

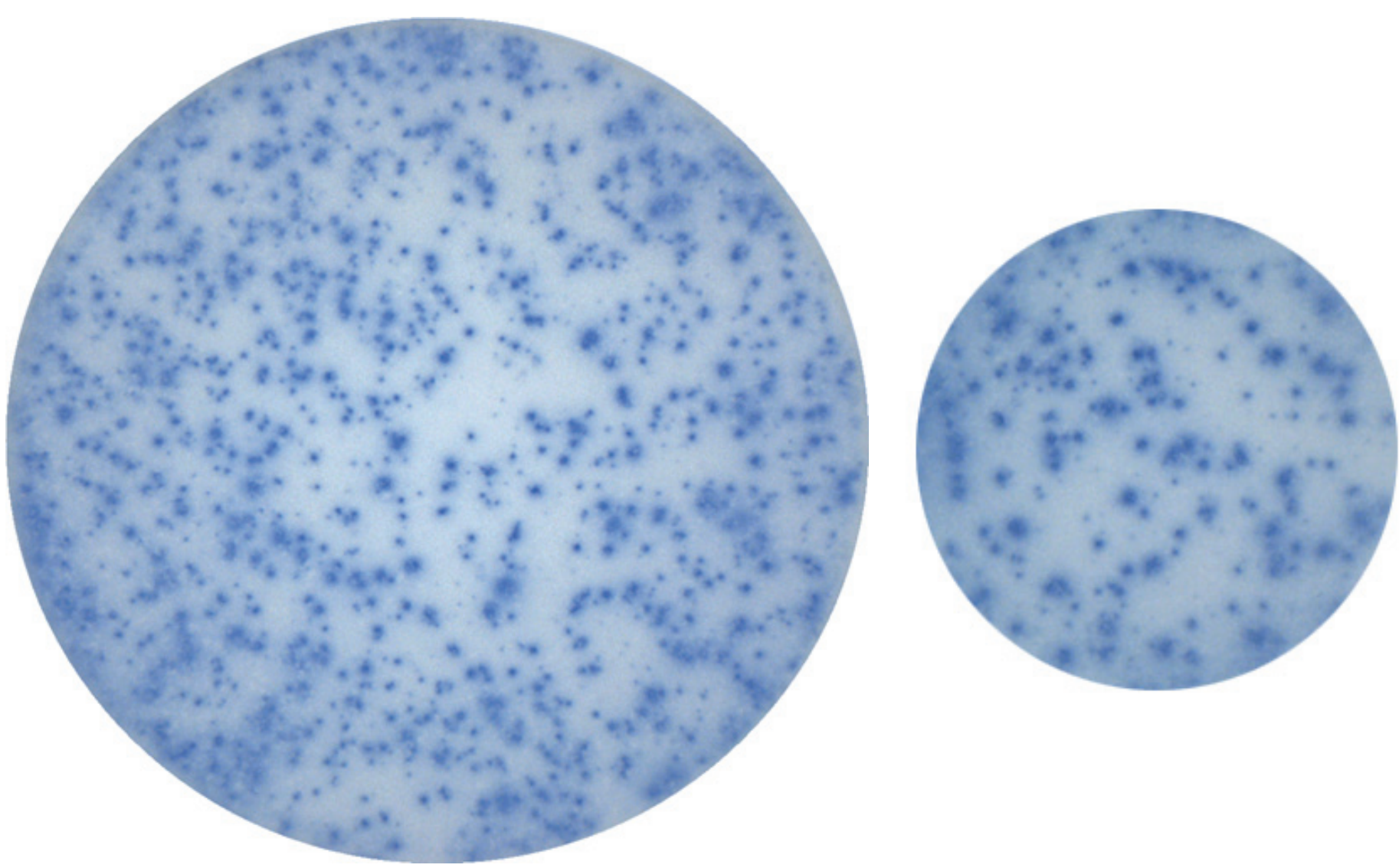
# ELISPOT assays in 384-well format allow analysis of 400 independent T cell tests with 10ml blood

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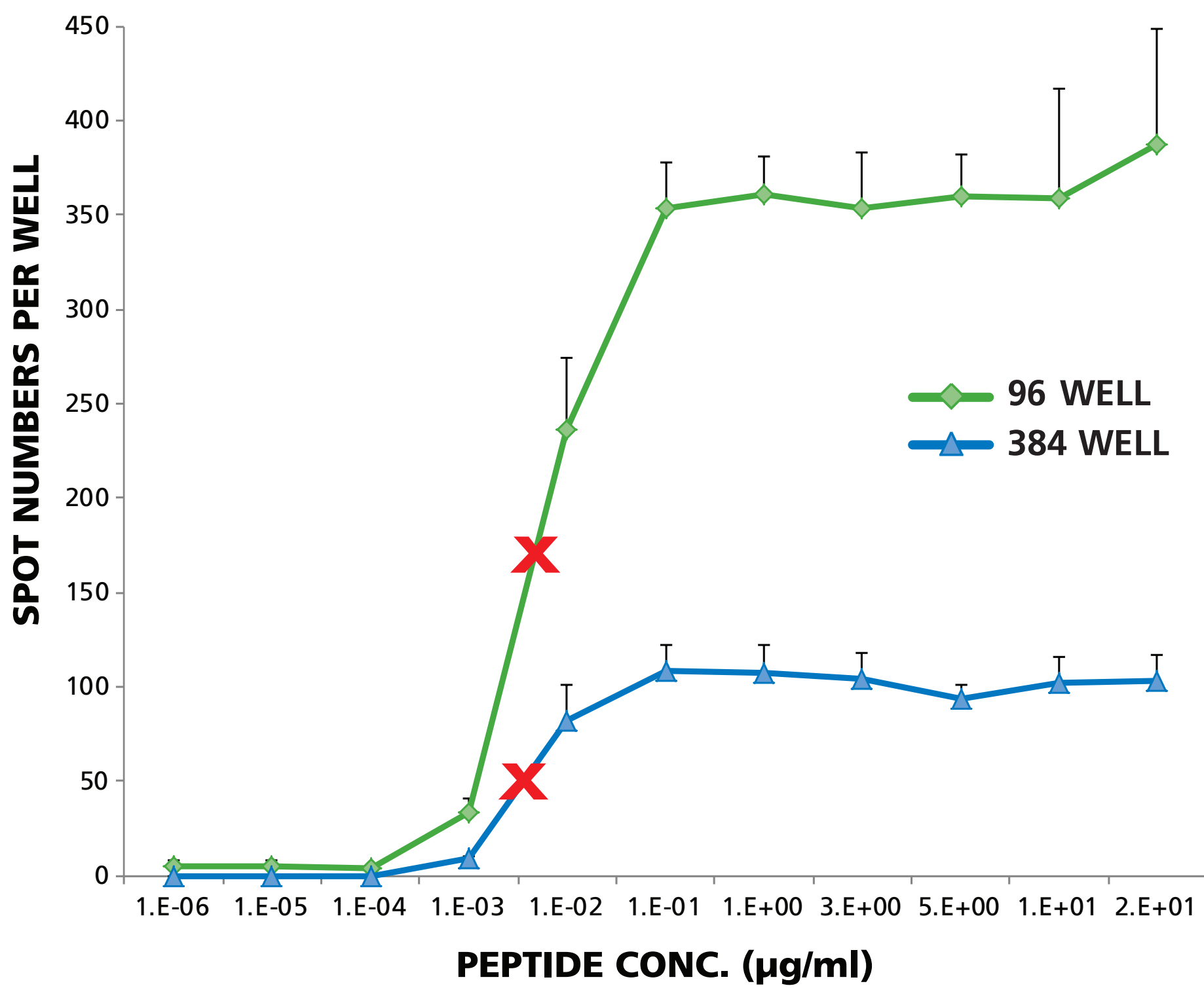
**INTRODUCTION:** The number of PBMC that can be obtained from human subjects is the limiting factor for comprehensive monitoring of antigen-specific T cell immunity, which requires that multiple tests are performed with those cells. Measuring frequencies of antigen-specific T cells producing IFN- $\gamma$ , IL-2, IL-4, IL-17, and Granzyme B provides information on the effector lineages. Titrating the antigen permits one to define the affinity of each the T cell subsets. Occasionally multiple antigens need to be tested to account for epitope spreading, or for the fine specificity of T cell immunity. Standard ELISPOT assays performed in 96-well format are already highly efficient in cell utilization because 100,000 PBMC per well suffice for measuring one of the above parameters. Here we report on increasing three-fold the efficiency of cell utilization by performing ELISPOT assays in 384-well format.

**METHODS:** The membrane surface of a well in a 384-well plate is one-third that of a 96-well plate. To address the question whether ELISPOT assays can be miniaturized to a 384-well format to further economize PBMC utilization, we scaled down the reagents to match the surface of the well. IFN- $\gamma$  was measured using ImmunoSpot® Test Kit, and to assure low background, CTL-Test™ Medium was used. The spots were counted using an ImmunoSpot® S6 Core reader.

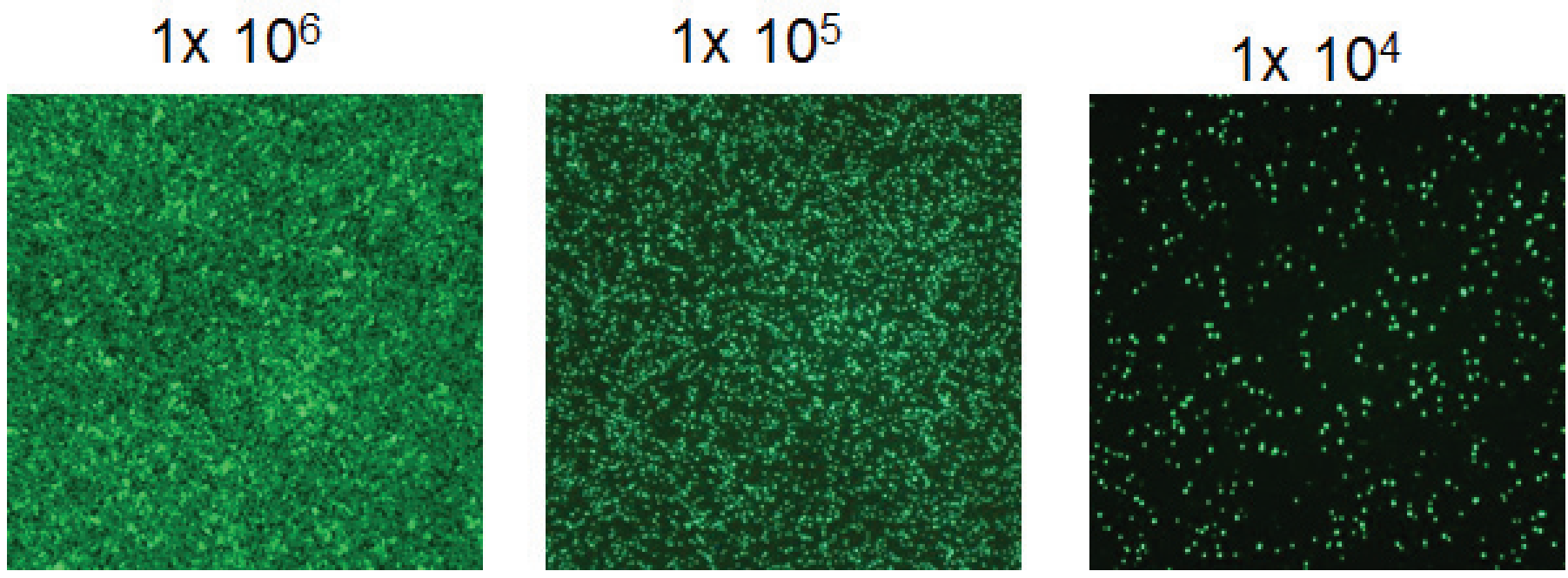
**RESULTS AND CONCLUSION:** We show that performing ELISPOT assays in 384-well format permits a one-in-three miniaturization of 96-well assays, that is, generating three times more single cell resolution data points for comprehensive characterization of T cell immunity: up to 400 tests with 10ml of blood. By miniaturizing the assay, multiple parameters of T cell immunity can be measured with the limited amount of blood that can be obtained from test subjects.



**Figure 1: Representative ELISPOT well in 96- and 384-well format.** For this figure, and for all ELISPOT data analyzed here, the respective images were captured with identical optical and digital resolution which permits the use of identical counting parameters, except for the adjusted region of interest for counting. The region of interest, that is, the membrane surface of a well of a 96-well plate is three times that of the 384-well plate. Note, the density and size of spots is identical for the two well formats.



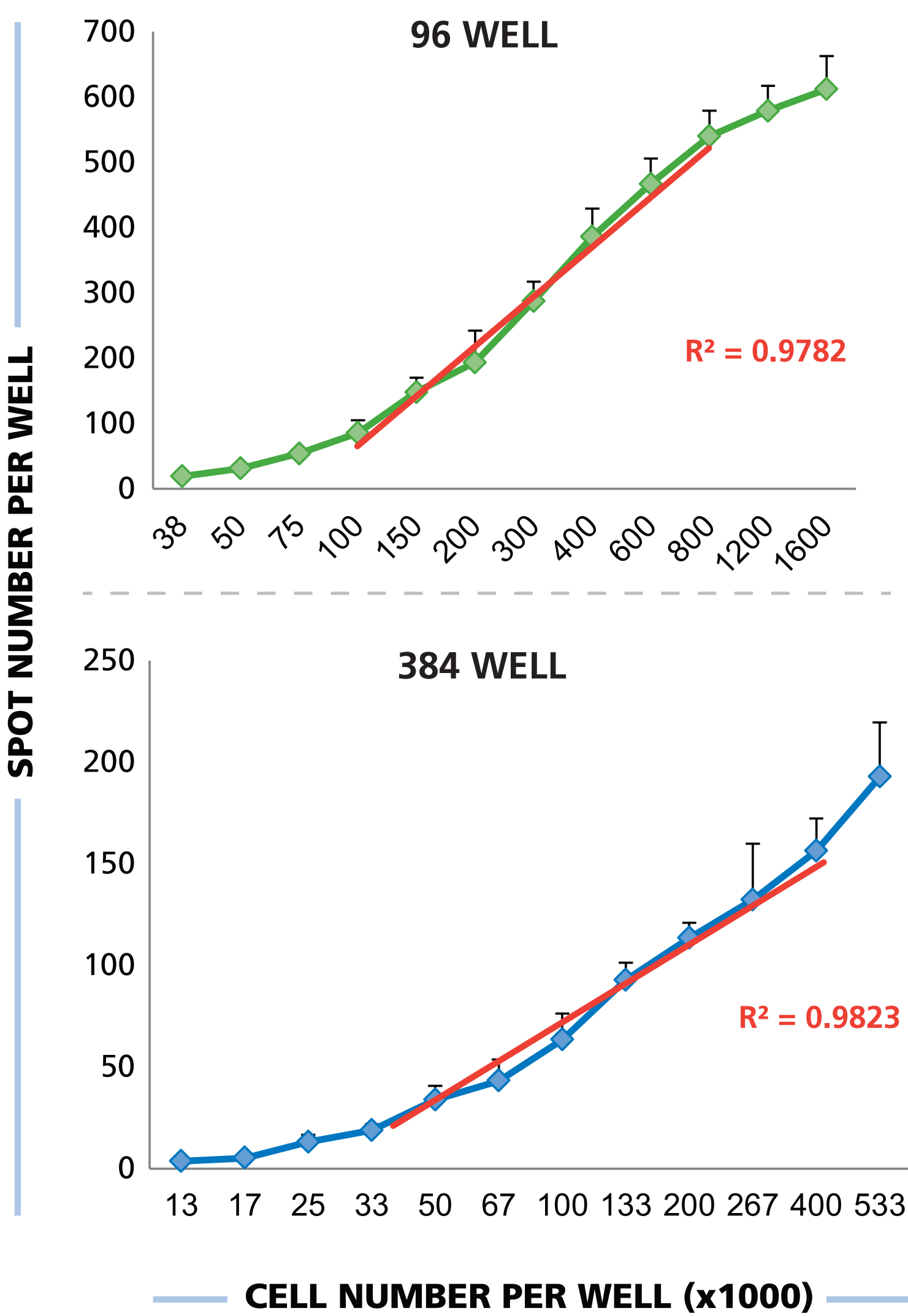
**Figure 4: Identical antigen dose response curves for 96- and 384-well plates.** HCMV peptide pp65-induced IFN- $\gamma$  production was measured in both plate types in parallel with one-third of the numbers of PBMC plated per well for 384-well plates. While the maximal spot counts induced by the peptide were approximately 3x higher for 96-well plates, the 50% maximally stimulatory peptide dose (marked by the red X) was identical for the two plate types. Thus, the 384-well format is equally suited for T cell affinity measurements.



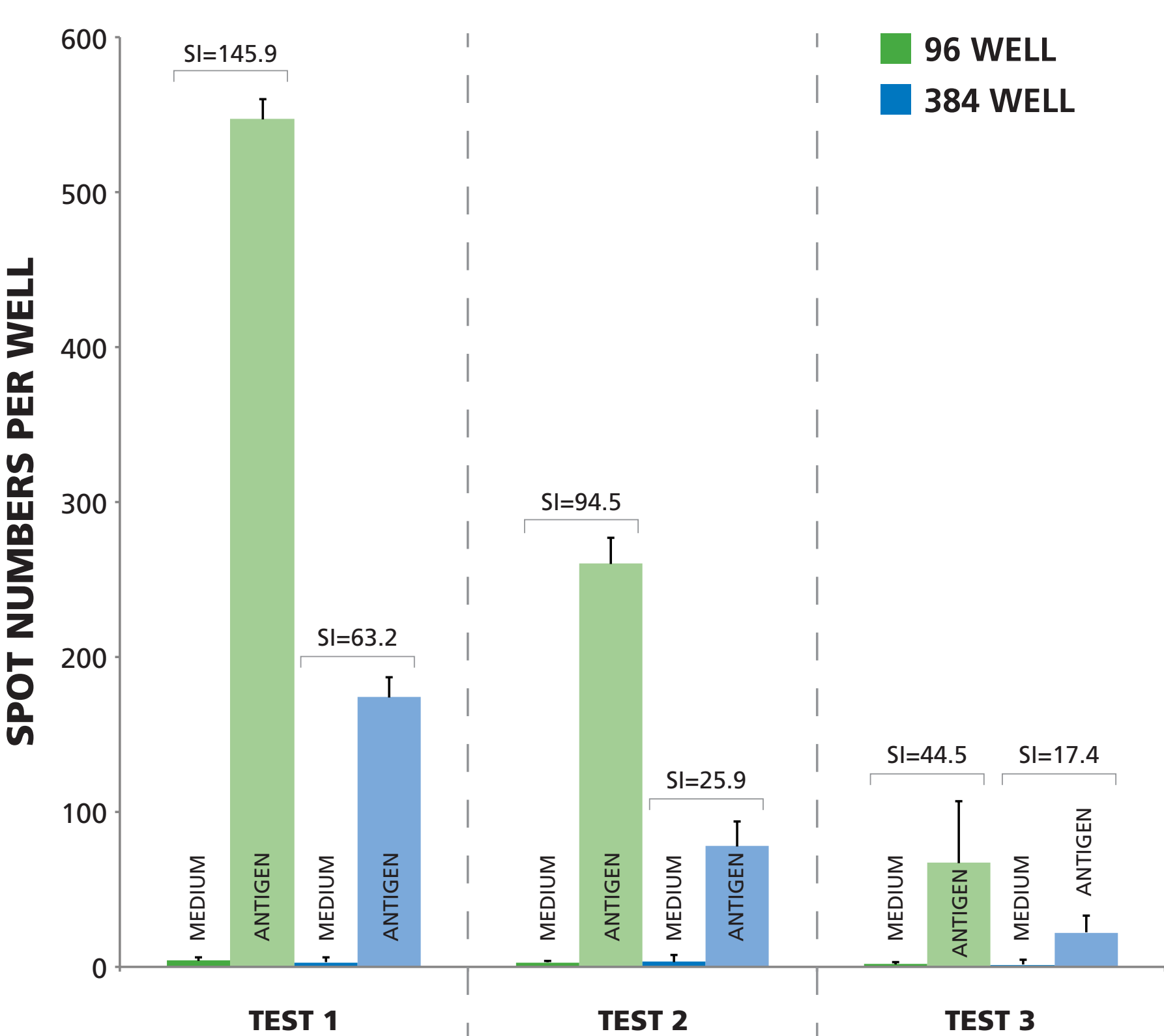
**Figure 3. PBMC number-dependent monolayer formation in 96-well plate format.** PBMC were stained with calcein and the specified numbers of cells were plated per well. Note, cell crowding starts at  $1 \times 10^6$  cells per well, and cells do not form a monolayer any more under  $1 \times 10^5$  cells per well corresponding to the range in which PBMC numbers and spot counts are linear in the ELISPOT assay (See Figure 2).

Test	96 W	384 W	96W/384W
1	670.0	177.5	<b>3.8</b>
2	577.5	177.3	<b>3.3</b>
3	547.3	173.8	<b>3.1</b>
4	499.3	164.0	<b>3.0</b>
5	423.0	118.5	<b>3.6</b>
6	344.5	102.8	<b>3.4</b>
7	260.0	77.8	<b>3.3</b>
8	140.3	52.3	<b>2.7</b>
9	126.0	35.5	<b>3.5</b>
10	66.8	21.8	<b>3.1</b>
11	26.0	7.5	<b>3.5</b>
MEAN 3.3 $\pm$ 0.3			

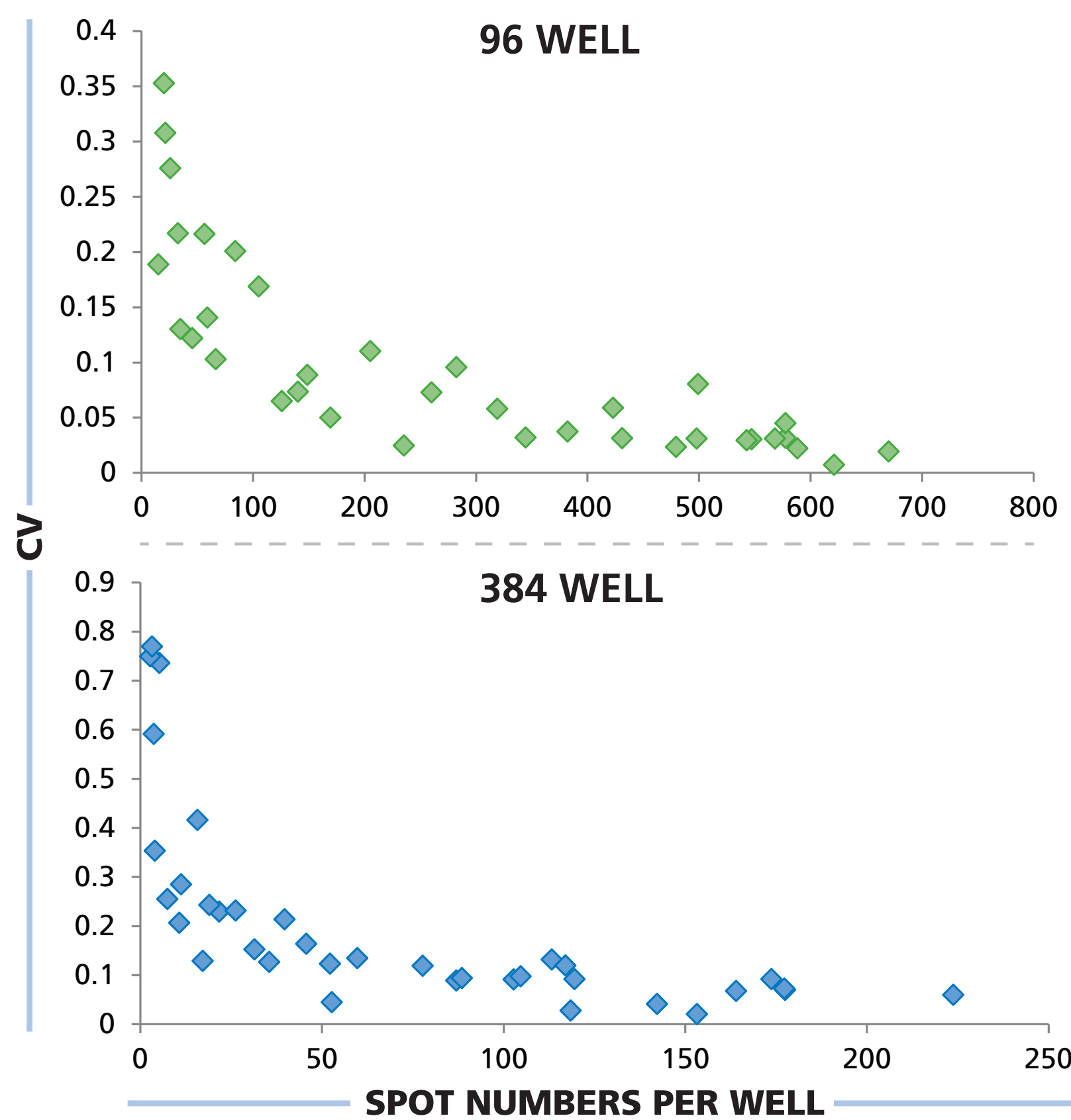
**Table 1: Spot counts in 96-well format are approximately three times the spot count in the 384-well plate format.** The membrane surface of each well in the 96-well plate is three times (3.031) that of the 384-well plate. Therefore, we tested whether the spot counts in both plate types would maintain this exact ratio if cells are plated at the same density (that is, if 1/3rd of PBMC are plated in the 384-well plate compared with 96-well plate). Of 36 tests performed, here representative data are shown for 11 tests. PBMC were tested in parallel in the two well formats using HCMV peptide pp65 to elicit IFN- $\gamma$  production in CD8 cells. The ratio of spots for each test is shown in bold. The mean ratio for all 11 tests was 3.3 with an SD of 0.3.



**Figure 2: Linearity between numbers of PBMC plated and numbers of ELISPOTs elicited for both plate types.** PBMC were plated in serial dilution in the numbers specified, and HCMV pp65 was used to elicit IFN- $\gamma$  production by the specific CD8 cells. For 96-well plates, a linear relationship was seen between  $1 \times 10^5$  and  $8 \times 10^5$  PBMC per well. For 384-well plates, this range was shifted by one third of the cell number, between  $0.3 \times 10^5$  and  $3 \times 10^5$  PBMC per well.



**Figure 5: The signal-to-noise ratio of 96-well plates is approximately three times higher than for 384-well plates.** Signal-to-noise (SI) is defined as the number of ELISPOTs induced by antigen (here HCMV pp65-elicited IFN- $\gamma$ ) divided by the number of spots in the medium background. Examples for medium control and antigen-induced spot counts are shown for three response levels with the SI specified. With the signal being approximately 3x higher for the 96-well plates, also the signal-to-noise ratio was approximately 3x higher for the 96-well plate.



**Figure 6: The coefficient of variation (CV) increases exponentially in both plate formats when spot counts are low.** The CV was calculated from quadruplicate wells tested in parallel in both plate formats for HCMV pp65-elicited IFN- $\gamma$  production. The data show that because the sampling volume for the 384-well plate is one third of the 96 well plate the CV will be larger for the 384-well plate for low spot counts.