Electronic Supplemental Material

Supplementary Table 1: Summary of results from the three CIP ELISPOT proficiency panel phases. (A) Replicates with high variation. The first column indicates the laboratory ID, the second column indicates the number of replicates performed and the third column indicates the number of replicates that had an extremely high variation, defined as being a variation above the 95th percentile. (B) Background spot production. The first column indicates the laboratory ID, the second column indicates the number of medium replicates performed and the third column indicates the mean number of spots per 100,000 PBMCs reported for medium control wells.

A) Replicates with High Variation

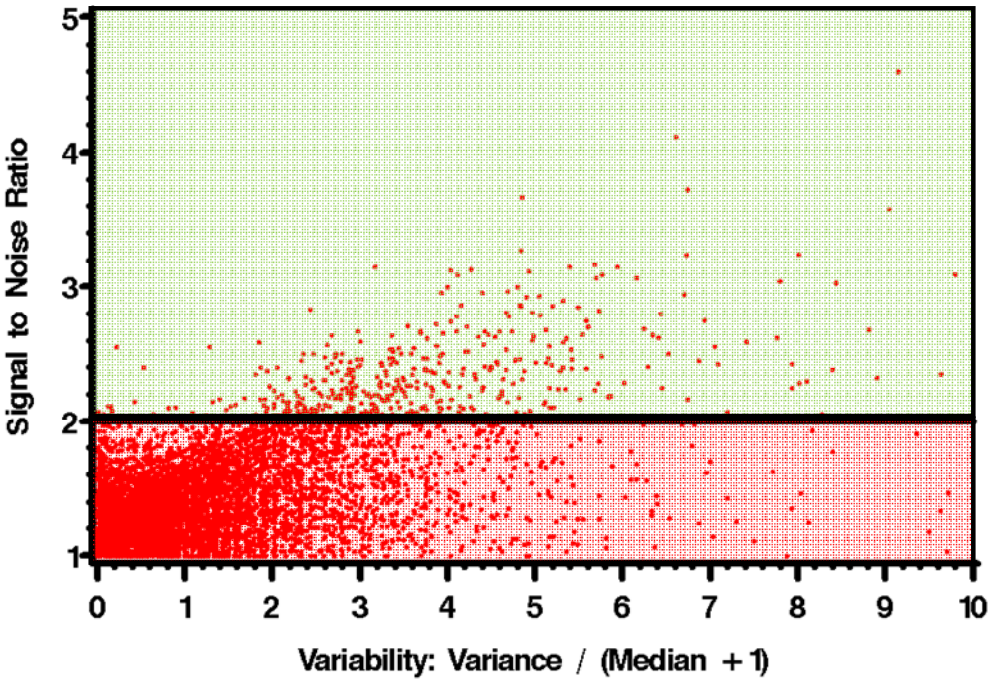
Lab ID	Number of Replicates	Replicates above 95th
1	52	**4**
2	54	**3**
3	54	0
4	54	**6**
5	54	**4**
6	39	2
7	54	**3**
8	54	0
9	54	1
10	24	**4**
11	54	**4**
12	39	1
13	39	2
15	15	0
16	15	0
19	15	0
21	15	1
23	15	0
24	15	1
19 labs	**717**	**36**

B) Background spot production

Lab ID	Number of Replicates	Mean Background
1	18	7.0
2	18	3.5
3	18	2.2
4	18	24.9
5	18	58.1
6	13	14.4
7	18	3.5
8	18	7.3
9	18	2.2
10	8	18.7
11	18	3.1
12	13	2.2
13	13	1.5
15	5	0.2
16	5	45.5
19	5	2.9
21	5	1.5
23	5	13.1
24	5	3.7
19 labs	**239**	11.5

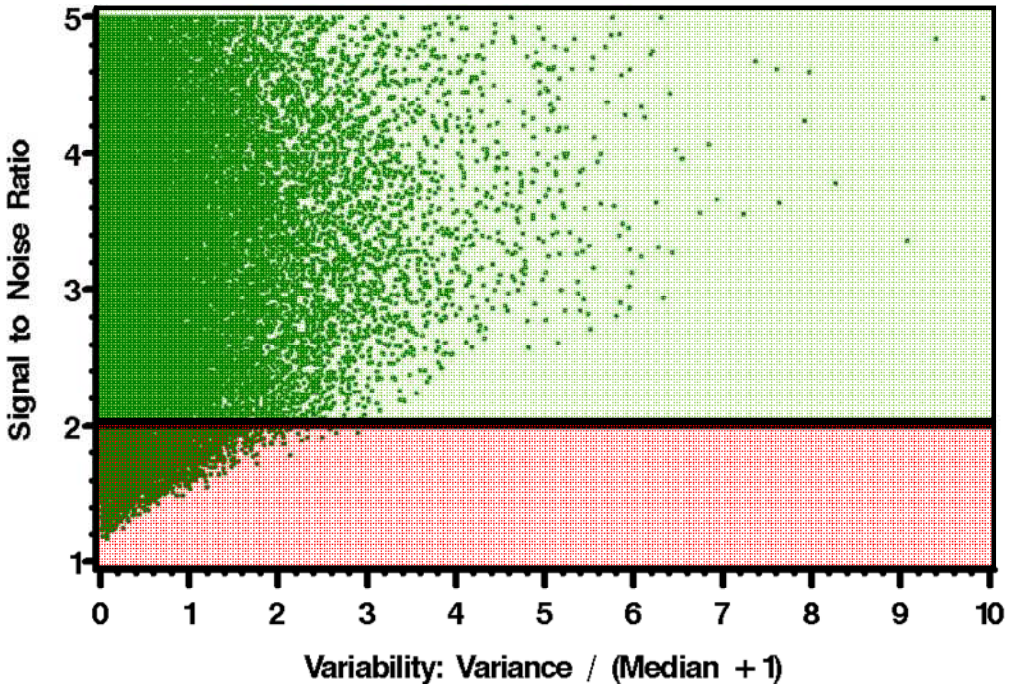
Supplementary Figure 1a: Simulation study (n=1000 donors) comparing response determination using statistical and empirical rules. Figure 1a displays the signal-to-noise ratio (y-axis) versus the intra-replicate variability of the experimental triplicate (x-axis). The red dots represent comparisons that would be considered by the ST not to be a response (one sided t-test p-value >0.05). Red dots that appear in the upper (green) part of the Figure would be considered as negative by the ST but positive by an empirical rule (> 2 fold mean of spot counts in medium control).

Figure 1a: Simulation: T-test not Significant



Supplementary Figure 1b: Simulation study (n=1000 donors) comparing response determination using statistical and empirical rules. Figure 1b displays the signal-to-noise ratio (y-axis) versus the intra-replicate variability of the experimental triplicate (x-axis). The green dots represent those comparisons that would be considered a response based on the ST (one sided t-test $p \leq 0.05$). Green dots that appear in the lower (red part) of the Figure would be considered as positive by the ST but negative by an ER (> 2 fold mean of spot counts in medium control).

Figure 1b: Simulation: T-test Significant



Instructions for the Web-based Statistical Response Determination Tool

An online tool to determine positive ELISPOT responses by the distribution free resampling methods (DFR(eq) and DFR(2x)) is available. Instructions regarding its use are provided below. The tool was created at the Statistical Center for HIV/AIDS Research and Prevention in collaboration with the authors and the CIMT Immunoguiding Program. Below are instructions on how to use the online tool to obtain response determination with both statistical DFR methods:

1. Point browser to http://www.scharp.org/zoe/runDFR/
2. Specify number of peptide pools/different antigens.
3. Specify number of experimental wells.
4. Specify number of negative control or mock wells.
5. Specify name of negative control exactly as it appears in data file.
6. Upload a .csv (comma delimited) data file formatted similar to the example file that is available for download on the website.

Column 1 must contain the participant identifier ("id"), column 2 must specify the visit day (enter 1 if participant's samples are from the same day), column 3 must list the antigen (be it peptide, peptide pool, protein or gene) used to stimulate the cells. The next columns correspond to the responses in each well. There should be as many columns of experimental well data as is specified in #3 above. There should be as many columns of negative control well data as is specified in #4 above.

7. Click on **Run Me** icon and wait for output to print on screen. It may also be downloaded as a .csv file.
8. DFR(eq) positivity calls are listed in the "DFR(eq) response" column and DFR(2x) calls in the "DFR(2x) response" column, The corresponding p-values are listed in the respective adjp (adjusted p-value) columns.

A more detailed explanation on how to correctly format a dataset for analysis using the web tool is presented below, illustrated using the data example provided online:

Scenario: ELISPOT experiment with 6 donors at one time point, testing 2 peptide pools (tyr and flu) in triplicate wells and 6 negative control wells for each donor. The goal is to determine for each donor if a positive response is detected for any of the two peptide pools using the DFR methods.

In order to utilize the web interface for the DFR tests, a data spreadsheet must be in the correct format and can be done as follows:

1. In the first column, enter donor id.
2. In the second column, enter the visit number. Since all of the data are from one time point, 1 should appear in each row of this column. (The purpose of this column is to allow data from the same individual at different time points to be analyzed separately.)
3. In the third column, indicate the antigens tested. Two peptide pools were tested: tyr and flu, and negative control wells which are denoted by “negctl". For each donor there will be three rows of data. One row for each donor must be the spot counts from the negative control wells. The other two rows will contain the spot counts from the two peptide pools tested. The order of the three rows within a donor does not matter. The spelling of the antigen and negative control names must be consistent across the donors. The program is case sensitive.
4. In the fourth to last columns, enter the spot counts for each well. For the negative control experiments there are 6 wells and therefore 6 columns of data. For the 2 peptide pool experiments there are 3 wells per peptide pool and therefore 3 columns of data.
5. To illustrate the data entry for donor 1, the spot counts for the 6 negative control wells were 9, 5, 8, 3, 5, 7. The spot counts for the tyr peptide pool were 23, 22, 24; the flu peptide pool were 11, 7, 8. Hence the spreadsheet would be as follows:

id	Day	antigen	well1	well2	well3	well4	well5	well6
1	1	negctl	9	5	8	3	5	7
1	1	tyr	23	22	24			
1	1	flu	11	7	8			

6. After completing the data entry in Excel save the file as a comma delimited file. To do this, select the 'File' menu and then select 'Save As'. The 'Save As' box will pop up. At the bottom of the pop up box it says 'Save as type'. Click on the down arrow across from where it says this. Scroll to the option 'CSV (comma delimited)(*.csv)' and select it. Then click on the 'Save' button. A text box will pop up alerting the user that 'The selected file type does not support workbooks that contain multiple sheets.' Click 'OK'. There may also be an alert stating 'myfile.csv may contain features that are not compatible with CSV (Comma delimited). Do you want to keep the workbook in this format?' Click 'Yes'. The comma delimited file should now be created and is ready for uploading.

Once the data file is ready, go to the website: http://www.scharp.org/zoe/runDFR/ and enter the relevant information.

Number of antigens: 2

Number of experimental wells: 3

Number of control wells: 6

Name of negative control: negctl

Submit your .csv data file here (view an example): myfile.csv file created as described above.

Click on **Run Me** icon and wait for output to print on screen.

The results may also be downloaded as a .csv file.

DFR(eq) positivity calls are listed in the "DFR(eq) response" column and DFR(2x) calls in the "DFR(2x) response" column, The corresponding p-values are listed in the respective adjp (adjusted p-value) columns.

There are 6 donors and two antigens of interest. Therefore there can be at most 12 positive responses. The results based on the output generated indicate 8/12 responses are positive by the DFR(eq) method and 4/12 are also positive by the DFR(2x) method.

Some tips to ensure correct output:

1. The antigens must be consistently named across donors. As stated above the program is case sensitive.
2. Missing data must be handled consistently, denoted either by a blank cell or NA. Text such as “nd” or “na” will not be recognized and will result in errors.