PBMC should respond to HCMV pp6, yet gave a borderline response when tested at 100,000 PBMC per well. The PBMC were plated at 100,000, 200,000, 400,000 and 800,000 cells per well, as specified on the X axis, and the HCMV pp6 induced response, as well as the medium control, was tested in an IFN-γ ELISPOT assay with 12 replicate wells. The test was done in standard CTL-Test™ Medium, and with signal-enhancing CTL-Test Plus™ Medium. Symbols specify these test conditions for both donors, shown in panels A and B, respectively. The mean and SD spot counts for 12 replicate wells for each condition are shown on the Y axis. The data show that both the increase of cell numbers, and the use of CTL-Test™ Plus Medium are suited to increase the antigen-specific signal. (C) To determine how many replicates are needed to unambiguously detect the positive response under the respective conditions, these data were subject to a 2-sample Student t-Test analysis requiring a significance level of 5%. The minimal required numbers of replicate wells to detect these responses with an α = 0.05 at different PBMC numbers are shown.