NCL Method ITA-42

Detection of Interferon gamma (IFNγ) and Interleukin 2 (IL2) by Fluorescent Enzyme-Linked Immunosorbent Spot (FLUOROSpot) Assay

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This protocol assumes an intermediate level of scientific competency with regard to techniques, instrumentation, and safety procedures. Rudimentary assay details have been omitted for the sake of brevity.

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1. Introduction

Activation of T-lymphocytes is a hallmark of adaptive immunity. Vaccines and immunotherapies activate T-cells as part of their intended mechanism of action [1, 2]. In contrast, undesirable activation of T-cells underlies hypersensitivity and autoimmune reactions [3, 4]. Therefore, monitoring T-cell activation provides a crucial mechanistic insight into both healthy and pathological conditions and is of interest for preclinical efficacy and safety studies along the drug development process. Activation of T lymphocytes can be detected by monitoring two cytokine biomarkers—interferon gamma (IFNγ) and interleukin 2 (IL-2). The detection of these cytokines, produced by immune cells exposed to drugs, immunotherapy, or vaccine products, aids in preclinical efficacy and safety studies.

IFN-γ and IL-2 can be detected using traditional single-plex enzyme-linked immunosorbent assay (ELISA), single-spot Enzyme-Linked Immunosorbent Spot (ELISpot) assay, or single-spot Fluorescent Enzyme-Linked Immunosorbent Spot (FLUOROSpot) assay, each of which analyzes one cytokine at a time. Alternatively, both cytokines can be detected simultaneously using either a multiplex ELISA or a dual-spot FLUOROSpot assay. Both the ELISpot and FLUROSpot formats are helpful to verify the results of traditional and multiplex ELISAs. Advantages and limitations of the ELISpot assay vs. multiplex ELISA have been discussed elsewhere [5]. This document describes a protocol for the simultaneous detection of IL-2 and IFNγ using a dual-spot FLUOROSpot assay, which is based on an interlaboratory study described earlier [6].

2. Principles

In this protocol, human peripheral blood mononuclear cells (PBMCs) from healthy donor volunteers are exposed to controls and test samples for 24 hours on 96-well FLUOROSpot plates pre-coated with IFNγ and IL-2 capture antibodies. At the end of the incubation, the cells are washed away, and cytokines captured by the antibodies immobilized on the FLUOROSpot plate are detected using two levels of detection reagents. First, FITC-labeled IFNγ detection antibody and biotin-labeled IL-2 detection antibody are added to the plates. Next, after plate washing, anti-FITC antibody conjugated to a green fluorescent label and Cy3-strepavidin are added to the plate. The result of these detection steps is the formation of green and red colored spots for IFNγ and IL-2, respectively, when the plate is analyzed using one fluorescent light at a time, detecting

green and red fluorescence. These colors co-localize and result in a yellow spot when IFNγ and IL-2 are produced by the same cell and analyzed using both red and green fluorescent channels simultaneously. The spots are quantified using a MultiSpot reader, which produces two readouts—the number of spots and activity—for each color separately, and allows for monitoring double-positive cells, i.e., those producing both cytokines. The number of spots reflects the number of cells that produce cytokines. The activity readout reflects the brightness of the fluorescent color and, therefore, indicates the quantity of the cytokine produced by a single cell. This protocol utilizes freshly isolated PBMCs, the AID IFNγ/IL-2 dual spot FLUOROSpot kit, and the AID multispot reader for plate analysis. Experimental steps involving instrument settings, plate reading, and analysis are based on a series of technical documents by Autoimmun Diagnostika GmbH [7-11]. When other cells, kits, and instruments are used for this analysis, the assay principle will not change; however, some experimental parameters such as seeding cell density, length of the incubation, fluorescent labels and detection channels, and spot analysis algorithm may need to be adjusted.

3. Reagents, Materials, Cell Lines, and Equipment

Note: The NCL does not endorse any of the suppliers listed below; these reagents were used in the development of the protocol and their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted. Please note that suppliers may undergo a name change due to a variety of factors. Brands and part numbers typically remain consistent but may also change over time.

3.1 Reagents

- 3.1.1 iSpot human IFNγ/IL-2 Kit (AID, ELSP 5710 or ELSP 5810)
- 3.1.2 RPMI-1640 (Cytiva, HyClone, SH30096.01)
- 3.1.3 Penicillin streptomycin solution (Cytiva, Hyclone, SV30010)
- 3.1.4 L-glutamine (Cytiva, Hyclone, SH30034.01)
- 3.1.5 Fetal bovine serum (FBS) (Cytiva, Hyclone, SH30070.03)
- 3.1.6 Phosphate buffered saline (PBS) (Cytiva, SH30256.01)
- 3.1.7 Trypan Blue solution (Gibco, 15250-061)
- 3.1.8 Phytohemaglutinin M (PHA-M) (Sigma, L8902)

3.1.9 Sterile distilled water

3.2 Materials

3.2.1 Pipettes covering the range of 0.05 to 10 mL

3.2.2 Polypropylene tubes, 50 and 15 mL

3.2.3 Reagent reservoirs

3.3 Equipment

3.3.1 Centrifuge

3.3.2 Refrigerator, 2–8ºC

3.3.3 Freezer, -20ºC

3.3.4 Cell culture incubator with 5% CO_2 and 95% humidity

3.3.5 37ºC water bath

3.3.6 Biohazard safety cabinet approved for level II handling of biological material

3.3.7 Inverted microscope

3.3.8 Vortex

3.3.9 Hemocytometer or automated cell counter

3.3.10 AID multispot reader

4. Preparation of Reagents and Controls for Cell Culture

4.1 Complete RPMI-1640 Medium

10% FBS (heat-inactivated)

2 mM L-glutamine

100 U/mL penicillin

100 µg/mL streptomycin sulfate

Store at 2–8ºC protected from light for no longer than 1 month. Before use, warm in a water bath.

4.2 Heat-inactivated Fetal Bovine Serum

Thaw a bottle of FBS at room temperature, or overnight at 2–8ºC, and allow to equilibrate to room temperature. Incubate 30 minutes at 56ºC in a water bath, mixing every 5 minutes. Fifty (50) mL single-use aliquots may be stored at 2–8ºC for up to one month or at a nominal temperature of -20ºC indefinitely.

4.3 Negative Control

Use PBS as a negative control. Process it the same way as your study samples.

4.4 Positive Control

PHA-M at a final concentration of 10 µg/mL is used as a positive control in this assay. The stock with a concentration of 1 mg/mL can be prepared in water and stored in small, single-use aliquots at -20°C. On the day of the experiment, prepare a working stock by thawing a frozen 1 mg/mL stock aliquot at room temperature and diluting it to 20 µg/mL in complete cell culture medium (e.g., by mixing 20 µL of the 1 mg/mL stock with 980 µL of the culture medium).
Note: Other T cell agonists, such as concanavalin A, PMA/Ca^{2+} ionophore, and anti-CD3 antibody alone or in combination with anti-CD28 antibody can also be used at relevant concentrations.

4.5 Vehicle Control

Vehicle control is the buffer or medium used to formulate test nanomaterials. Common excipients used in nanoformulations are trehalose, sucrose, and albumin. However, other reagents and materials are also used alone or in combination. Vehicle control should match the formulation buffer of the test nanomaterial by both composition and concentration. This control can be skipped if nanoparticles are stored in PBS.

5. Preparation of Study Samples

The required amounts of nanoparticles will depend on the number of concentrations tested, the number of replicates, and the nanoparticle stock concentration. The minimum required dilution (MRD) of this assay is 2; therefore, the working stock concentration of the test sample should be twice as high as the final concentration desired for analysis in this assay. For example, if one desires to test nanoparticles at 1 mg/mL, the working stock concentration should be 2 mg/mL prepared in the cell culture medium. The recommended minimum volume of the working stock is 800 µL for analysis of cultures derived from 3 donors (100 µL per well in duplicate, plus extra to prepare dilutions and account for dead volume of pipette tips and sample tubes). A strategy for the estimation of nanoparticle test concentrations for in vitro testing has been discussed elsewhere [12].

6. PBMC Preparation

6.1 Place freshly drawn blood into 15- or 50-mL conical centrifuge tubes, add an equal volume of room-temperature PBS and mix well.

6.2 Slowly layer the Ficoll-Paque solution underneath the blood/PBS mixture by placing the tip of the pipet containing Ficoll-Paque at the bottom of the blood sample tube. Alternatively, the blood/PBS mixture may be slowly layered over the Ficoll-Paque solution. Use 3 mL of Ficoll-Paque solution per 4 mL of blood/PBS mixture. For example, 15 mL Ficoll-Paque per 20 mL of diluted blood in a 50 mL tube.

Note: To maintain the Ficoll-blood interface, it is helpful to hold the tube at a 45º angle.

6.3 Centrifuge 30 min at 900x*g*, 18–20ºC, without brake.

Note: Depending on the type of centrifuge, one may also need to set the acceleration speed to a minimum.

6.4 Using a sterile pipet, remove the upper layer containing plasma and platelets and discard it.

6.5 Using a fresh sterile pipet, transfer the mononuclear cell layer into another centrifuge tube.

6.6 Wash cells by adding an excess of HBSS and centrifuge for 10 min at 400x*g*, 18–20ºC. The HBSS volume should be ~3 times the volume of mononuclear layer.

Note: Typically, 4 mL of blood/PBS mixture results in ~ 2 mL of mononuclear layer and requires at least 6 mL of HBSS for the wash step. We use 10 mL of HBSS per each 2 mL of cells.

6.7 Discard supernatant and repeat wash step once more.

6.8 Re-suspend cells in complete RPMI-1640 medium. Dilute cells 1:5 or 1:10 with trypan blue, count cells, and determine viability using trypan blue exclusion. If viability is at least 90%, proceed to step 6.9.

6.9 Dilute the cells to a concentration of 1,000,000 viable cells per 1 mL and add 1 mL of anti-human CD28 per each milliliter of the cell suspension or as directed in the kit instructions. 100 µL of this suspension will be added per well to the FLUOROSpot plate in step 7.2 (100,000 viable cells/well).

7. Setting up the FLUOROSpot Plate for Cell Treatments

7.1 Add 100 µL of test samples and controls prepared in complete cell culture medium to the appropriate wells on the FLUOROSpot plate.

Notes:

a. The minimum number of replicates per each control and sample is 2. A higher number of replicates can also be used.

b. It is advisable to use positive control samples in wells A1, A12, and H12 to facilitate stage calibration. Please consider the plate map in the Appendix of this protocol as an example of plate set-up.

7.2 Add 100 µL of cells from step 6.9 to each well of the FLUOROSpot plate from step 7.1 and incubate in a cell culture incubator (37°C, 5% CO_2, and 90–95% humidity) for 20–24 hours.

Note: It is important to avoid disturbing the plates during the incubation step.

7.3 At the end of the incubation, remove the plates from the incubator and continue with the detection of INFγ and IL-2 using the IFNγ/IL-2 FLUOROSpot kit according to the manufacturer's instructions.

Note: The procedure described in the following step is specific to the AID kit. When kits from other manufacturers are used, the steps detailed below may be different.

8. Experimental Procedure for IFNγ and IL-2 Detection

8.1 FLUOROSpot Reagent Preparation

8.1.1 Warm all reagents to room temperature immediately before use, checking all buffers for the presence of precipitates which may form at low temperature during storage to ensure that they are fully dissolved.

8.1.2 Wash Buffer: Make sure any visible crystals have been dissolved by gentle mixing. Add 50 mL of wash buffer to 450 mL of deionized water.

8.1.3 Anti-human IFNγ and anti-human IL-2 **secondary antibodies**: Add 20 µL of anti-IFNγ and 20 µL of anti-IL-2 detection antibodies to 10 mL of the dilution buffer provided with the kit and mix well.

Note: Prepare this reagent immediately before step 8.2.2.

8.1.4 Streptavidin-Cy3 and anti-FITC-green **detection reagents**: Add 50 µL of streptavidin-Cy3 and 33 µL of anti-FITC-green to 10 mL of the dilution buffer provided with the kit and mix well.

Note: Prepare this reagent immediately before step 8.2.4.

8.2 FLUOROSpot Experimental Procedure for IFNγ and IL-2 Detection

8.2.1 Empty the wells of the culture plate from step 7.3 and wash the plate 5 times with 200 µL per well of the wash buffer prepared in step 8.1.2, blotting the plate against a paper towel after the last wash to remove excess buffer.

8.2.2 Add 100 µL/well of the secondary antibodies prepared in step 8.1.3 and incubate in a humid chamber at room temperature for 2 hours in the dark.

8.2.3 Wash the plate 5 times with 200 µL per well of the wash buffer prepared in step 8.1.2, blotting the plate against a paper towel after the last wash to remove excess buffer.

8.2.4 Add 100 µL of the detection reagents prepared in step 8.1.4 and incubate in a humid chamber at room temperature for 1 hour in the dark.

8.2.5 Wash the plate 5 times with 200 µL per well of the wash buffer prepared in step 8.1.2, blotting the plate against a paper towel after the last wash to remove excess buffer.

8.2.6 Add 100 µL per well of the Enhancer solution provided with the kit and incubate in the dark at room temperature for 15 minutes.

8.2.7 Empty wells and blot the plate against a paper towel to remove excess enhancer solution.

8.2.8 Turn the plate upside down and dry it uncovered overnight or at least for 3 hours at room temperature in the dark.

8.2.9 Read plate on the MultiSpot Reader.

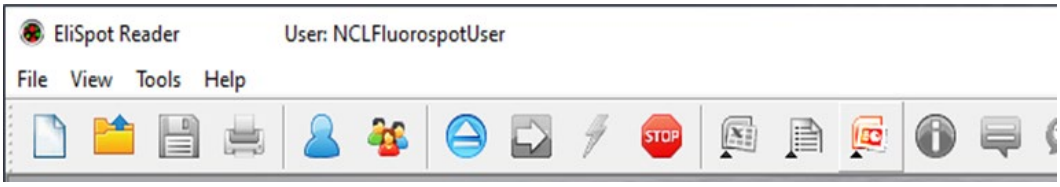
8.3 AID Reader Start-up and Plate Read (*Refer to the instrument and software manuals for more detailed instructions.)*

8.3.1 Turn on the AID multispot reader, light sources, and computer.

8.3.2 Launch the AID software and choose the **EliSpot** button.

8.3.3 Above the menu bar, check the user profile. If it reads **User: NCL FluorospotUser** go to step 8.3.6. If not, under **File** on the menu, click on **Options**, then click **User Profiles** in the newly opened Options window.

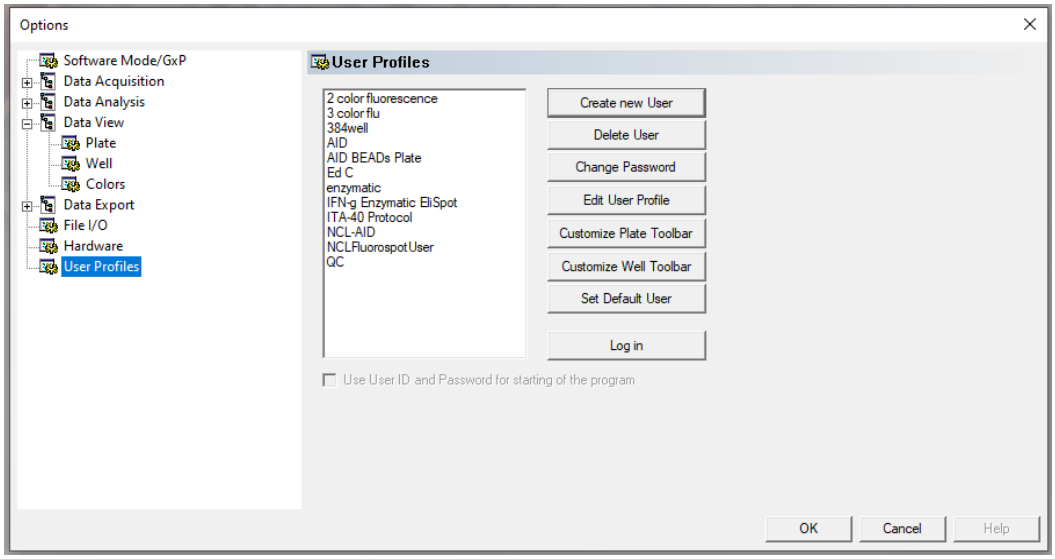
Note: This protocol shares the software menu structure customized for the NCL. Other users will have similar structures specific to their laboratories. This instruction should be treated as an example to help new users with mastering the software in their laboratories.



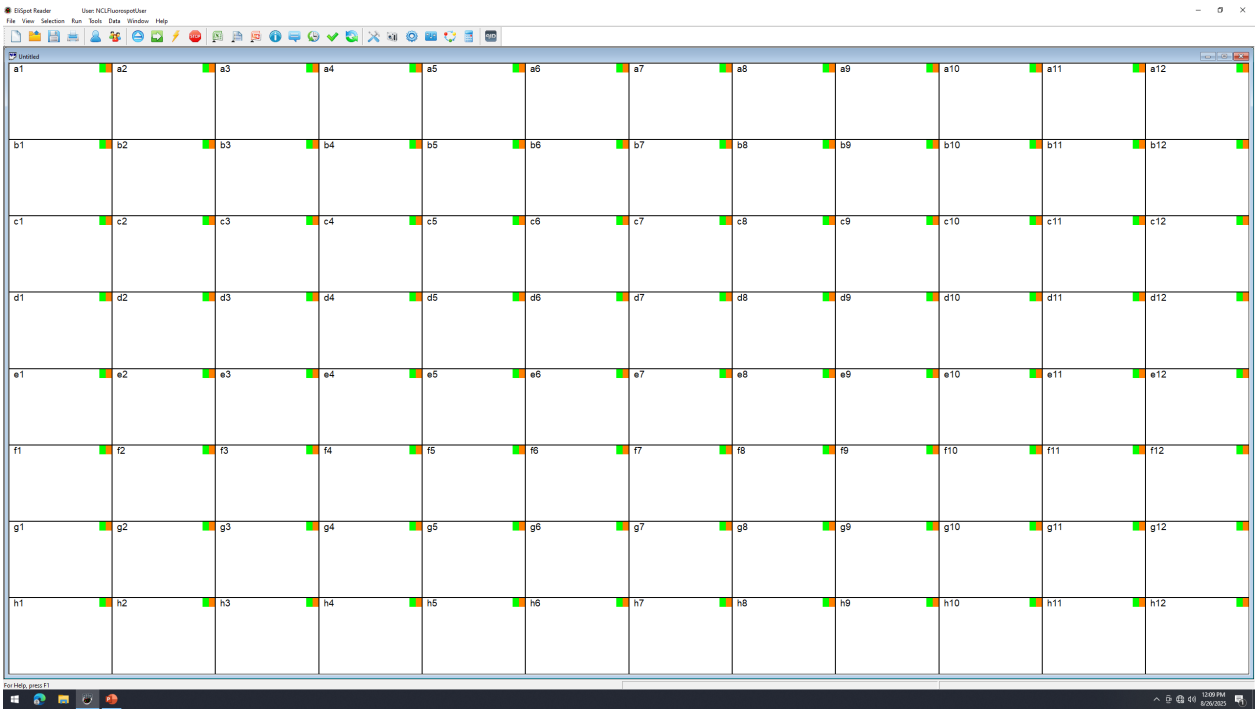
8.3.4 In the user profile window, highlight **NCLFluorospotUser**, click **Log In**, enter the user password, and then click **OK**.

Note: Each user should have their own password to operate this software on their computer. New users should create their own user profiles following the software's manual.

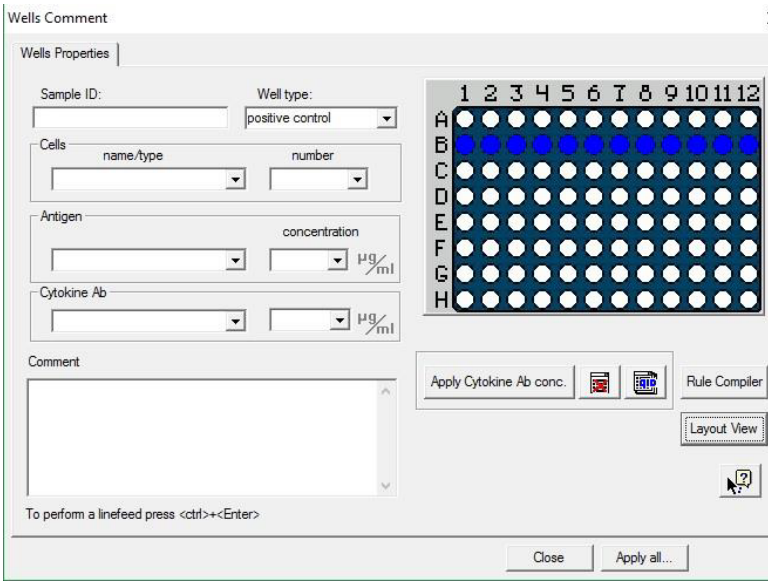
8.3.5 Click **OK** in the Options window to close.



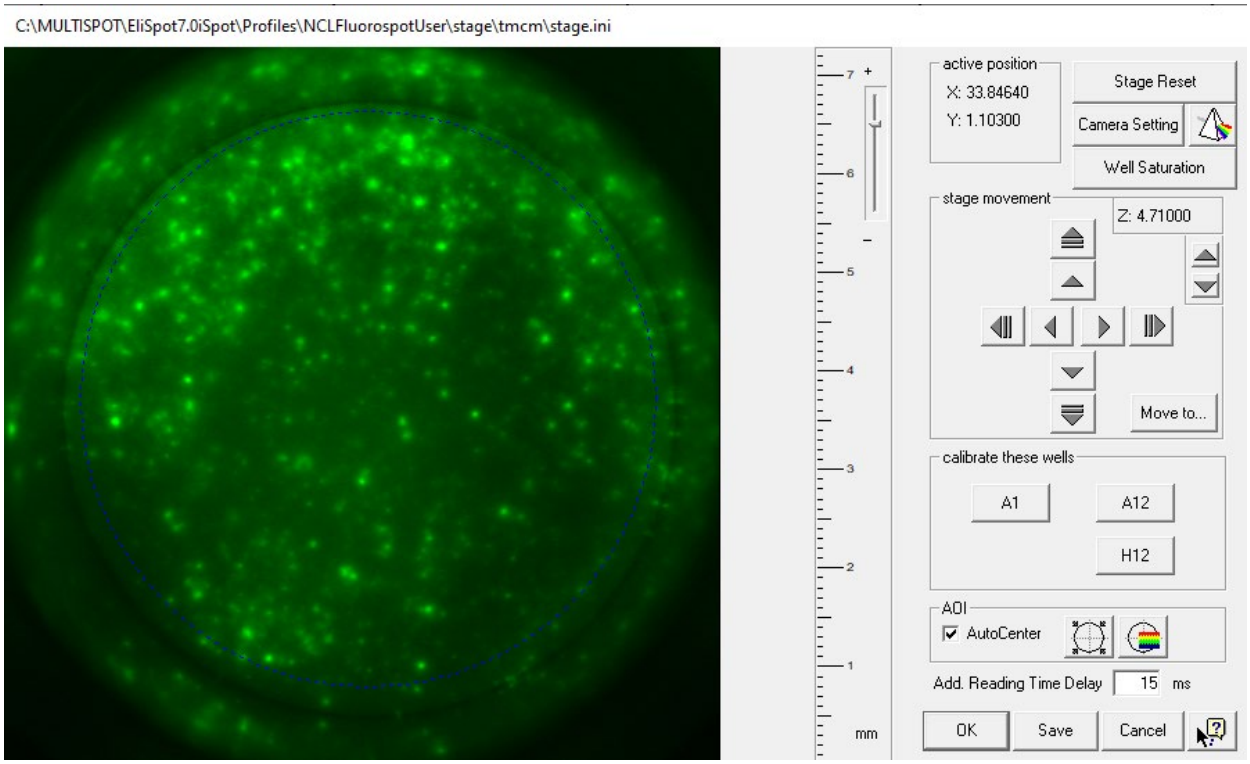
8.3.6 Under the **File** menu click **New**. This should open a plate template with the ITA-42 settings. Remember to save the file under a new name



8.3.7 To add information on cell lines used, cells/well, and treatments, click on the **Tools** menu, click **Plate Layout**, then click **Edit Layout**. This will open the **Wells Properties** window.



8.3.8 Under the **Tools** menu, choose **Calibrate Stage** and choose well **A1**; center and adjust imaging ring to encompass most of the well. Do the same for wells **A12** and **H12**. Click **Save** and **OK** to save the stage calibration.



8.3.9 On the plate map, use the mouse to select the wells to be read, then right click and highlight **Read, Count & Analyze** on the drop-down menu.

8.3.10 After the plate has been read, click the **Save** button.

9. Results and Analysis

If necessary, the analysis algorithms and count settings may be changed from the AID default. This may be done before or after the plate is read.

9.1 Once the plate has been read and analyzed, click the **Excel** button on the menu bar. This will open an Excel file with the spot counts and activities for each of the selected wells.

9.2 Images of the plate and individual wells may be selected and captured using the **Power Point** button on the menu bar or by using the Print Screen function.

9.3 Plate and well information may be captured by clicking **Tools**, **Plate Layout**, then **View Layout**.

10. References

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11. Abbreviations

AID	Advanced Imaging Devices (Autoimmun Diagnostika)
CF	cell-free
FBS	fetal bovine serum
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PC	positive control
PHA-M	Phytohemaglutinin M
RT	room temperature
T	test sample
UNC	untreated negative control

12. Appendix

Example Plate Map

	1	2	3	4	5	6	7	8	9	10	11	12
A	PC	Cell-free	CF PC	CF T1	CF T2	CF T3	CF T4					PC
B	Untreated	Untreated	PC	PC	T1	T1	T2	T2	T3	T3	T4	T4
C	Untreated	Untreated	PC	PC	T1	T1	T2	T2	T3	T3	T4	T4
D	Untreated	Untreated	PC	PC	T1	T1	T2	T2	T3	T3	T4	T4
E	Untreated	Untreated	PC	PC	T1	T1	T2	T2	T3	T3	T4	T4
F	Untreated	Untreated	PC	PC	T1	T1	T2	T2	T3	T3	T4	T4
G	Untreated	Untreated	PC	PC	T1	T1	T2	T2	T3	T3	T4	T4
H		Cell-free	CF PC	CF T1	CF T2	CF T3	CF T4					PC

Wells A1, A12, H12: PBMC + PC for stage calibration

Rows A, H: Cell-free (CF) controls

Rows B, C: Donor 1

Rows D, E: Donor 2

Rows F, G: Donor 3

PC, positive control; CF, cell-free well;
T, test sample (at 4 different concentrations, i.e., T1, T2, T3, T4)