



## TRAINING GUIDE

For *In Vitro* Diagnostic Use Only

This Training Guide covers use of:  
T-SPOT<sup>®</sup>.TB8 (Multi-use 8-Well Strip Plate Format. Catalogue number: TB.300)

**To be used in conjunction with the T-SPOT.TB Package Insert  
[PI-TB-IVD-UK-V3]**

## INTRODUCTION

This training guide is intended to give guidance and information to the operator in preparing and running the T-SPOT.*TB* test. This guide explains sample collection, separation of Peripheral Blood Mononuclear Cells (PBMCs) prior to running the assay, performing the T-SPOT.*TB* test, interpreting test results and troubleshooting.

### Using this document

Instructions on performing the T-SPOT.*TB* test are provided in the top section of each relevant page. Additional information such as notes, corresponding visual aids and examples are shown in blue text

Table of Contents	Page
Introduction	2
Reagent & Storage	3
Materials provided	4
Storage & stability	4
Equipment and materials required, but not provided	5
Sample Collection and Handling	6
Blood collection tubes	7
Blood draw volumes	8
T-Cell Xtend® procedure	8
Cell Isolation	9
Leucosep procedure	9
Standard FICOLL procedure	9
Cell preparation tube (BD vacutainer CPT) procedure	9
PBMC removal	12
Cell washing	13
Cell Counting and Dilution	14
Manual counting of PBMCs	15
Automated counting of PBMCs	17
Plate Setup and Incubation	19
Spot Development and Counting	23
Spot development	24
Spot Interpretation	26
Quality Control	32
Nil controls	33
Positive controls	33
Results Interpretation	34
Results interpretation and assay criteria	35
Borderline & invalid results	35
Spot Interpretation: Panel A & B Antigen	36
Troubleshooting Images	38

# **REAGENT & STORAGE**



## Instructions For Use

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### MATERIALS PROVIDED

T-SPOT.TB8 (Multi-use 12 x 8-well strip) contains:

- 1 microtiter plate: 96 wells, supplied as 12 x 8-well strips in a frame, coated with a mouse monoclonal antibody to the cytokine interferon gamma (IFN- $\gamma$ )
- 2 vials (0.8mL each) Panel A: contains ESAT-6 antigens, bovine serum albumin and antimicrobial agents
- 2 vials (0.8mL each) Panel B: contains CFP10 antigens, bovine serum albumin and antimicrobial agents
- 2 vials (0.8mL each) Positive Control: contains phytohaemagglutinin (PHA), for use as a cell functionality control, bovine serum albumin and antimicrobial agents
- 1 vial (50 $\mu$ L) 200 x concentrated Conjugate Reagent: mouse monoclonal antibody to the cytokine IFN- $\gamma$  conjugated to alkaline phosphatase
- 1 bottle (25mL) Substrate Solution: ready-to-use BCIP/NBT<sup>plus</sup> solution
- CD containing the package insert and other information.

### STORAGE & STABILITY

Store the unopened kit at 2-8°C. The components of the kit are stable up to the expiration date printed on the kit box, when stored and handled under the recommended conditions. The kit must not be used beyond the expiration date on the kit label.

Store opened kit components at 2-8°C. Opened components must be used within 8 weeks of opening, such period ending no later than the expiration date on the kit label. Do not mix components between different kits.

Avoid prolonged exposure of the Substrate Solution to light.

## Instructions For Use

### EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- 8-well strip plate frame (available from Oxford Immunotec).
- Class II microbiological cabinet (recommended but not necessary)
- Blood collection tubes, such as Vacutainer®, CPT™ or Heparin Tubes (Note: CPT tubes are available from Oxford Immunotec).
- T-Cell Xtend if processing blood stored for longer than 8 hours post collection.
- \*FICOLL-PAQUE PLUS or alternative PBMC separation material (such as Leucosep tubes, also available from Oxford Immunotec) if not using CPT tubes.
- A centrifuge for separation of PBMCs (capable of at least 1800 RCF (g) and able to maintain the samples at room temperature (18-25°C) if using density centrifugation methods to separate the PBMCs.
- 15ml centrifuge tubes.
- Equipment and reagents to enable counting of PBMCs; either manually using Trypan Blue (or other appropriate stain) and a haemocytometer on a microscope or automatically using a suitable haematology analyser.
- A humidified incubator capable of  $37 \pm 1^\circ\text{C}$  with a 5% CO<sub>2</sub> supply.
- An automatic microtiter plate washer or an 8 channel or stepper pipette to manually wash plates.
- Adjustable pipettes to cover a range of volumes from 1-1000µL (such as four Gilson pipettes capable of delivering volumes of 1-10µL, 2-20µL, 20-200µL and 100-1000µL) and sterile pipette tips. Pasteur Pipettes may also be used where appropriate.
- Sterile D-PBS solution: such as GIBCO® 1x D-PBS (Invitrogen; catalogue number 14040-091).
- Distilled or deionised water.
- A means of visualising the wells, or capturing a digital image of the well, such as a stereomicroscope, magnifying glass or plate imager to allow counting of spots.
- Sterile cell culture medium such as GIBCO AIM V® (Invitrogen; catalogue number 31035-025 research grade). (Note: AIM-V medium is available from Oxford Immunotec). The use of this serum free medium for the incubation step is strongly recommended. RPMI 1640 (Invitrogen; catalogue number 21875-034) may be used in the initial sample preparation steps only (Other culture media can be used but must be validated by the laboratory). It is recommended that cell culture media are stored in appropriate aliquots and excess material is discarded after use. Cell culture media should be pre-warmed to 37°C before use with the T-SPOT.TB test.

# **SAMPLE COLLECTION & HANDLING**

## Instructions For Use

### BLOOD COLLECTION TUBES

Oxford Immunotec Ltd have validated three FICOLL\* based cell separation methods; Standard Method, Leucosep tubes, and Cell Preparation Tubes (CPT™). Individual laboratories should validate their procedures for collection and separation of PBMCs for use in the T-SPOT.TB assay.

Blood samples can be collected in heparin or citrate blood collection tubes with PBMCs being subsequently separated using standard separation techniques. Alternatively, blood samples can be collected in CPTs from Becton Dickinson (BD).

Patient's cells can be pooled if necessary to obtain sufficient cells from multiple tubes of blood that are collected and processed concurrently.

Note: EDTA tubes are **NOT** recommended.

## Training Notes and Visual Aids

Blood collection tubes which are suitable for use with the T-SPOT.TB assay are highlighted in Figure 1.

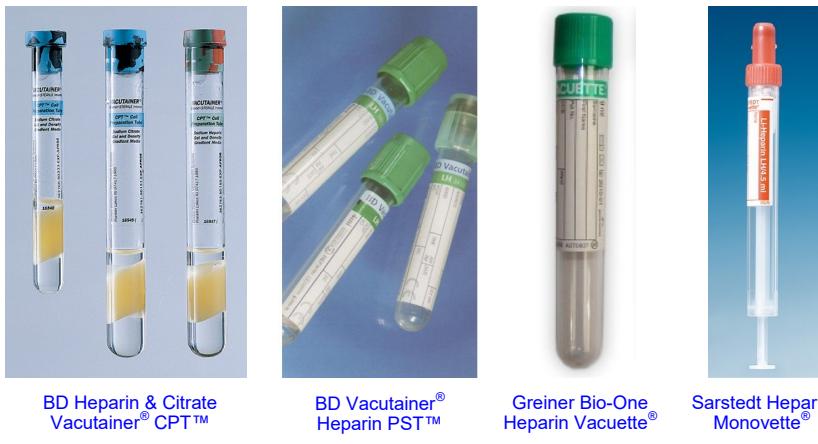


Figure 1: Examples of blood collection tubes which are suitable for use in the T-SPOT.TB assay.

**Note:** Tubes from alternative suppliers that contain heparin or citrate should be validated before being used for specimen collection.

Blood collection tubes which are NOT suitable for use with the T-SPOT.TB assay are highlighted in Figure 2.



Figure 2: Examples of blood collection tubes which are unsuitable for use in the T-SPOT.TB assay.

**Note:** Tubes from alternative suppliers that do not contain heparin or citrate should not be used for specimen collection.

## Instructions For Use

### BLOOD DRAW VOLUMES

Typically, for an immunocompetent patient, sufficient PBMCs to run the assay can be obtained from venous blood samples according to the following guidelines:

- Adults and children 10 years old and over: one 6mL heparin tube, or one 8mL CPT, or two 4mL CPT.
- Children 2-9 years old: one 4mL heparin tube or 4ml CPT.
- Children up to 2 years old: one 2mL heparin tube.

Blood samples should be collected according to the instructions supplied with the collection device. Immediately after the blood is drawn, the tube must be inverted (8-10 times) to ensure that the blood is mixed thoroughly with the anticoagulant. Do **NOT** refrigerate or freeze blood samples.

If a low cell yield is suspected, then collect: 2 x 8mL CPTs, 2 x 6mL Heparin Vacutainer tubes or similar. This will ensure sufficient PBMC yield to run the assay.

Blood samples to be processed with the T-SPOT.TB test must be used within 8 hours post venepuncture. Samples can be used up to 32 hours post venepuncture with the addition of the T-Cell Xtend reagent prior to running the T-SPOT.TB test. Whole blood samples should be maintained between 18°C and 25°C until processed.

### T-Cell Xtend PROCEDURE

To allow greater flexibility of the T-SPOT.TB test, the T-Cell Xtend reagent can be used in conjunction with the T-SPOT.TB test allowing samples to be used up to 32 hours post venepuncture.

The T-Cell Xtend reagent is an antibody complex which cross links cell surface markers on inhibitory white blood cells and red blood cells. This cross-linking increases the density of inhibitory white blood cells so that they pellet when a density gradient is applied.

- T-Cell Xtend reagent should be added to the sample immediately prior to processing. The blood should be poured into a 15ml centrifuge tube, and the volume checked. Add 25µL of T-Cell Xtend reagent per mL of whole blood and pipette up and down to mix (Figure 4).
- Replace the cap of the centrifuge tube and invert 8-10 times to mix (Figure 5).
- Incubate the whole blood with T-Cell Xtend reagent for 20 ± 5 minutes at room temperature (18-25°C).
- Continue with normal cell isolation procedures such as FICOLL or Leucosep density centrifugation methods (detailed on page 9 of this document).



Figure 3: T-Cell Xtend reagent



Figure 4: Adding 25µL of T-Cell Xtend reagent per mL of blood



Figure 5: Mixing blood and incubating at Room Temperature for 20 minutes before proceeding to the T-SPOT.TB test

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## Instructions For Use

### CELL ISOLATION

#### LEUCOSEP PROCEDURE

Leucosep tubes (available from Oxford Immunotec Ltd) facilitate the collection and separation of peripheral blood mononuclear cells (PBMC) from whole blood. They are more convenient and easier to use than the traditional FICOLL gradients methods. These tubes contain a frit or porous barrier above the FICOLL-PAQUE PLUS, thereby eliminating the need to gently layer the sample.

- Ensure the FICOLL-PAQUE PLUS is below the frit. If not, centrifuge for one minute at 350 RCF (g).
- Add 3mL of pre-warmed (37°C) RPMI 1640 to 5mL of whole blood and invert the tube 3 times to mix. Pour the diluted blood into the Leucosep tube.
- Centrifuge, in a swing out rotor, at 1000 RCF (g) for 10 minutes at room temperature (18-25°C), with the brake off.
- Ensure the tubes are balanced before centrifugation in an aerosol-resistant bucket.

#### STANDARD FICOLL PROCEDURE

- Dilute blood 1:1 with RPMI 1640 (pre-warmed to 37°C) and invert the tube 3 times to mix.
- Carefully layer diluted blood sample onto the FICOLL at a ratio of 3 volumes of diluted blood to 1 volume FICOLL. Do not allow the layers to mix.
- Centrifuge, in a swing out rotor, at 1000 RCF (g) for 22 minutes at room temperature (18-25°C), with the brake off.
- Ensure the tubes are balanced before centrifugation in an aerosol-resistant bucket.

#### CELL PREPARATION TUBE (BD VACUTAINER CPT) PROCEDURE\*

- Collect blood in 4mL or 8mL CPTs following the manufacturer's instructions.
- 8mL CPT tubes should be centrifuged in a swing out rotor, at 1600 RCF (g) for 28 minutes at room temperature (18-25°C).
- 4mL CPT tubes should be centrifuged in a swing out rotor, at 1800 RCF (g) for 30 minutes at room temperature (18-25°C).
- Ensure the tubes are balanced before centrifugation in an aerosol-resistant bucket.

\* The T-Cell Xtend reagent is **NOT** compatible with the BD CPT system.

## Training Notes and Visual Aids

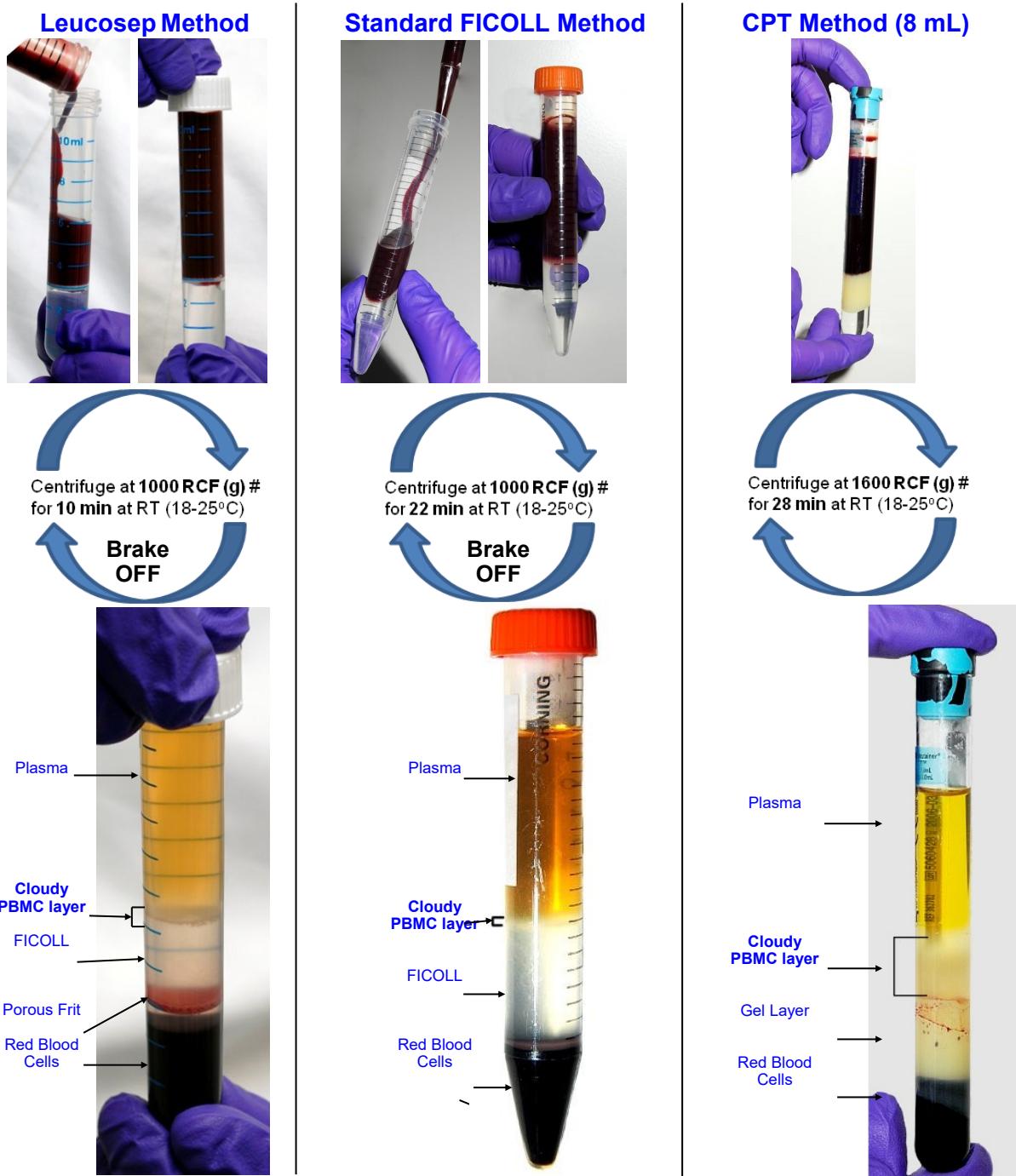


Figure 6: Images showing the validated cell separation methods

**Notes:**

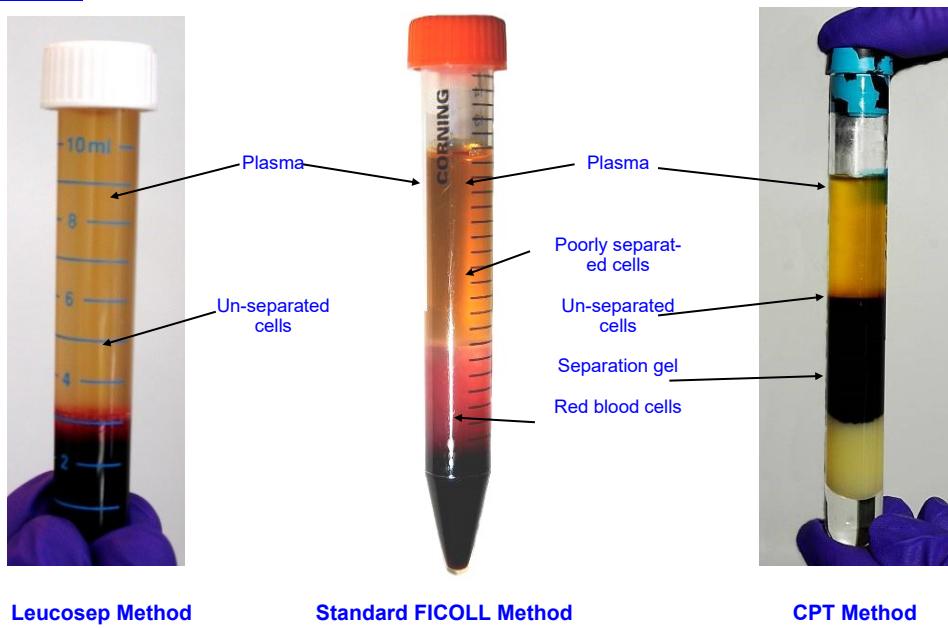
# RCF (g)= Relative Centrifugal Force. The term RCF, and not RPM, is used throughout this document.

A refrigerated centrifuge is not essential; if a refrigerated centrifuge is not available take additional care to ensure the rotor is well balanced as any slight vibration will cause a build up of heat which will alter the density of the gel layer in the CPT and the PBMCs will migrate into the red cell layer.

To simplify patient identification, after cell separation label tubes towards the top to allow easy viewing of labels above the Red Blood Cell and PBMC layers.

Warning: It is recommended that PBMC separation steps are performed in a BL II Safety Cabinet to protect the user and prevent contamination of the samples.

## Incorrect separation



Leucosep Method

Standard FICOLL Method

CPT Method

Figure 7: Incorrectly separated Leucosep, FICOLL and CPT methodologies

If correct separation has not been achieved, it may be caused by the use of the wrong centrifugation speed. If the centrifuge was set to RPM instead of RCF (as required), centrifuge again using the correct RCF.

Where separation is still not achieved, the following should also be checked:

Were the appropriate blood collection tubes used and were the tubes stored appropriately before use?

Were the blood samples stored at room temperature (18–25°C)?

Were the tubes inverted to mix the samples thoroughly with anticoagulant when the blood was drawn?

Was the FICOLL gradient set up according to the method described in this section?

Was the appropriate centrifugation speed used and was the centrifuge brake turned off?

Was the blood sample processed on the day of blood collection (within 8 hours) or within 32 hours if using the T-Cell Xtend reagent?

Note: Please consult the electronic T-SPOT.TB Centrifuge Speed Calculator available on the CD provided with each kit (Figure 8). Or use the manual slide calculator (Figure 9) available from Oxford Immunotec upon request.

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### Centrifuge Speed Calculator

This centrifuge speed calculator can be used to obtain the correct centrifugation speed for the three centrifugation steps used in T-SPOT.TB

#### Directions for use:

To obtain the correct radius of your rotor, place a tube in the appropriate centrifuge bucket and swing the rotor out to its vertical (see diagram below). Measure from the centre of the rotor to the top of the tube (A). Then measure the length of the tube (B). Add these two values together. This is the overall length of the radius of your centrifuge rotor.

Enter this value into the large box below. The converted speeds for each step will automatically be calculated and displayed in red. Please adjust your centrifuge to as close to these speeds as possible for each step of T-SPOT.TB.

NB: Ensure the correct speed is used for the cell separation step depending on the cell separation method you are using.

**Radius of Rotor in cm**  
(centre of rotor to tip of tube when vertical)

**0.0** cm

	Force (RCF or g)			Speed (RPM)		
Cell separation method	4ml CPT	8ml CPT	Ficoll	4ml CPT	8ml CPT	Ficoll
Cell separation speed	1800	1600	1000	0	0	0
First wash speed		600			0	
Second wash speed		350			0	

Figure 8: T-SPOT.TB Centrifuge Speed Calculator

**CENTRIFUGATION SPEED**

Instructions: Put the slide to set the radius of the rotor in cm and read the info in the second window.

10	20	30	40	50	
Radius of rotor in cm	Axes: CPT	Bins: CPT	Leucosep	First Wash	Second Wash

Force (RCF or g): 1800 1600 1000 600 350

Time (min): 30 20 10 5 1

Radius = A + B

Please see Visual Procedure Guide for full details of assay procedure

**T-SPOT®.TB**

This slide rule can be used as a calculation aid to identify the centrifugation speed, cell and conjugate dilutions required in the T-SPOT.TB assay procedure.

**CONJUGATE DILUTION**

Instructions: Put the slide to set the number of samples being developed and read the info in the second window.

1	2	3	4	5	6	7	8
Samples being developed	Conjugate required (μl)	Vessel of PBS required (ml)	Final volume of Conjugate made (μl)	Conjugate made (μl)			

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Figure 9: T-SPOT.TB Manual Slide Calculator

## Instructions For Use

### PBMC REMOVAL

Insert a Pasteur or pipette tip down through the plasma to aspirate the cloudy PBMC layer (Figure 1,2,3). Transfer the PBMC layer to a 15mL conical centrifuge tube. Ensure that all of the cloudy PBMC layer is collected. It is better to take some of the plasma layer than to leave any of the PBMCs in the blood collection tube.

An alternative is to decant everything above the frit. In that case transfer everything above the frit into a 15mL conical centrifuge tube

## Training Notes and Visual Aids

### 1. Leucosep Method



### 2. Standard FICOLL



### 3. CPT Method(8mL)



### 4. Decanting Method



Figure 10: Removal of PBMC's after centrifugation

#### Notes:

The use of a 1mL pipette tip or other wide bore pipette, such as a Pasteur, is recommended to reduce any possible damage to the cells.

If using CPTs, avoid transferring any of the separation gel, which can block the pipette tip. If this happens, transfer the cells already in the tip into a centrifuge tube and then use a new pipette tip to transfer the remaining PBMCs.

If using Leucosep tubes, the fill level in the tube is high. It can be helpful to remove some of the plasma first to avoid overspill when inserting the pipette tip.

## Instructions For Use

### CELL WASHING

Cell washing with medium is performed to remove any extra-cellular cytokine and therefore reduce background.

#### WASH 1

- Make the volume up to 10mL using AIM V or RPMI 1640 (Figure 11).
- Centrifuge in a swing out rotor at 600x RCF (g) for 7 minutes at 18-25°C.
- After removal from the centrifuge, carefully pour off the supernatant and gently resuspend the pellet in 1mL AIM V or RPMI 1640.



Figure 11. Fill the 15mL tube up to 10mL with culture medium.

#### WASH 2

- Make the volume up to 10mL again using AIM V or RPMI 1640.
- Centrifuge in a swing out rotor at 350x RCF (g) for 7 minutes at 18-25°C.
- After removal from the centrifuge, pour off the supernatant and gently resuspend the pellet in 0.7mL of AIM V (do **NOT** use RPMI 1640 for this step).

Note: For immunocompromised patients, cells may be resuspended in 0.5mL of AIM V to increase cell concentration.

## Training Notes and Visual Aids

### Important notes for washing PBMCs

- Cell culture medium for the washing steps should be pre-warmed to 37°C for at least 1 hour before contact with PBMCs.
- RPMI 1640 is less expensive than AIM V and so is more cost effective for the PBMC washing steps.
- It is strongly recommended that cells are resuspended in AIM V for the overnight incubation (Note: Although RPMI 1640 is suitable for washing PBMCs, it should not be used for overnight incubation).
- Cells should be mixed by gently pipetting up and down several times. This will ensure that the cells are evenly distributed.
- Different media can be used in this step, but each must be validated by the user laboratory for use in the T-SPOT.TB assay.

#### Notes:

To avoid problems with contaminated media, dispense 500mL bottles of AIM-V or RPMI 1640 aseptically into smaller aliquots.

If preferred, the set-up of the plate can be started during the centrifugation steps (see page 18 Plate Set Up and Incubation).

After centrifugation, check for a cell pellet at the bottom of the tube. If a pellet hasn't formed check that the correct centrifugation speed was used and repeat the step (see page 11 for further information on calculating centrifuge speeds).

Use a 1mL pipette to resuspend the cells. Expel 1mL of media with force, with the tip aimed at the wall near the pellet. This will create a vortex which should break up the pellet. If this fails to break up the pellet, media can be aspirated and expelled using the same pipette tip.

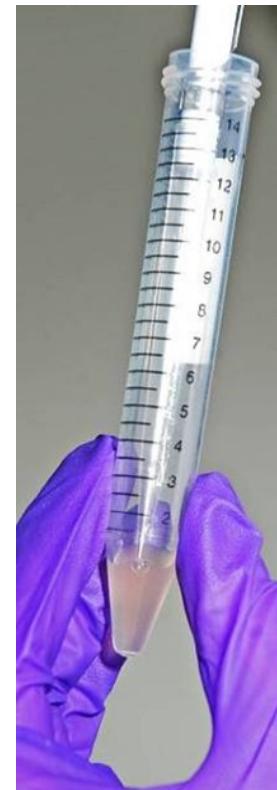


Figure 12: Resuspending the cell pellet in AIM V

# **CELL COUNTING AND DILUTION**

## Instructions For Use

### CELL COUNTING AND DILUTION

The T-SPOT.TB assay requires  $250,000 \pm 50,000$  PBMCs per well. A total of four wells are required for each patient sample; thus  $1 \times 10^6$  PBMCs are required per patient. The number of *M. tuberculosis* responsive T cells in the specimen is normalised to a fixed number of PBMCs.

- Perform a PBMC count. Cells can be counted by a variety of methods, including manual counting using Trypan Blue (or other appropriate stain) and a haemocytometer, or using an automated haematology analyser.
- Briefly, for manual counting with a Neubauer haemocytometer using Trypan Blue, add  $10\mu\text{L}$  of the final cell suspension to  $40\mu\text{L}$   $0.4\%(\text{w/v})$  Trypan Blue solution. Place an appropriate aliquot onto the haemocytometer and count the cells in the grid. For other types of haemocytometer and for automated devices, follow the manufacturers' instructions.

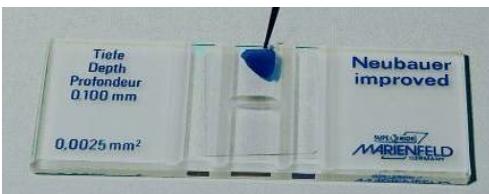
## Training Notes and Visual Aids

### MANUAL COUNTING OF PBMC's

Cell concentration can be determined by counting the number of cells within a defined volume. A Neubauer haemocytometer contains two chambers, which are divided into nine major squares (Figure 13 (a)). These have a volume of  $0.1\text{mm}^3$  or  $1 \times 10^{-4}\text{mL}$  each. Disposable counting slides are an optically clear plastic slide consisting of 10 separate counting chambers with integral coverslip. Each of these counting areas consists of ten major squares that have a volume of  $0.1\text{mm}^3$  or  $1 \times 10^{-4}\text{mL}$  each (Figure 13 (b)).

Count the cells in one major square (highlighted in red in Figure 13) of the counting grid using the low-power ( $\times 10$ ) objective to count the number of cells within the appropriately defined area. When counting the cells that touch the perimeter lines, count only those cells that touch the left and upper outside lines and disregard those that touch the right and lower lines. This is to avoid counting the same cells twice. An example of cells that have been stained with Trypan blue is shown in Figure 13 (c).

Neubauer Haemocytometer



Disposable Counting Slide (FAST-READ)

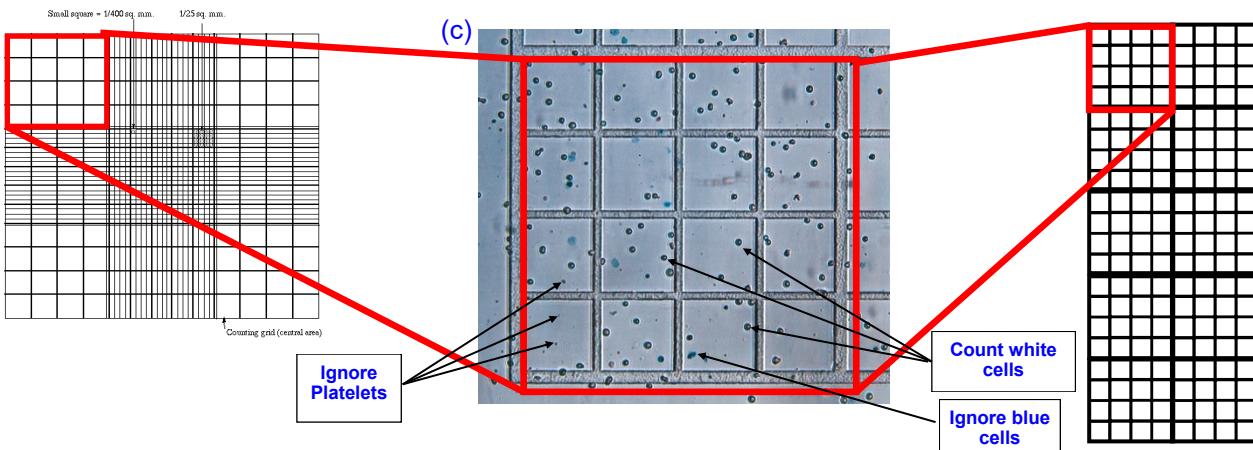
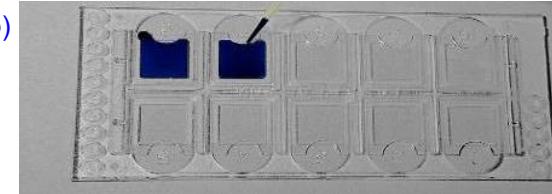


Figure 13: Sample chamber and counting areas of (a) Neubauer haemocytometer and (b) Disposable Counting Slide. Cells stained with Trypan Blue as seen under a microscope using the  $\times 10$  objective (c) NB: counting area representations not to scale.

## Instructions For Use

- Calculate the concentration of PBMCs present in the stock cell suspension.
- Prepare 500µL of the final cell suspension at a concentration of  $2.5 \times 10^6$  cells/mL.

### Notes:

Care should be taken to ensure that the cell suspension is well mixed immediately prior to removal of aliquots for dilution or for counting. Cells can settle towards the bottom of the tube leading to a misinterpretation of the true cell number. Mixing should be carried out by either gentle swirling of the tube by hand, or by gently agitating the suspension by pipetting the suspension up and down several times.

Ensure the calculation is correct for the cell counting system used as the use of either insufficient or excess cells may lead to an incorrect interpretation of the result.

PBMC numbers between 200,000 and 300,000 per well have been shown to give consistent T-SPOT.TB test results.

## Training Notes and Visual Aids

### Manual Counting example

Calculate the concentration of PBMCs in the stock cell suspension by using the following equation:

$$\frac{\text{Volume of cell suspension required to prepare cell dilution (mL)}}{\text{Number of cells counted}} = \frac{25}{125}$$

This can only be used when the dilution factor is 5 and the volume of the area counted is 0.1µL.

### Example:

$$\text{For a cell count of 125: } \frac{25}{125} = 200\mu\text{L cell suspension}$$

Bring this volume of cell suspension to the required volume of 500µL by adding 300µL AIM V medium or other serum-free cell culture medium. This gives a final solution of 250,000 cells/100µL for use in the assay.

Note: For assistance please consult the electronic T-SPOT.TB cell dilution calculator (manual) available on the CD provided with each kit (Figure 14). Or use the manual slide calculator (Figure 15) available from Oxford Immunotec upon request.

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### Cell Dilution Calculator (Manual counting)

This calculation template can be used to aid in the cell count calculation and subsequent cell dilution to 250,000 cells per well for T-SPOT.TB

#### Directions for use:

For each patient sample perform the 1 in 5 dilution with Trypan Blue and count 16 squares (4x4 grid) of the haemocytometer as detailed in the Visual Procedure Guide. Enter the patient ID in the field along with its corresponding cell count (must be 50 or above). The next column (column D) states the number of microliters needed to be removed from the 0.7ml cell suspension and added to a new tube. The next column (column E) states the number of microliters of AIM-V medium required for the dilution to produce 250,000 cells per 100µl (2,500,000 cells per ml) required for each well of the T-SPOT.TB assay.

NB: This calculation template can only be used if a 1 in 5 dilution is made with the cell viability stain Trypan Blue (i.e. 10µl of cell suspension in 40µl Trypan Blue) and the samples are counted using a FAST-READ disposable haemocytometer or a reusable Neubauer glass haemocytometer.

Sample No.	Patient ID.	Cell Count (>49)	Vol. in microliters (µl) of cell suspension required to produce appropriate dilution [ 25 / Cell Count x 1000 ]	Vol. in microliters (µl) of AIM-V required to produce the correct dilution - 250,000 cells per 100µl
Example	1234	125	200	300
1			0	0
2			0	0



Figure 14: (left)  
T-SPOT.TB  
electronic cell  
dilution calculator  
(manual counting)

Figure 15: (right)  
T-SPOT.TB manual  
slide calculator

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## AUTOMATED COUNTING OF PBMC's

A haematology analyser (automated cell counter) can be used to calculate the number of White Blood Cells (WBC) per mL (cells/mL) in the stock cell suspension in order to determine cell concentration.

The following equation should be used when using a haematology analyzer:

$$\text{Volume of cell suspension required to prepare cell dilution (mL)} = \frac{1.25}{N}$$

where N is the initial cell concentration represented as millions of cells/mL.

Example:

For a cell concentration of 10 million cells/mL:

$$\frac{1.25}{10} = 125\mu\text{L cell suspension}$$

Bring this volume of cell suspension to the required volume of 500μL by adding 375μL AIM V medium. This gives a final solution of 250,000 cells/100μL for use in the assay.

Notes:

When using a haematology analyzer, check the length of the probe on the analyzer. It may not be long enough to reach the cell suspension in the centrifuge tube. In this case, transfer some of the liquid into a small vial (ideally with a capacity of 2 – 5mL) for sampling on the analyzer. Ensure that the analyzer is programmed to count white blood cells only.

Check the volume of sample that the haematology analyzer requires. Analyzers typically use between 100μL and 400μL. 4 x 100μL of cell suspension will be added to the microtiter plate wells when running the assay so if the analyzer uses 400μL, at least 900μL of cell suspension will be needed, allowing for some dead volume. In this case, the final volume of the cell suspension should be 1mL rather than 0.7mL.

Note: For assistance, please consult the electronic T-SPOT.TB cell dilution calculator (automated) available on the CD provided with each kit (Figure 16). Or use the manual slide calculator (Figure 17) available from Oxford Immunotec upon request.

**T-SPOT® TB**

**Cell Dilution Calculator (Automated counting)**

This calculation template can be used to aid in the cell count calculation and subsequent cell dilution to 250,000 cells per well for T-SPOT.TB

Directions for use:

If an automated cell counter or haematology analyser is available for counting cells (see Visual Procedure Guide) this calculator maybe used. After analysis enter the patient ID in the field (column B) along with the Total White Blood Cell (WBC) count number in millions per ml into (column C). The next column (column D) states the number of microliters needed to be removed from the 1.0ml cell suspension and added to a new tube. The next column (column E) states the number of microliters of AIM-V medium required for the dilution to produce 250,000 cells per 100μl (2,500,000 cells per ml) required for each well of the T-SPOT.TB assay.

NB: This calculation template can only be used if the Total WBC count is entered and is in millions per ml.

Sample No.	Patient ID.	Cell Concentration (million cells / ml) Must be ≥2.5	Vol. in microliters (μl) of cell suspension required to produce appropriate dilution [ 1.25 / Cell Conc. x 1000 ]	Vol. in microliters (μl) of AIM-V required to produce the correct dilution - 250,000 cells per 100μl
Example	1234	10.00	125	375
1			0	0
2			0	0
3			0	0
4			0	0

Figure 16: T-SPOT.TB electronic cell dilution calculator (automated counting)



Figure 17: T-SPOT.TB manual slide calculator

**Please see table below for cell counts out of range:**

**Cell counts out of range**

Manual count [cells]	Automatic count [cells/mL]	Action	Cell count after re- spin/dilution
40-50	$2-2.5 \times 10^5$	Plate neat	n/a
<40	$<2.0 \times 10^6$	<p>Re-spin sample on final spin settings.</p> <p>Re-suspend in minimum volume (400ml).</p> <p>Re-check cell count on cell counter or on the Microscope (If counting manually). If cell count after re-spin is <math>&lt;2 \times 10^6</math> cells/ml, discard sample and report result as 'Non-reportable due to insufficient cells' with appropriate comments.</p>	<p><b>&gt;60</b> cells dilute as necessary. (manual count)  <b>&gt;2.5x10<sup>6</sup></b> cells/mL dilute as necessary. (automated count)</p> <p><b>40-50</b> cells, plate neat (manual count)  <b>2.0—2.5x10<sup>6</sup></b> cells/mL, plate neat (automated count)</p> <p><b>&lt; 40</b> cells. Discard sample and report result as 'Non-reportable due to insufficient cells' with appropriate comments. (manual count)  <b>&lt; 2.0x10<sup>6</sup></b> cells/mL. Discard sample and report result as 'Non-reportable due to insufficient cells' with appropriate comments. (automated count)</p>
For cell counts $>20 \times 10^6$ cells/mL on cell counter dilute sample and re-count the re-diluted sample.  For cell counts >200 on Microscope, dilute sample by 500µl increments until cell count <200 is obtained.		Rescan and re-count the diluted sample on cell counter. If re-count is $>3.0 \times 10^6$ cells/mL, re-dilute the sample as per the re-count. If re-count is $2-2.5 \times 10^6$ cells/mL, plate cells neat. If sample is over diluted (i.e. re-count is $<2 \times 10^6$ cells/ml), go back to neat cells and dilute with AIM-V in 500µl increments to re-count until cell counts within specification is obtained.	

## **PLATE SET UP AND INCUBATION**

## Instructions For Use

### PLATE SET UP AND INCUBATION

The T-SPOT.*TB* assay requires four wells to be used for each sample. A Nil Control and a Positive Control should be run with each individual sample. It is recommended that the samples be arranged vertically on the plate as illustrated below.

- Nil Control
- Panel A (ESAT-6)
- Panel B (CFP10)
- Positive Control

Each 96-well plate can process up to 24 patient samples. Use the number of plates required for the numbers of samples that are to be processed. Each strip will process 2 samples, use only the number of strips that is required.

The T-SPOT.*TB* assay measures T cell function; no standard curves are required. Therefore, each patient will only require 4 wells to be used for each sample. The recommended plate layout for 24 samples is shown below:

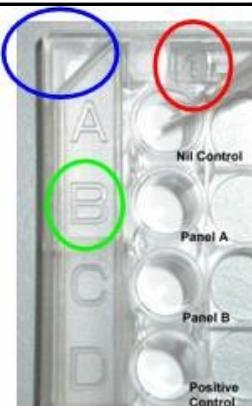
Row	1	2	3	4	5	6	7	8	9	10	11	12
A	1N	3N	5N	7N	9N	11N	13N	15N	17N	19N	21N	23N
B	1A	3A	5A	7A	9A	11A	13A	15A	17A	19A	21A	23A
C	1B	3B	5B	7B	9B	11B	13B	15B	17B	19B	21B	23B
D	1M	3M	5M	7M	9M	11M	13M	15M	17M	19M	21M	23M
E	2N	4N	6N	8N	10N	12N	14N	16N	18N	20N	22N	24N
F	2A	4A	6A	8A	10A	12A	14A	16A	18A	20A	22A	24A
G	2B	4B	6B	8B	10B	12B	14B	16B	18B	20B	22B	24B
H	2M	4M	6M	8M	10M	12M	14M	16M	18M	20M	22M	24M

Key: N=nil control, A=Panel A, B=Panel B, M=Mitogen Positive Control)

- Remove the pre-coated 8-well strips from the packaging, clip into a plate frame (available from Oxford Immunotec) and allow to equilibrate to room temperature. Remove the required number of strips only, reseal any remaining unused strips and the desiccant pouch in the outer foil packaging and return to storage at 2-8°C. Add the Panels and the Controls;
- Add 50µL AIM V cell culture medium to each Nil Control well
- Add 50µL Panel A solution to each well required
- Add 50µL Panel B solution to each well required
- Add 50µL Positive Control solution to each cell functionality control well.

Do not allow the pipette tip to touch the membrane. Indentations in the membrane caused by pipette tips may cause artifacts in the wells. (See example 5 on page 38)

## Training Notes and Visual Aids



The plate orientation should be as shown (Figure 18). Position the A1 well at the top left hand corner. This corner is clearly distinguishable by the flattened section shown in the blue circle. The column numbers are positioned along the top edge of the plate, shown in the red circle. The row letters are shown in the green circle down the left hand side of the plate.

Figure 18 shows 4 wells used for one patient sample positioned in the top four wells on the first strip of an 8 well strip plate. The suggested orientation of wells to be used are shown e.g. A1 Nil Control, B1 Panel A, C1 Panel B and D1 Positive Control. Patient wells should be arranged in this order to avoid cross contamination when adding the positive control and patient cells.

Note: Label opened items with the date. After 8 weeks, remaining components should be discarded.

Figure 18: 4 wells used for one patient. A1 to D1, Nil Control, Panel A, Panel B and Positive control respectively

### Instructions For Use

- To each of the 4 wells to be used for a patient sample, add 100µL of the patient's final cell suspension (containing 250,000 cells). Use a new tip for the addition of each individual patient's cells to avoid cross-contamination between patients.

Note: Take care not to contaminate adjacent wells, by passing liquid from one well to another if pipette tips are reused for multiple wells.

### Training Notes and Visual Aids

Clip the strips to be used into an empty plate frame fitted with a bottom plate and lid. Figure 19 (left), shows a breakdown of the 8 well strip plate (TB.300) containing 2 strips: Plate frame (A), Lid (B), bottom plate (C), and the 2x 8 well strips (D). Figure 19 (right), shows the complete frame containing 2 strips (16 wells) sufficient for 4 patient samples. The frames, bottom plate, and lids can be retained and reused.

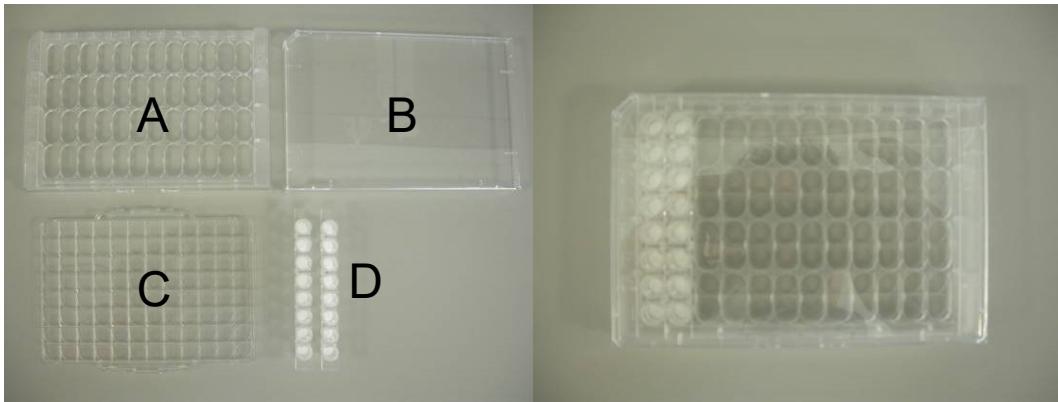


Figure 19: Left: Breakdown of the 8 well strip plate. Right: Final assembly with 2 strips. Enough wells for 4 patient samples (e.g. 16 wells).

Plate, Panel A, Panel B, and Positive Control (PHA) should all be at room temperature (18-25°C). AIM V must be pre-warmed to 37°C.

Prior to pipetting, gently swirl cell suspension or gently pipette up and down to ensure cells are evenly distributed.

Do **NOT** allow the pipette tip to touch the membrane. Indentations in the membrane caused by pipette tips may cause damage to the wells. If necessary, rest the tip on the side of the well.

Change tip when changing from one reagent to the next.

### Instructions For Use

- Incubate the plate with the lid on in a humidified incubator at  $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$  with 5% CO<sub>2</sub> supply for 16-20 hours. Avoid disturbing the plate once in the incubator. Do not stack plates as this may lead to uneven temperature distribution and ventilation.

### Training Notes and Visual Aids



Figure 20: Place plate into incubator for 16-20 hours

Incubate plate in a humidified incubator at  $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$  with 5% CO<sub>2</sub> supply (Figure 20). Check the water dish for sufficient water to ensure that a humid atmosphere is achieved throughout the incubation period.

## **SPOT DEVELOPMENT AND COUNTING**

## Instructions For Use

### SPOT DEVELOPMENT

Remove the plate from the incubator and discard the cell culture medium by flicking the contents into an appropriate container.

Note: At this point, remove the Substrate Solution from the kit and allow to equilibrate to room temperature for 1 hour.

- Add, with force, 200 $\mu$ L Dulbecco's Phosphate Buffered Saline (D-PBS) solution to each well. Do not use PBS containing Tween® or other detergents, as this causes high background counts.
- Discard the D-PBS solution. Repeat the well washing an additional 3 times with fresh D-PBS solution for each wash.

## Training Notes and Visual Aids



Figure 21: Aspirate 200 $\mu$ L of D-PBS from a reagent tray

Note: For washing, an 8 channel or stepper pipette and a plastic reservoir to hold D-PBS may be used as shown in Figure 21.



Figure 22: Dispense 200 $\mu$ L of D-PBS into each well

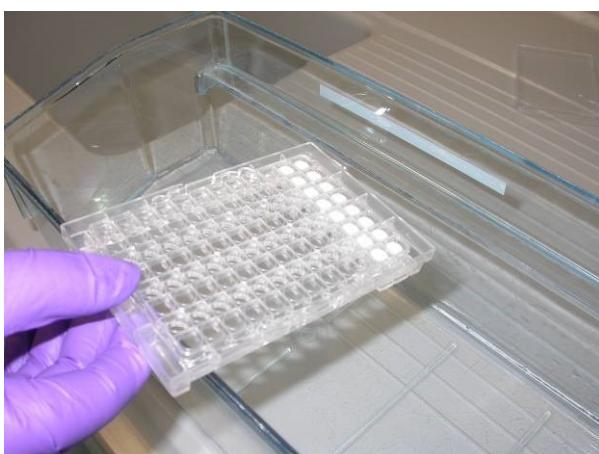


Figure 23: Invert plate into a container

Note:

Do not use pipettes to remove the D-PBS as this risks damaging the membrane.

Discard D-PBS by inverting the plate over a suitable container after each wash (Figure 23).

## Instructions For Use

- Before opening the Conjugate Reagent vial, ensure all liquid is at the bottom of the tube. Centrifuge if necessary.
- Dilute concentrated Conjugate Reagent 200 x in D-PBS to create the working strength solution.
- Add 50 $\mu$ L working strength Conjugate Reagent solution to each well and incubate at 2-8°C for 1 hour.
- Discard the Conjugate Reagent and perform four 200 $\mu$ L D-PBS washes as described on the previous page.
- Add 50 $\mu$ L Substrate Solution to each well and incubate at room temperature for 7 minutes.
- Wash the plate thoroughly with distilled or deionised water to stop the detection reaction.
- Allow the plate to dry in a well ventilated area or in an oven at up to 37°C. Spots become more visible as the plate dries; therefore ensure that the plate is thoroughly dry before reading. Allow up to 4 hours drying time at 37°C or overnight if left at room temperature.

Note:

In order to load all wells quickly, a multi-channel pipette and a plastic reservoir is recommended. Ensure all residual D-PBS is removed prior to adding the Substrate Solution. It is recommended that an aliquot of Substrate Solution be removed from the reagent bottle to avoid risk of contamination.

## Training Notes and Visual Aids



Figure 24: Perform Conjugate Reagent dilution (1:200)

### Example Dilution:

Each patient sample will have 4 wells. 50 $\mu$ L diluted Conjugate Reagent will be added to each well. Thus, for one strip (2 samples, 8 wells), prepare 500 $\mu$ L of working strength solution by adding 2.5 $\mu$ L of concentrated Conjugate Reagent (use a 1-20  $\mu$ L pipette) to 497.5 $\mu$ L D-PBS. Mix by inverting 5-6 times.

For one 96-well plate (24 samples) prepare 5mL of working strength solution by adding 25 $\mu$ L of concentrated Conjugate Reagent to 4975 $\mu$ L D-PBS.

Note: Twice as much Conjugate Reagent than is required is provided with each kit. Care should be taken to limit the amount of excess solution prepared (for wastage) to avoid running out of Conjugate Reagent.

Care should be taken to ensure that the Conjugate Reagent is added to every well as the solution is clear and colourless.



Figure 25: Add Substrate Solution and incubate at 18-25°C for 7 mins



Figure 26: Wash wells with distilled or deionised water

After washing the plate with distilled or deionised water (Figure 26), tap out any residual liquid onto absorbent paper. As the wells dry, the background decreases and the spots become more sharply defined and thereby, easier to count. Ensure the plate is thoroughly dry before reading.

# **SPOT INTERPRETATION**

### Instructions For Use

- Count and record the number of distinct, dark purple spots on the membrane of each well. Please see the results interpretation section of this document to determine whether a patient sample is 'Positive' or 'Negative'.
- A spot counting training guide called T-SPOT<sup>®</sup>.Tutor (Figure 27) can be obtained via the Oxford Immunotec website at <http://www.oxfordimmunotec.com/international/resources>.

Once developed, the completed assay plates remain stable and they do not, therefore, need to be read immediately. The plates may be archived for retrospective quality control or re-examination for up to 12 months if kept in a dry, dark environment at room temperature.

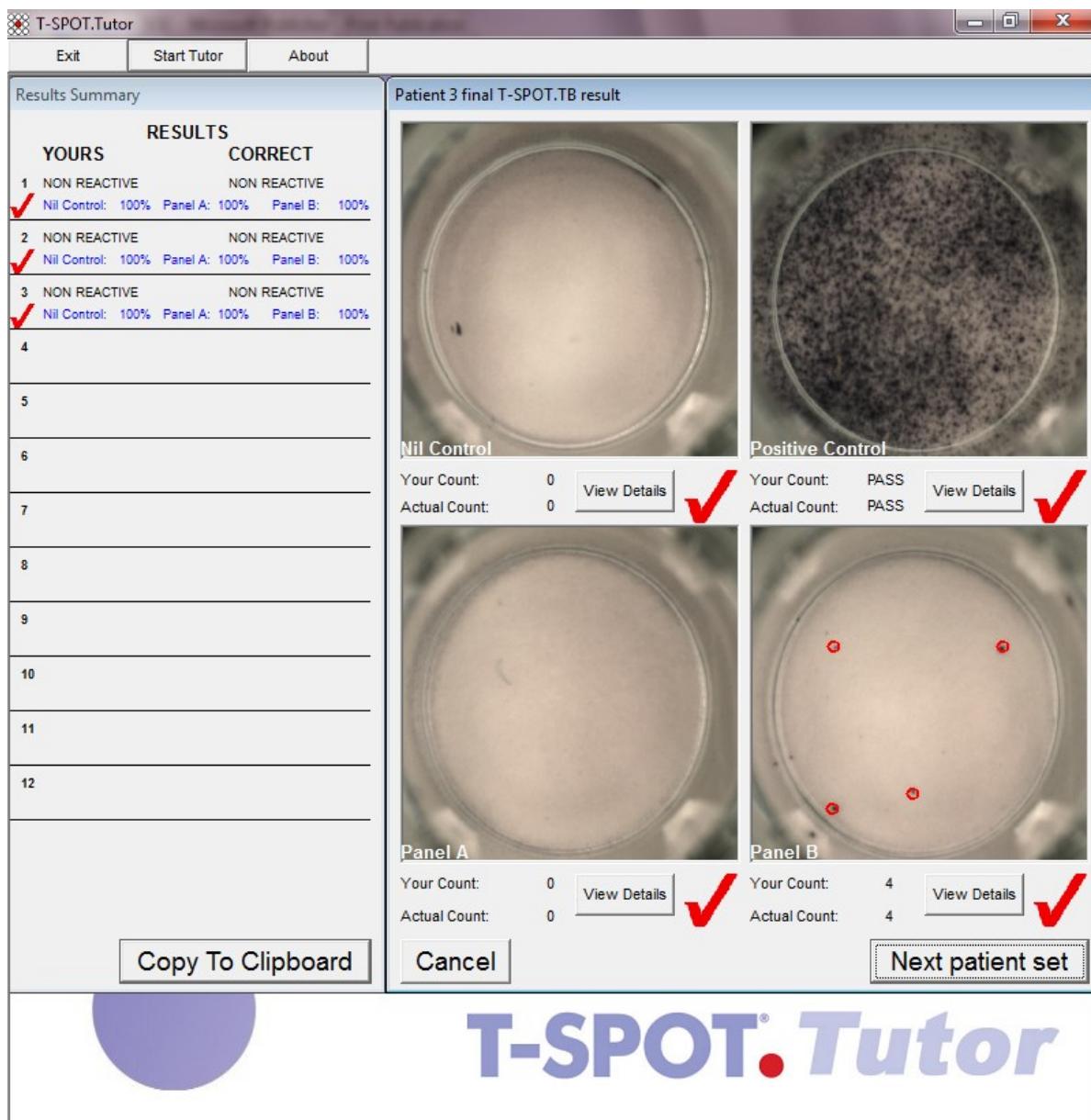


Figure 27: Screen shot from T-SPOT.Tutor

## **Training Notes and Visual Aids**

### **What are the recommended methodologies for interpreting T-SPOT.TB test results?**

A USB microscope is an excellent option for visualising spots produced by the T-SPOT.TB test, but other validated manual methods may also be used, such as the use of a magnifying glass or stereomicroscope. Where available, an automated ELISPOT plate imager can be used to visualise the spots. A variety of automated imagers are available to aid in the counting of spots formed in the T-SPOT.TB assay.

Examples of suppliers include BIO-SYS, AID, CTL™, and Zeiss™. All plate imagers should be validated before being used for diagnostic purposes and have a calibration routine established.

### **Must I count every spot?**

Wells that clearly contain either sufficient spots or an absence of spots, such that the assay result can be determined with confidence, do not need to be counted accurately. Based on results from our clinical data, we concluded that most of the results are clearly positive (Panel - Nil Control is more than 10 spots) or negative (Panel - Nil Control is 2 or less spots). In these cases, you may report a result as being positive or negative without individual spot enumeration. The Positive Control well does not require individual spot enumeration provided it passes the quality control criteria (please see quality control section on page 31).

### **How do I differentiate between valid spots and non-specific artefacts that may appear in the well?**

The spots produced as a result of antigen-stimulation appear as large, round, dark spots, and often a gradient effect can be observed with a darker centre and a more diffused periphery (halo). In comparison to these valid spots, non-specific artefacts appear to be either smaller or lighter in colour, and may be irregular in shape.

### **How do I count spots?**

Ensure the plate is dry before beginning the interpretation, as spots become more visible as the plate dries. Drying can take place in an oven at up to 37°C, or in a well ventilated area at room temperature. Allow up to 4 hour drying time at 37°C or overnight at room temperature.

Count and record the number of large, round, dark spots on the membrane of each well. This should be performed blind (i.e. without patient details). The results for all four wells (i.e. Nil Control, Panel A, Panel B, and Positive Control) must be recorded, with the reporting of results following the guidelines set out in the T-SPOT.TB Package Insert. We recommend the use of T-SPOT®.Reporter if you count spots manually (contained on the CD accompanying each T-SPOT.TB kit). This software implements the T-SPOT.TB algorithm to determine the result of a sample.

In the following pictures, the spots which should be counted in Nil Control, Panel A and Panel B wells are shown. Pictures were taken using a USB powered Microscope and an automated plate imager. Valid spots are indicated with a black ring, invalid spots are indicated by a red ring.

**Training Notes and Visual Aids**

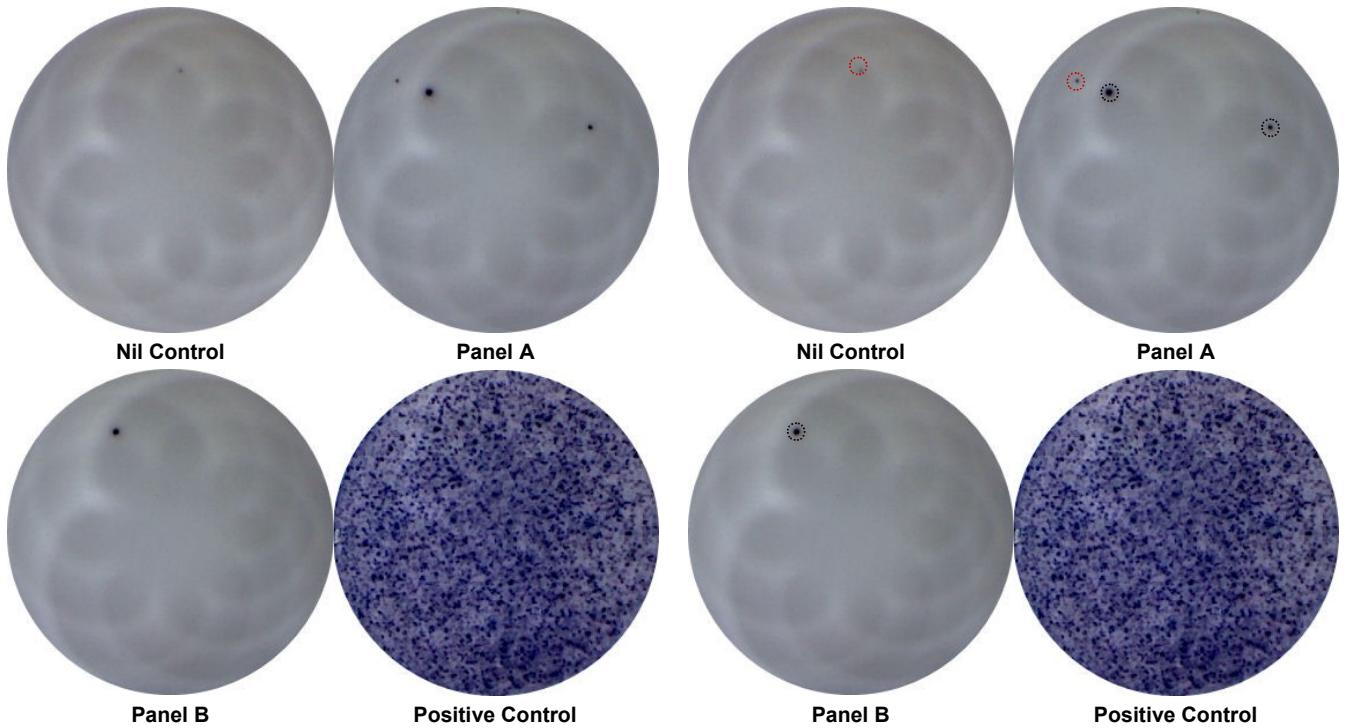


Figure 28: Patient 1 viewed with a USB Microscope (invalid spots shown in red)

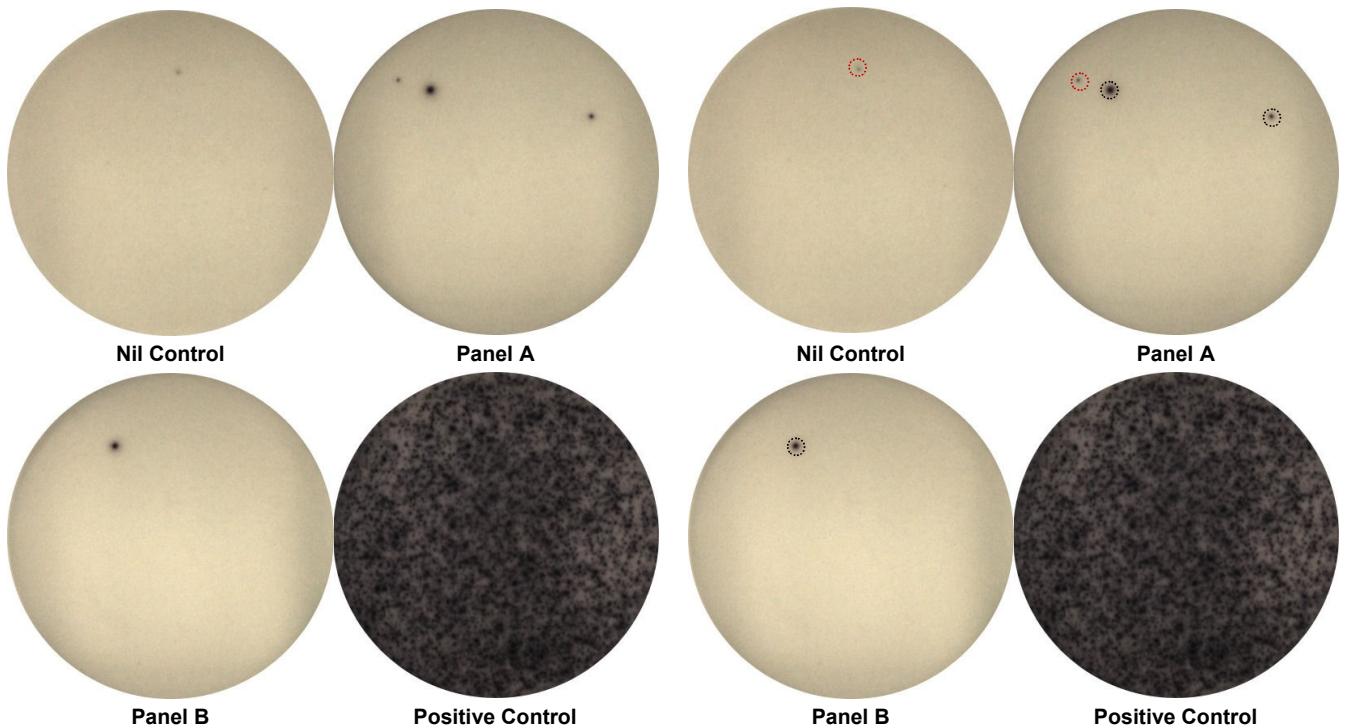


Figure 29: Patient 1 viewed with an AID plate reader (invalid spots shown in red)

**Training Notes and Visual Aids**

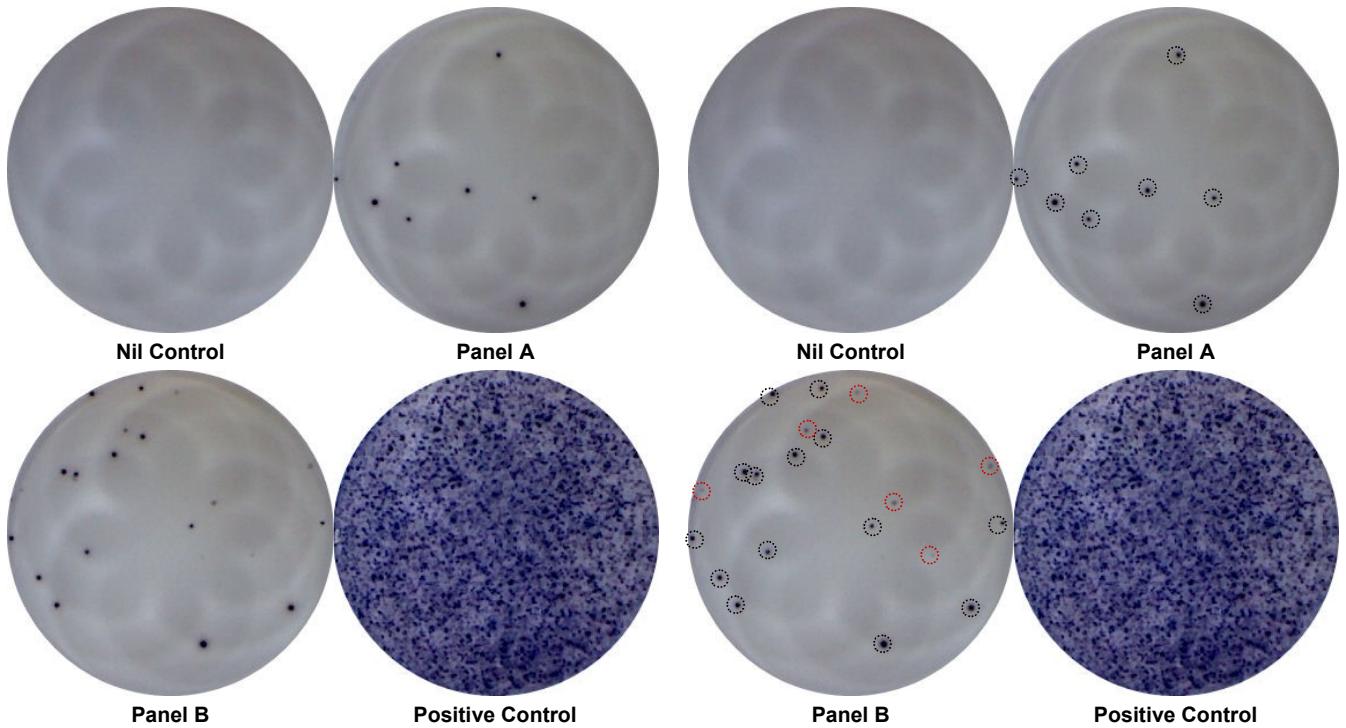


Figure 30: Patient 2 viewed with a USB Microscope (invalid spots shown in red)

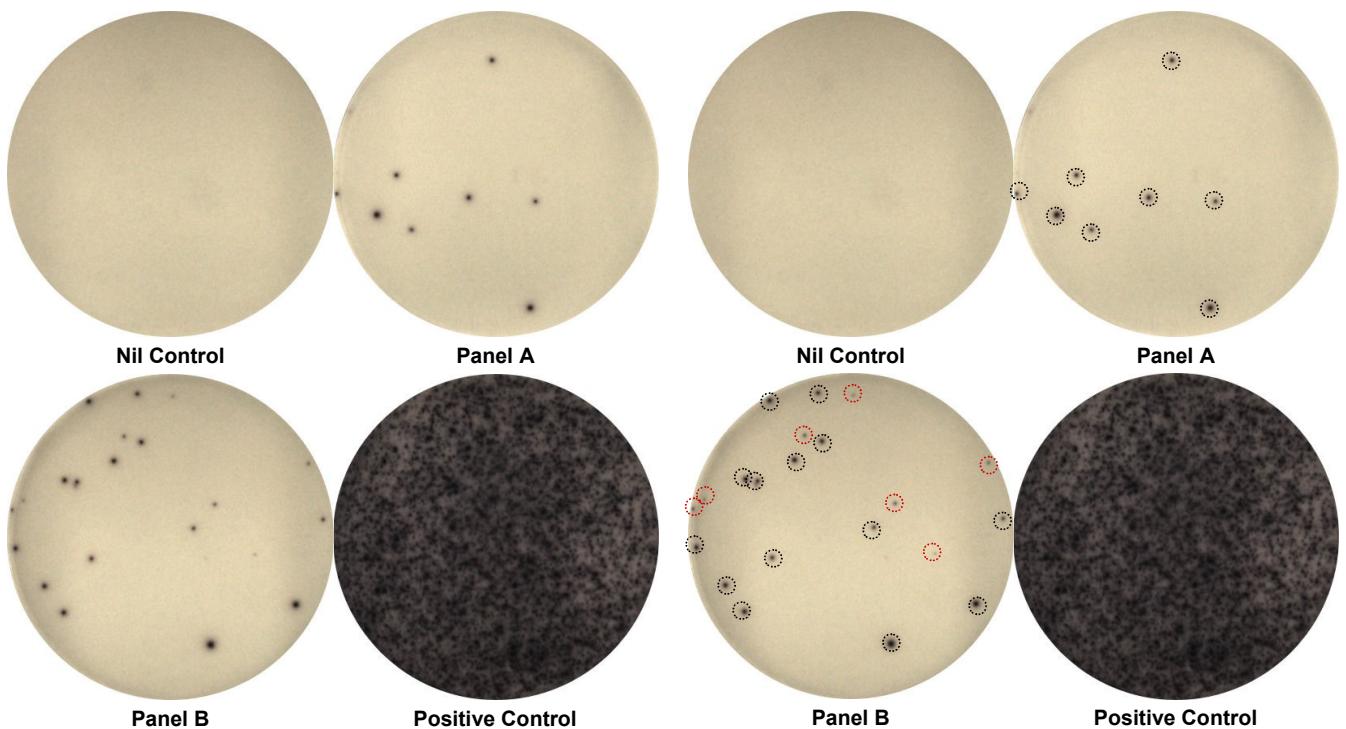


Figure 31: Patient 2 viewed with an AID plate reader (invalid spots shown in red)

**Training Notes and Visual Aids**

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Figure 32: Images taken with a USB microscope showing a well with seven valid spots, (Black Ring) three weak invalid spots (Red Ring) and



Figure 33: Images taken with a USB microscope showing a well with twenty six valid spots (Black Ring) and fifteen weak invalid spots (Red

# **QUALITY CONTROL**

## Instructions For Use

### QUALITY CONTROL

A typical result would be expected to have few or no spots in the Nil Control and 20 or more spots in the Positive Control.

#### NIL CONTROLS

High numbers of spots in the Nil Control may occur. In addition, high background staining in one or more wells may occur which makes counting of spots difficult. If high background staining occurs such that discrimination of the spots from the background is hindered, the test should be considered "Indeterminate". These results are usually due to operator issues such as: sub-optimal plate washing, medium contamination or inappropriate specimen handling and PBMC separation methods. It is, however, possible that the state of health of the patient may produce this effect in a small number of cases.

A Nil Control spot count in excess of 10 spots should be considered as "Indeterminate".

#### POSITIVE CONTROLS

Typically, the cell functionality Positive Control spot count should be  $\geq 20$  or show saturation (too many spots to count). A small proportion of patients may have T cells which show only a limited response to PHA<sup>1</sup>. Where the Positive Control spot count is  $< 20$  spots, it should be considered as "Indeterminate", unless either Panel A or Panel B are 'Positive' as described in the Results Interpretation and Assay Criteria (see below), in which case the result is valid.

## Training Notes and Visual Aids

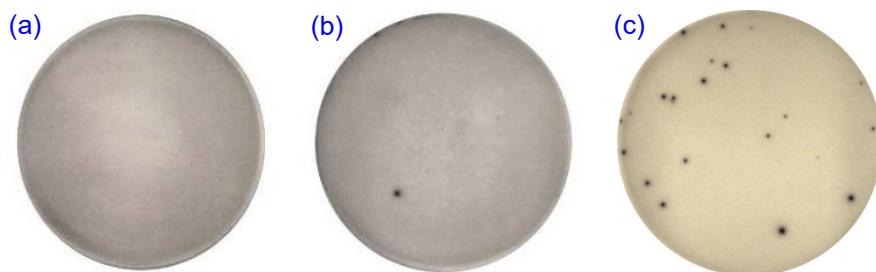


Figure 34: Typical images from Nil Control wells: (a) shows a well with 0 spots (b) shows a well with 1 spot and (c) shows an invalid Nil Control well of  $>10$  spots

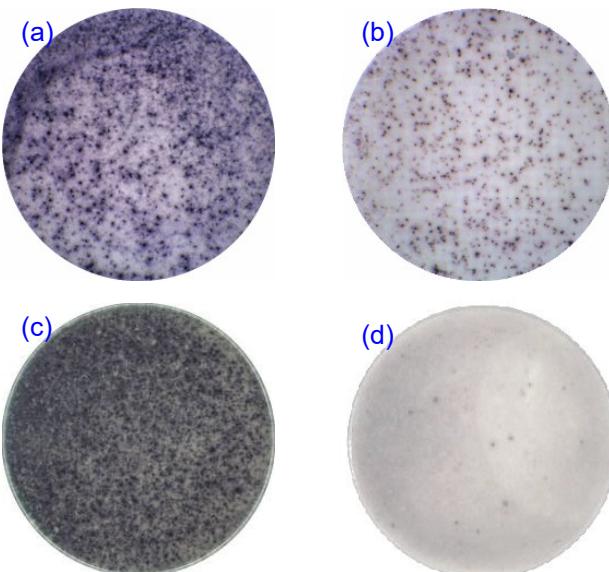


Figure 35: Typical images from Positive Control wells (a) (b) and (c) all shown acceptable responses for patient samples. Figure (d) shows an invalid Positive Control well due to having  $<20$  spots.

# **RESULTS INTERPRETATION**

## Instructions For Use

# RESULTS INTERPRETATION AND ASSAY CRITERIA

Refer to the Quality Control section before applying the following criteria.

NOTE: Diagnosing or excluding tuberculosis disease, and assessing the probability of latent tuberculosis infection (LTBI), requires a combination of epidemiological, historical, medical and diagnostic findings that should be taken into account when interpreting the T-SPOT.TB test. Refer to local medical guidance for detailed recommendations about diagnosing TB infection (including disease) and selecting persons for testing.

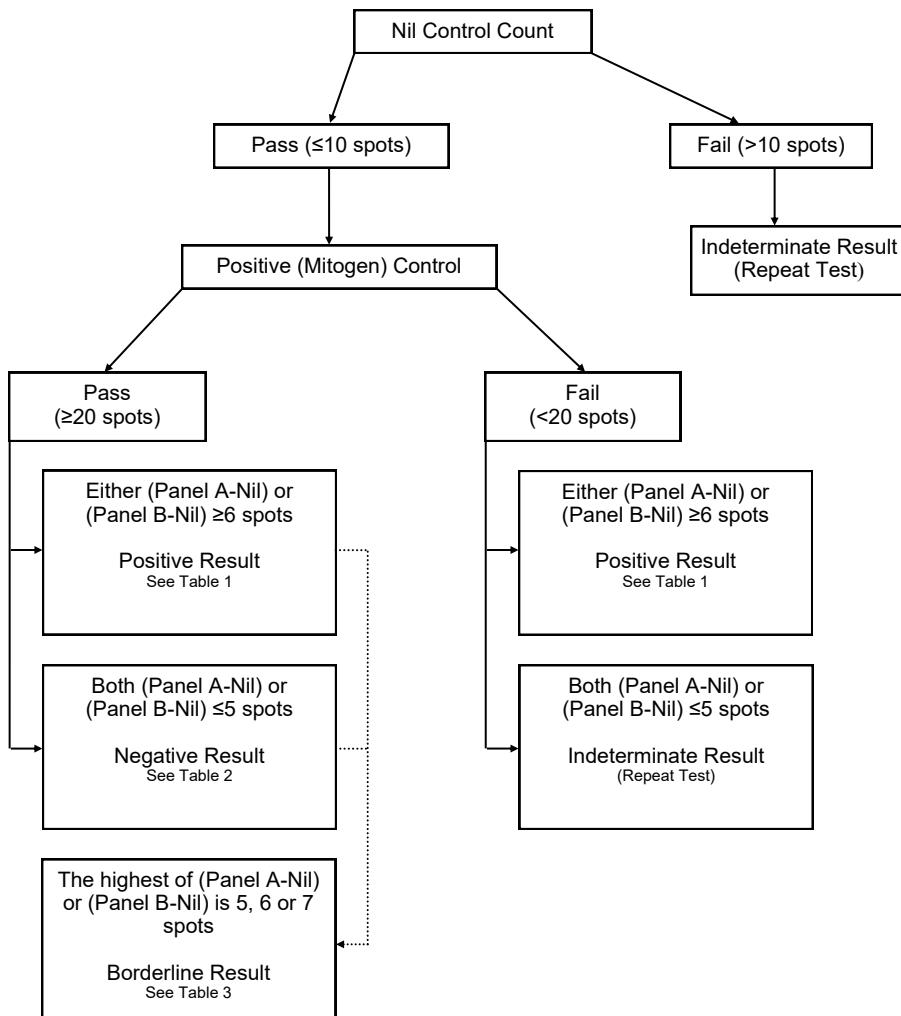
Results for the T-SPOT.TB test are interpreted by subtracting the spot count in the Nil Control well from the spot count in each of the two Panels, according to the following algorithm:

The test result is Positive if (Panel A-Nil) and/or (Panel B-Nil)  $\geq 6$  spots

The test result is Negative if both (Panel A-Nil) and (Panel B-Nil)  $\leq 5$  spots. This include results less than zero.

## BORDERLINE AND INVALID RESULTS

Results where the highest of the Panel A or Panel B spot count is such that the (Panel minus Nil) spot count is 5, 6 or 7 spots should be considered Borderline (equivocal) and re-testing by collecting another patient specimen is recommended.



## **SPOT INTERPRETATION PANEL A & B ANTIGEN**

## Instructions For Use

Table 1: Positive Interpretation: Either (Panel A-Nil) or (Panel B-Nil)  $\geq 6$  spots

Nil Control Well Count	Either Panel A or Panel B has the following number of spots <sup>†</sup>	Result Interpretation
0	$\geq 6$	<b>Positive</b>
1	$\geq 7$	<b>Positive</b>
2	$\geq 8$	<b>Positive</b>
3	$\geq 9$	<b>Positive</b>
4	$\geq 10$	<b>Positive</b>
5	$\geq 11$	<b>Positive</b>
6	$\geq 12$	<b>Positive</b>
7	$\geq 13$	<b>Positive</b>
8	$\geq 14$	<b>Positive</b>
9	$\geq 15$	<b>Positive</b>
10	$\geq 16$	<b>Positive</b>
>10 spots	n/a	<b>Indeterminate**</b>

<sup>†</sup>Note: The Panel with the highest number of spots is used for the calculation.

Table 2: Negative Interpretation: Both (Panel A-Nil) and (Panel B-Nil)  $\leq 5$  spots

Nil Control Well Count	Both Panel A and Panel B has the following number of spots	Result Interpretation
0	$\leq 5$	<b>Negative</b>
1	$\leq 6$	<b>Negative</b>
2	$\leq 7$	<b>Negative</b>
3	$\leq 8$	<b>Negative</b>
4	$\leq 9$	<b>Negative</b>
5	$\leq 10$	<b>Negative</b>
6	$\leq 11$	<b>Negative</b>
7	$\leq 12$	<b>Negative</b>
8	$\leq 13$	<b>Negative</b>
9	$\leq 14$	<b>Negative</b>
10	$\leq 15$	<b>Negative</b>
>10 spots	n/a	<b>Indeterminate**</b>

Table 3: Borderline (equivocal) Interpretation: The highest of (Panel A-Nil) or (Panel B-Nil) is 5, 6 or 7 spots

Nil Control Well Count	The highest of Panel A or Panel B has the following number of spots	Result Interpretation
0	5, 6 or 7	<b>Borderline (equivocal)*</b>
1	6, 7 or 8	<b>Borderline (equivocal)*</b>
2	7, 8 or 9	<b>Borderline (equivocal)*</b>
3	8, 9 or 10	<b>Borderline (equivocal)*</b>
4	9, 10 or 11	<b>Borderline (equivocal)*</b>
5	10, 11 or 12	<b>Borderline (equivocal)*</b>
6	11, 12 or 13	<b>Borderline (equivocal)*</b>
7	12, 13 or 14	<b>Borderline (equivocal)*</b>
8	13, 14 or 15	<b>Borderline (equivocal)*</b>
9	14, 15 or 16	<b>Borderline (equivocal)*</b>
10	15, 16 or 17	<b>Borderline (equivocal)*</b>
>10 spots	n/a	<b>Indeterminate**</b>

\*Results where the highest of the Panel A or Panel B spot count is such that the (Panel minus Nil) spot count is 5, 6 or 7 spots should be considered Borderline (equivocal) and re-testing by collecting another patient specimen is recommended.

\*\* In the case of "Indeterminate" results, these should be reported as "Indeterminate" and it is recommended to collect another sample and re-test the individual.

## **TROUBLE SHOOTING IMAGES**

## Instructions For Use

### TROUBLESHOOTING

This assay should be performed using the principles of Good Laboratory Practice and by strictly adhering to these instructions for Use.

#### Borderline (equivocal) Results

Borderline (equivocal) results are those where the maximum of the two (Panel minus Nil) spot count results are within  $\pm 1$  spots from the ROC-determined assay cut-off of  $\geq 6$  spots. Borderline (equivocal) results, although valid, are less reliable than results where the spot count is further from the cut-off. Re-testing of the patient, using a new sample, is therefore recommended. If the result is still Borderline (equivocal) on re-testing, then other diagnostic tests and/or epidemiologic information should be used to help determine TB infection status of the patient.

#### Invalid Results

Invalid results are uncommon and may be related to the immune status of the individual being tested<sup>2&3</sup>. They may also be related to a number of technical factors, potentially resulting in "high background", "low mitogen", and "high nil" results such as:

- Use of inappropriate blood collection tubes
- Storage of blood greater than 8 hours (greater than 32 hours using the T-Cell Xtend reagent) prior to processing
- Storage of blood outside the recommended temperature range (18-25°C prior to processing)
- Contamination of the cell culture media
- Incomplete plate washing

Repeating the test using a new patient sample is recommended for invalid results. Technical documents are available covering key troubleshooting points. These are available by contacting Oxford Immunotec.

For Product Support contact: +44 (0) 810 100245.

## Training Notes and Visual Aids

### SPOT INTERPRETATION — ADDITIONAL INFORMATION

#### Panel A and B Antigens

The number of spots in the antigen wells can vary from zero to several hundred. High spot numbers will be difficult and time-consuming to count so may be recorded as >20 spots. Examples of typical wells are shown in Figure 34-36

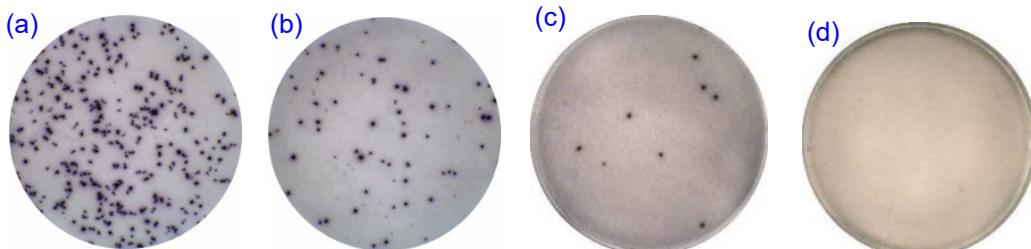


Figure 36: Typical images from Panel A and B antigen wells, (a) Positive sample (>20 spots); (b) Positive sample (>20 spots); (c) Positive sample (8 spots) and (d) Negative sample (0 spots)

#### Example patient interpretations:

	Well	Spot count	Valid?
<b>Patient 1.</b>	Nil Control	= 2	✓
	Panel A	= 11	✓
	Panel B	= 1	✓
	Positive Control	= >20	✓

Panel A value subtract the Nil Control value = 9 ( $9 \geq 6$ ) therefore: **Result = Positive**

<b>Patient 2.</b>	Nil Control	= 1	✓
	Panel A	= 0	✓
	Panel B	= 2	✓
	Positive Control	= >20	✓

Panel B value subtract the Nil Control value = 1 ( $1 \leq 5$  therefore: **Result = Negative**

<b>Patient 3.</b>	Nil Control	= 11	X
	Panel A	= 13	✓
	Panel B	= 12	✓
	Positive Control	= >20	✓

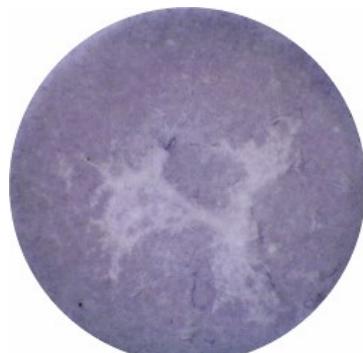
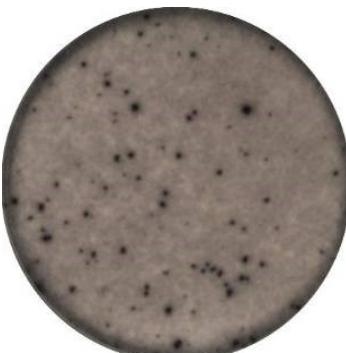
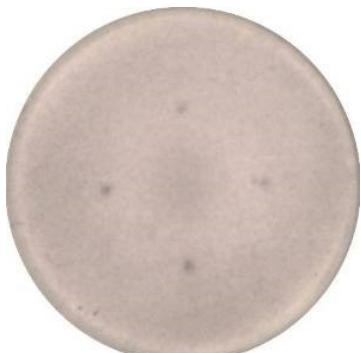
Nil Control value >10 therefore: **Result = Indeterminate (Repeat Test)**

<b>Patient 4.</b>	Nil Control	= 0	✓
	Panel A	= 13	✓
	Panel B	= 12	✓
	Positive Control	= 18	X

Positive Control fail BUT Panel A & B (subtract Nil 0) = 13 & 12 (>6) therefore: **Result = Positive**

## SPOT INTERPRETATION — TROUBLESHOOTING

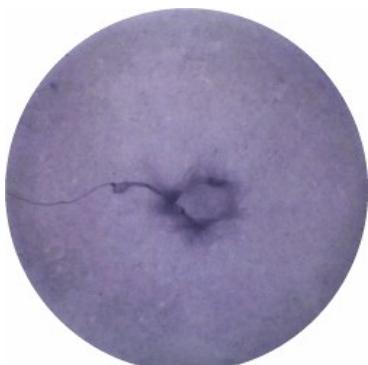
If wells contain debris or have a high background care should be taken when counting spots.  
See the examples below:



**Example 1: 4 Mark Effect.** These marks can be attributed to excessive pressure on the back of the plate caused when tapping solutions out of the plate during the plate washing steps. [In this case the spot count = 0].

**Example 2: High background in Panel well.** Although a high background is uncommon with the T-SPOT.TB test, spots can still be seen over background. [In this case the spot count  $\geq 20$ ].

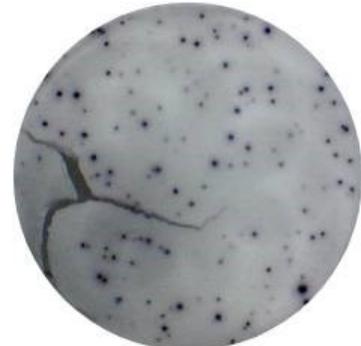
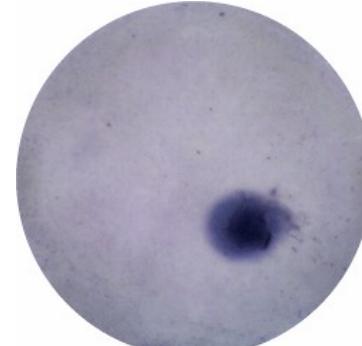
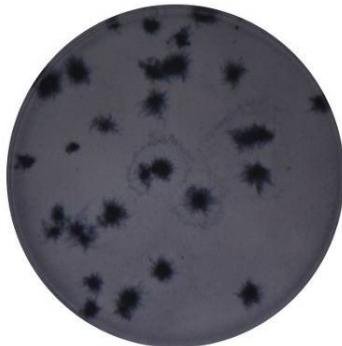
**Example 3: Excessively high background** can be produced, possibly due to: suboptimal plate washing, medium contamination or inappropriate specimen handling and PBMC separation. It is also possible that the state of health of the patient may produce this effect in a small number of cases. [In this case, the result for this Nil control well was invalid]



**Example 4:** Debris in well may be visible e.g. a hair. However, spots will still be evident. [In this case the spot count = 0]. Care should be taken when pipetting cells and reagents to not allow debris to fall into the assay wells.

**Example 5:** Pipette tips can cause dark marks on the membrane. However spots will still be evident. [In this case the spot count = 0]. Care should be taken when pipetting reagents and cells to not touch the membrane with pipette tips.

**Example 6:** Automated Washer tips also cause dark marks on the membrane. However spots will still be evident. [In this case the spot count = 0]. Care should be taken that the tips do not touch the membrane during operation.



**Example 7:** Fungal contamination in the well (media). These growths are not round in shape. Rather they appear as non-uniform and have fibrous edges. They are also much larger than ELISPOT spots. [In this case spot count = 0]. Care should be taken not to allow contamination to occur.

**Example 8:** Debris in well. Spots if present will still be evident. [In this case spot count = 0]. Care should be taken when pipetting cells and reagents into the plates to not allow environmental debris to fall into the assay wells.

**Example 9:** Broken membrane. Spots are still evident in this well. Care should be taken when pipetting into the wells to ensure that the membranes are not damaged.

## References

1. Köller MD *et al.*, Functional and molecular aspects of transient T cell unresponsiveness: role of selective Interleukin-2 deficiency. *Clin Exp Immunol.* 2003 May;132(2):225-31.
2. NCCLS. Performance of single cell immune response assays; approved guideline. NCCLS document I/LA26-A.
3. Meier T *et al.* Sensitivity of a new commercial enzyme-linked immunospot assay (T SPOT-TB) for diagnosis of tuberculosis in clinical practice. *Eur. J. Clin. Microbiol. Infect. Dis.*, 2005; 24: 529-536.

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US7575870, US8617821, US9360480, EP941478, JP4094674, AU728357, CA2272881; US7115361; US7632646, EP1144447, JP4633931, ZA2001-3356; US7901898, US8216795, US8507211, US9005902; US7579141, US8021832, US9238066, US9482671, US 15/019,532, EP1214088, EP2087906, JP4820489, AU773268, CA2372583

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