

# T-SPOT<sup>®</sup>. TB

## TRAINING GUIDE

For *In Vitro* Diagnostic Use Only

This Visual Procedure Training Guide covers use of both:  
T-SPOT. TB 8 (Multi-use 8-Well Strip Plate Format. Catalogue number: TB.300)  
T-SPOT. TB 96 (Single-use 96-well Plate Format. Catalogue number: TB.200)

**To be used in conjunction with the T-SPOT. TB Package Insert [PI-TB-US-V1]**

## 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, preparation of Peripheral Blood Mononuclear Cells (PBMCs) prior to running the assay, performing T-SPOT.*TB*, interpreting test results and troubleshooting.

### Using this document

Instructions from the T-SPOT.*TB* package insert 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 beneath these instructions.

<b>Table of contents</b>	<b>Page</b>
Introduction	1
Reagent Storage	2
Materials Provided	3
Storage & Stability	3
Equipment and Materials Required But Not Provided	4
Sample collection and handling	5
Blood collection tubes	6
Blood draw volumes	7
Isolation of PBMCs with CPT	7
Isolation of PBMCs with Ficoll	8
Cell washing	10
Important notes for washing PBMCs	11
Cell counting and dilution	12
Manual counting of PBMCs	13
Dilution Calculations	14
Automated counting of PBMCs	15
Plate set-up and incubation	16
Spot development and counting	21
Spot development	21
Counting spots	23
Quality Control	24
Nil controls	25
Positive controls	25
Results Interpretation	26
Results Interpretation and assay criteria	27
Borderline & Invalid Results	29
Spot Interpretation: Panel A and B antigen	30
Troubleshooting images	31

# Reagent & Storage

## Package Insert Instructions For Use

### MATERIALS PROVIDED

T-SPOT.*TB* 8 (Multi-use 12 x 8-well strip version) contains:

1. 1 microtiter plate: 96 wells, supplied as 12x 8-well strips in a frame, coated with a mouse monoclonal antibody to the cytokine interferon gamma (IFN- $\gamma$ ).
2. 2 vials (0.8mL each) Panel A: contains ESAT-6 antigens, bovine serum albumin and antimicrobial agents.
3. 2 vials (0.8mL each) Panel B: contains CFP10 antigens, bovine serum albumin and antimicrobial agents.
4. 2 vials (0.8mL each) Positive Control: contains phytohemagglutinin (PHA), for use as a cell functionality control, bovine serum albumin and antimicrobial agents.
5. 1 vial (50 $\mu$ L) 200x concentrated Conjugate Reagent: mouse monoclonal antibody to the cytokine IFN- $\gamma$  conjugated to alkaline phosphatase.
6. 1 bottle (25mL) Substrate Solution: ready-to-use BCIP/NBT<sup>plus</sup> solution.
7. CD containing the package insert

T-SPOT.*TB* 96 (Single-use solid 96-well plate version) contains:

1. 1 microtiter plate: 96 wells coated with a mouse monoclonal antibody to the cytokine interferon gamma (IFN- $\gamma$ ).
2. 2 vials (0.7mL each) Panel A: contains ESAT-6 antigens, bovine serum albumin and antimicrobial agents.
3. 2 vials (0.7mL each) Panel B: contains CFP10 antigens, bovine serum albumin and antimicrobial agents.
4. 2 vials (0.7mL each) Positive Control: contains phytohemagglutinin (PHA), for use as a cell functionality control, bovine serum albumin and antimicrobial agents.
5. 1 vial (50 $\mu$ L) 200x concentrated Conjugate Reagent: mouse monoclonal antibody to the cytokine IFN- $\gamma$  conjugated to alkaline phosphatase.
6. 1 bottle (25mL) Substrate Solution: ready-to-use BCIP/NBT<sup>plus</sup> solution.
7. CD containing the package insert

### 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.

For T-SPOT.*TB* 8, 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.

**Avoid prolonged exposure of the Substrate Solution to light.**

T-SPOT.*TB* 96 is a single-use kit and, once opened, all materials should be used immediately and not reused. Do not mix components between different kits.

## EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

1. 8-well strip plate frame (available from Oxford Immunotec).
2. BLII cabinet (recommended).
3. Blood collection tubes, such as Vacutainer<sup>®</sup> CPT<sup>™</sup> or heparinized tubes (Note: CPT tubes are available from Oxford Immunotec).
4. Ficoll (if not using CPT tubes)
5. A centrifuge for preparation 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.
6. Equipment and reagents to enable counting of PBMCs; either manually using Trypan Blue (or other appropriate stain) and a hemocytometer on a microscope or automatically using a suitable hematology analyzer.
7. A humidified incubator capable of  $37 \pm 1^\circ\text{C}$  with a 5% CO<sub>2</sub> supply.
8. An automatic microtiter plate washer or an 8 channel or stepper pipette to manually wash plates.
9. Adjustable pipettes to cover a range of volumes from 1-1000 $\mu\text{l}$  (such as four Gilson pipettes capable of delivering volumes of 1-10 $\mu\text{L}$ , 2-20 $\mu\text{L}$ , 20-200 $\mu\text{L}$  and 100-1000 $\mu\text{L}$ ) and sterile pipette tips.
10. Sterile PBS solution: such as GIBCO<sup>™</sup> 1x D-PBS (Invitrogen; catalogue number 14040-133).
11. Distilled or deionized water.
12. A means of visualizing the wells, or capturing a digital image of the well, such as a stereomicroscope, magnifying glass or plate imager to allow counting of spots.
13. Sterile cell culture medium such as GIBCO AIM-V<sup>™</sup> (Invitrogen; catalogue number 31035-025 research grade). (Note: AIM-V media is available from Oxford Immunotec). **The use of this serum free medium for the incubation step is strongly recommended.** RPMI 1640 (Invitrogen; catalogue number 11875-093) may be used in the initial sample preparation steps only. 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 T-SPOT.TB.** To avoid problems with contaminated media, it can be helpful to dispense bottles of AIM-V or RPMI 1640 into smaller aliquots.

# SPECIMEN COLLECTION & HANDLING

## Package Insert Instructions For Use

### SPECIMEN COLLECTION & HANDLING

Individual laboratories should validate their procedures for collection and separation of PBMCs to obtain sufficient numbers. It is recommended that:

- Blood samples are collected into either sodium citrate or sodium heparin Vacutainer® CPT tubes (Becton Dickinson) and then the PBMCs separated in the tube using the manufacturer's instructions.
- Blood samples are collected into lithium heparin blood collection tubes with PBMCs being subsequently separated using standard separation techniques such as Ficoll-Paque®. Alternative methods to purify the PBMC fraction may be employed if desired. **Note EDTA tubes are not recommended.**
- Patient's cells can be pooled, if necessary to obtain sufficient cells from multiple tubes of blood which were collected and processed concurrently.

## Training Notes and Visual Aids

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



BD Sodium Citrate  
Vacutainer® CPT™



BD Vacutainer® Lithium  
Heparin PST™

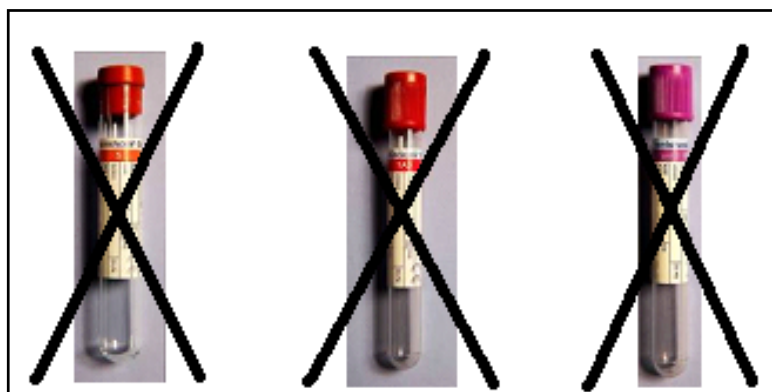


Greiner Bio-One  
Lithium Heparin Vacuette®

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

**Note:** Tubes from alternative suppliers that contain lithium heparin, sodium citrate or sodium heparin may also be used for specimen collection.

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



BD Vacutainer® Z

BD Vacutainer® CAT

BD Vacutainer®  
EDTA (K2E or K3E)

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

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

## Package Insert Instructions For Use

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 8mL or two 4mL tubes (CPT) or two lithium heparin 6mL tubes
- Children 2-9 years old: one 4mL tube (both methods)
- Children up to 2 years old: one 2mL pediatric tube (both methods)

Blood samples should be processed within 8 hours. Samples may be successfully stored for longer periods of time but users should validate this in their own setting<sup>1-2</sup>. **Whole blood samples should be maintained between 18°C and 25°C until processed.** The instructions below give more information on specimen collection steps:

1. Collect a blood sample according to the instructions supplied with the collection device. The tube contents must be inverted (8 – 10 times) to ensure that the whole blood is mixed thoroughly with the anticoagulant. Store collected blood at room temperature (18-25°C). **Do not refrigerate or freeze.**
2. For CPT blood collection tubes, centrifuge 8mL CPT tubes at 1600 RCF(g) for 28 minutes or 4mL CPT tubes at 1800 RCF (g) for 30 minutes at room temperature (18-25°C). If using Ficoll-Paque™ Plus, dilute the blood with an equal volume of RPMI 1640 medium (1 part blood to 1 part RPMI). Layer carefully the diluted blood onto Ficoll-Paque Plus (2-3 parts diluted blood to 1 part Ficoll-Paque) and centrifuge at 1000 RCF (g) for 22 minutes at room temperature (18-25°C).

## Training Notes and Visual Aids

### [Separating PBMCs by BD Vacutainer® Cell Preparation Tube \(CPT™\)](#)

Collect blood in 4mL or 8mL CPTs following the manufacturer's instructions (Figure 3).

Centrifuge CPTs at 18-25°C.

- 4mL - 1800 RCF (g) for 30 minutes
- 8mL - 1600 RCF (g) for 28 minutes

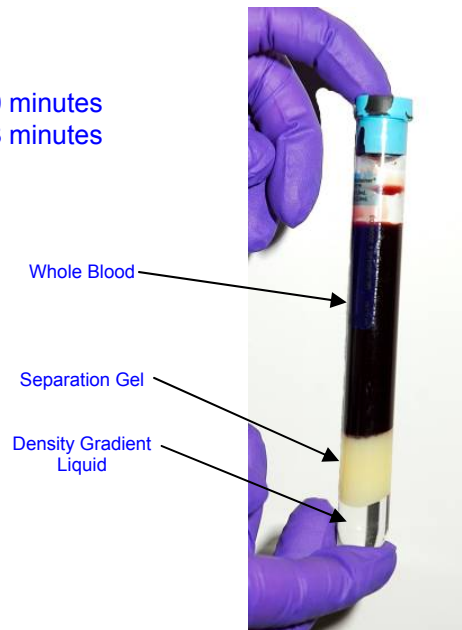


Figure 3. CPT filled with whole blood ready for centrifugation

### Notes:

*RCF = Relative Centrifugal Force. The term RCF, and not RPM, is used to separate aqueous solutions in the centrifuge.*

*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.*



### Isolation of PBMCs by Ficoll Extraction Method

Collect appropriate volume of blood into lithium heparin tubes following the manufacturer's Instructions and dilute 1:1 with RPMI 1640 pre-warmed to 37°C. Carefully layer diluted blood sample onto Ficoll-Paque™ Plus (Figure 4) at a ratio of 3 volumes of diluted blood to 1 volume Ficoll-Paque Plus. Do not allow the layers to mix (Figure 5).

Centrifuge at 1000 RCF (g) for 22 minutes at room temperature (18-25°C).

Ensure the tubes are balanced before centrifugation in an aerosol-resistant bucket, with the brake off.

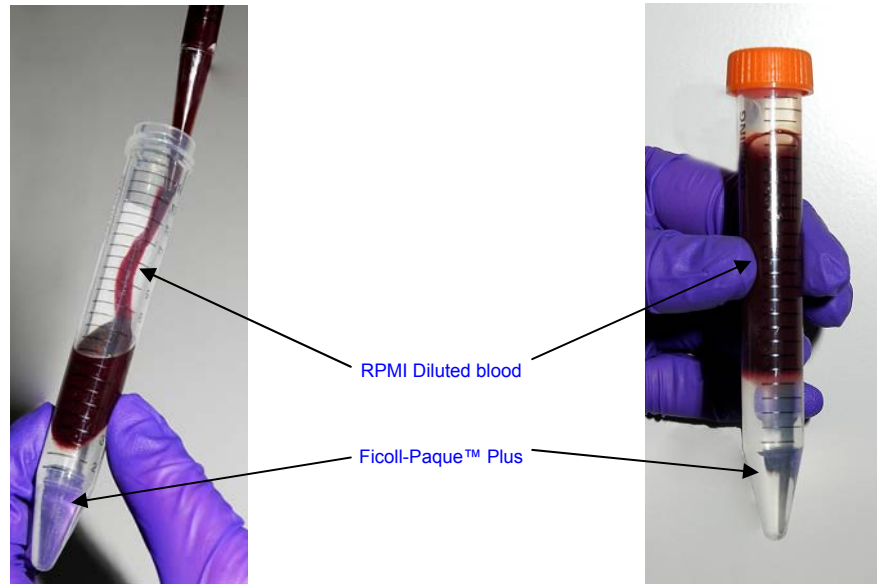


Figure 4 & 5. layering diluted whole blood on Ficoll-Paque and tube ready for centrifugation, respectively

#### **Notes:**

*If a low cell yield is suspected then collect: 2x 8mL CPTs, 2x 6mL Lithium Heparin Vacutainer tubes or similar (see page 3). This will ensure sufficient PBMC yield to run the assay.*

*PBMC isolation can be achieved using CPTs or Ficoll gradients. Leucosep tubes (Greiner Bio-One) offer a time-saving approach to Ficoll gradients. These tubes contain a porous barrier that enables the blood sample to be poured onto the Ficoll gradient, thereby eliminating the need to gently layer the sample on Ficoll-Paque.*

### BD Cell Preparation Tube (CPT™)

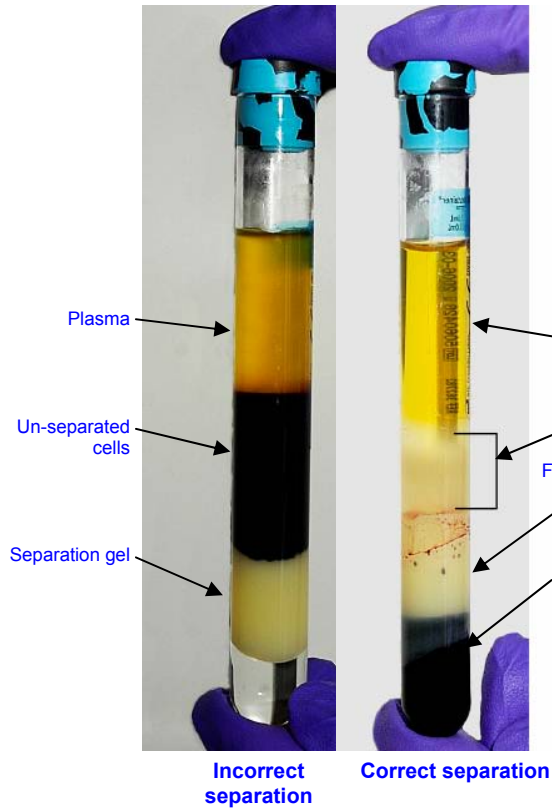


Figure 6. Incorrectly and correctly separated CPT method

### Ficoll Extraction

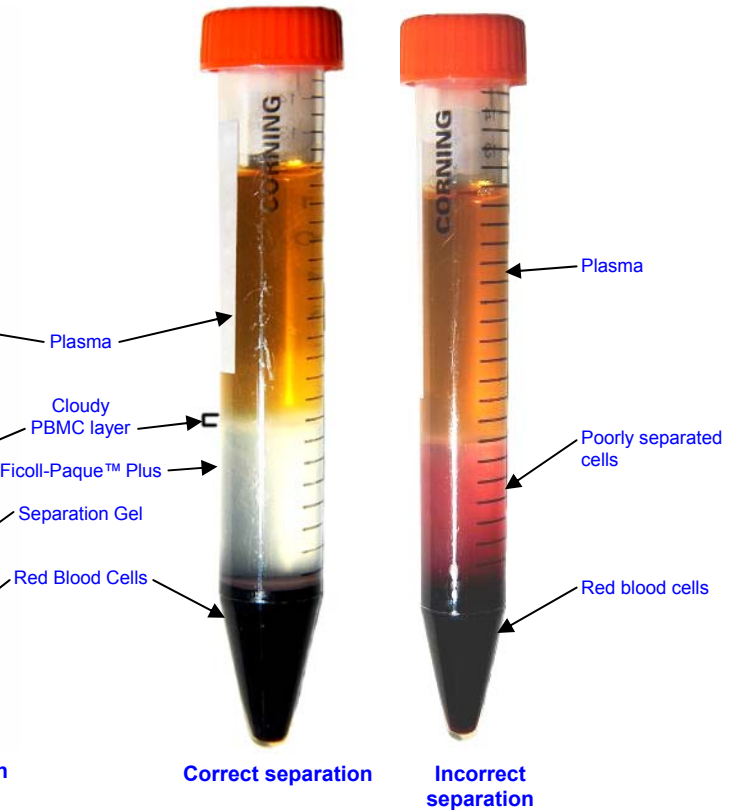


Figure 7. Incorrectly and correctly separated Ficoll-Paque method

If correct separation has not been achieved, it may be caused by the use of the wrong centrifugation speed. If the tubes were centrifuged in RPM instead of RCF (as required), assure appropriate RCF to RPM conversion and centrifuge again.

Where separation is not achieved the following should 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?
- 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)?

### Package Insert Instructions For Use

3. Collect the white, cloudy band of PBMCs using a pipette and transfer to a 15mL conical centrifuge tube. Bring the volume to 10mL with cell culture medium.

**Cell culture media for the washing steps should be pre-warmed to 37°C before contact with PBMCs.**

**Cell culture media for the washing steps should be pre-warmed to 37°C before contact with PBMCs.**

### Training Notes and Visual Aids

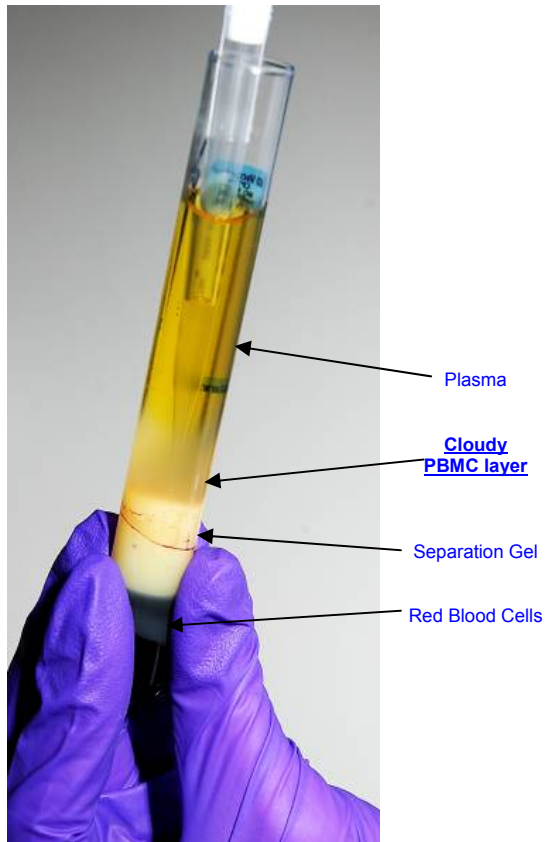


Figure 8. Removal of the cloudy PBMC layer from a CPT with a 1mL pipette

Insert a pipette tip down through the plasma to the cloudy layer and aspirate the liquid into the pipette tip (Figure 8). Transfer to a 15 mL conical centrifuge tube.

The centrifuge tubes require a conical bottom (see example in Figure 10) so that a pellet is formed when centrifuged in the next step. Falcon (blue top) and Corning (orange top) centrifuge tubes are known to perform well.

#### Notes:

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

*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. However, 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.*

*A variety of media can be used for washing the cells during steps 3-5; both AIM-V and RPMI 1640 have been used successfully and are recommended.*

## Package Insert Instructions For Use

4. Centrifuge at 600 RCF (g) for 7 minutes. Pour off the supernatant and resuspend the pellet in 1mL medium.
5. Bring the volume to 10mL with fresh medium and centrifuge at 350 RCF (g) for 7 minutes.
6. Pour off the supernatant and resuspend the pellet in 0.7mL cell culture medium.

**The serum-free medium AIM-V has been used successfully and is strongly recommended**

## Training Notes and Visual Aids



Figure 9. Fill the 15mL tube up to 10mL with culture medium.

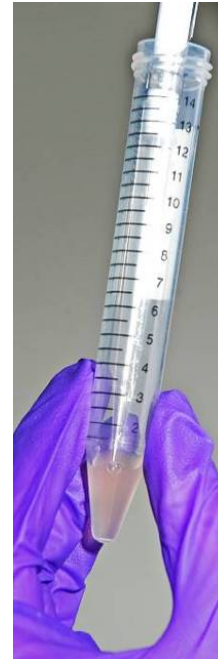


Figure 10. Resuspend the cell pellet in 0.7mL of AIM-V

## Important notes for washing PBMCs

- Cell culture media for the washing steps should be pre-warmed to 37<sup>0</sup>C before contact with PBMCs
- 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 either gentle swirling of the tube by hand, or by gently agitating the suspension by pipetting the suspension up and down several times. This will ensure that the cells are evenly distributed

### Notes:

To avoid problems with contaminated media, dispense 500mL bottles of AIM-V or RPMI 1640 aseptically into smaller aliquots e.g. 10x 50mL aliquots. For instance, this allows a 50mL aliquot of AIM-V to be used per 24 tests (1x T-SPOT.TB kit) when RPMI is used for cell washing.

If preferred, the set-up of the plate can be started during the centrifugation steps (see page 14 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.

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.

If too few cells are suspected, resuspend the pellet in 0.5mL AIM-V.

# CELL COUNTING AND DILUTION



Harnessing the power of T cell measurement

## Package Insert Instructions For Use

### CELL COUNTING AND DILUTION

T-SPOT.TB 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* T cells in the specimen is normalized to a fixed number of PBMCs.

1. 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 hemocytometer, or using an automated hematology analyzer.
2. Briefly, for manual counting with a Neubauer hemocytometer using Trypan Blue, add  $10\mu\text{L}$  of the final cell suspension to  $40\mu\text{L}$  0.4%(w/v) Trypan Blue solution. Place an appropriate aliquot onto the hemocytometer and count the cells in the grid. For other types of hemocytometer and for automated devices, follow the manufacturers' instructions.

### Training Notes and Visual Aids

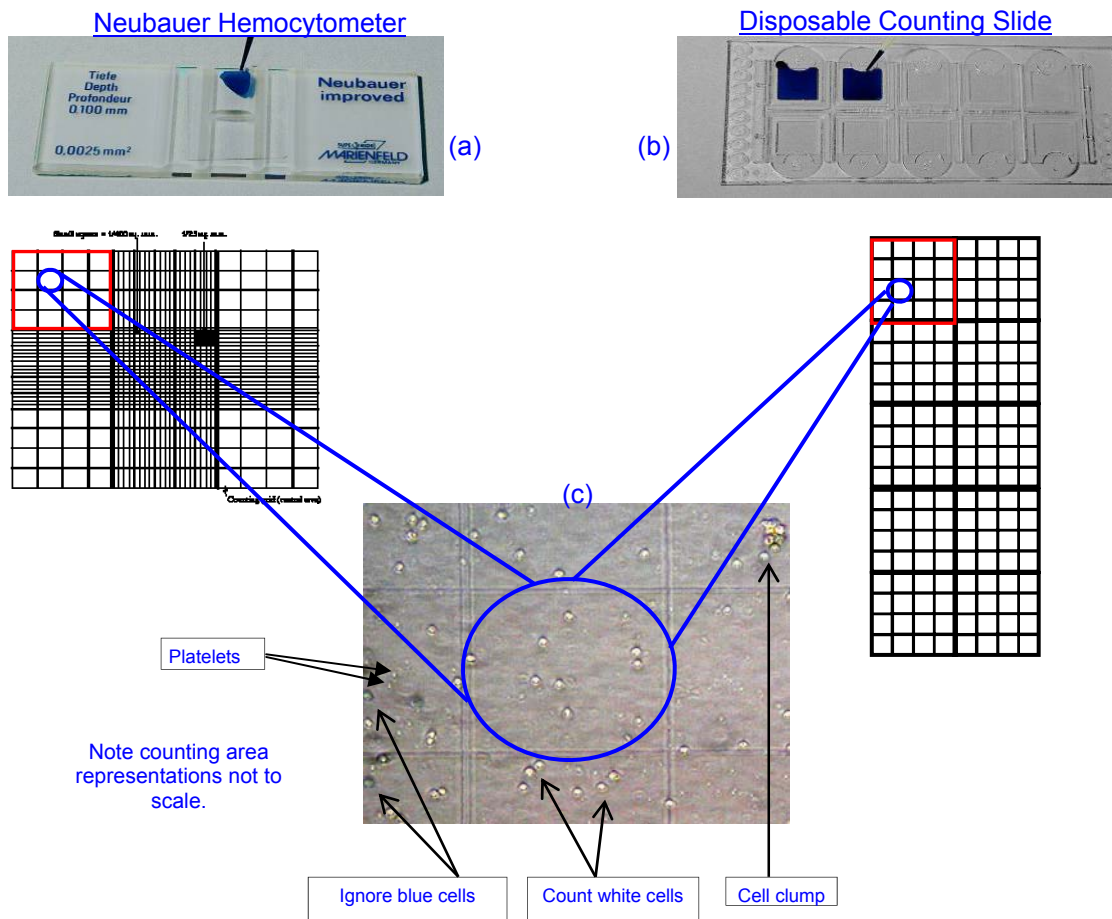


Figure 11: Sample chamber and counting areas of (a) Neubauer hemocytometer and (b) Disposable Counting Slide. Cells stained with Trypan Blue as seen under a microscope using the x10 objective (c)

Cell concentration can be determined by counting the number of cells within a defined volume. A Neubauer hemocytometer contains two chambers, which are divided into nine major squares (Figure 11 (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 11 (b)).

Count the cells in one major square (highlighted in red in Figure 11) of the counting grid using the low-power (x10) 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 11 (c).

---

<b>Package Insert Instructions For</b>
--

3. Calculate the concentration of PBMCs present in the stock cell suspension.

4. Prepare 500µL of the final cell suspension at a concentration of  $2.5 \times 10^5$  cells/100µL (giving  $1.25 \times 10^6$  PBMCs in total).

**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 done 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.*

*Ensure cells are thoroughly mixed, by gently agitating the suspension by pipetting the suspension up and down several times, before removing an aliquot for dilution. PBMC numbers between 200,000 and 300,000 per well have been shown to give consistent T-SPOT.TB results.*

---

<b>Training Notes and Visual Aids</b>
---------------------------------------

**Manual Counting example**

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

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

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

**Example:**

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.

## Automated Counting

A hematology analyzer (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 hematology 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 **500 $\mu$ L** by adding **375 $\mu$ L** AIM-V medium. This gives a final solution of 250,000 cells/100 $\mu$ L for use in the assay.

### Notes:

*When using a hematology 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 hematology analyzer requires. Analyzers typically use between 100 $\mu$ L and 400 $\mu$ L. 4 x 100 $\mu$ L of cell suspension will be added to the microtiter plate wells when running the assay so if the analyzer uses 400 $\mu$ L, you will need at least 900 $\mu$ L of cell suspension, allowing for some dead volume. In this case, the final volume of the cell suspension should be 1mL rather than 0.7mL.*

### Precautions:

Where insufficient cells are obtained to perform the assay the following should be checked:

- Was the appropriate volume of blood drawn from the patient?
- Was the counting chamber used correctly?
- Were the correct calculations used when calculating number of cells/mL and dilutions?
- Were the cells thoroughly mixed prior to removing an aliquot for counting?



# PLATE SET UP AND INCUBATION

## Package Insert Instructions For Use

### PLATE SET UP AND INCUBATION

T-SPOT.*TB* requires four wells to be used for each patient 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 numbers of plates required for the numbers of samples that you wish to process. The 8-well strip version of T-SPOT.*TB* (T-SPOT.*TB* 8) provides the additional flexibility of 12 x 8-well strips. Each strip will process 2 samples. If you have purchased this version, use only the numbers of strips that you require.

T-SPOT.*TB* is an assay that 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)

1. Remove the pre-coated 8-well strips from the packaging, clip into a plate frame 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. Alternatively, if the 96-well version is being used, remove the plate from the pouch and allow it to equilibrate to room temperature.
2. Add in 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.

### Training Notes and Visual Aids

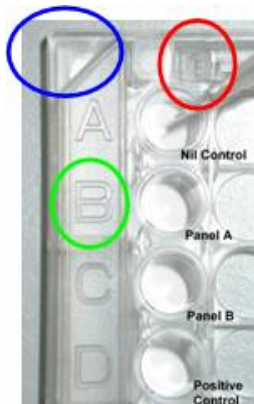


Figure 13. 4 wells used for one patient. A1 to D1, Nil Control, Panel A, Panel B and Positive control respectively

The plate orientation should be as shown (Figure 13). 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 13 shows 4 wells used for one patient sample positioned in the top four wells on the first strip of an 8 well strip plate (TB.300). 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.

### Package Insert Instructions For Use

3. To each of the 4 wells to be used for a patient sample, add 100 $\mu$ L 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 wells. **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 14 (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 14 (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.

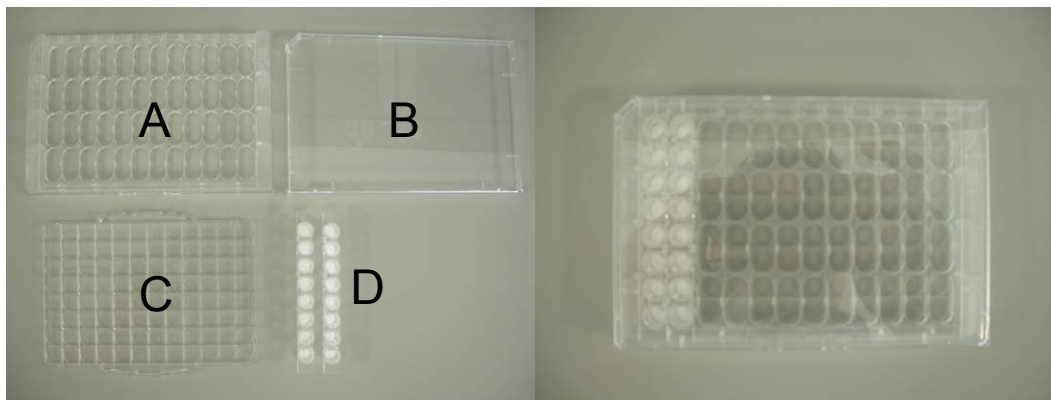


Figure 14: Left: Breakdown of the 8 well strip plate (TB.300). Right: Final assembly with 2 strips. Enough wells for 4 patient samples (e.g. 16 wells).

Plate, Panel A, Panel B, 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.

### Package Insert Instructions For Use

4. Incubate the plate with the lid on in a humidified incubator at  $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$  with 5%  $\text{CO}_2$  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 15: 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%  $\text{CO}_2$  supply (Figure 15). Check the water dish for sufficient water to ensure that a humid atmosphere is achieved.

# SPOT DEVELOPMENT AND COUNTING



Harnessing the power of T cell measurement

## SPOT DEVELOPMENT AND COUNTING

1. 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.*
2. Add 200µL Phosphate Buffered Saline (PBS) solution to each well. **Do not use PBS containing Tween™ or other detergents, as this causes high background counts.**
3. Discard the PBS solution. Repeat the well washing an additional 3 times with fresh PBS solution for each wash.

*Note: For washing, an automatic plate washer or an 8 channel or stepper pipette to manually wash plates may be used. Discard PBS into a suitable container after each wash. Do not use pipettes to remove the PBS as this risks damaging the membrane. If using a plate washer, ensure the manifold is adjusted so that the tips do not touch the membrane. After the final wash, tap the plate on lint-free towel to ensure all PBS is removed – any*

### Training Notes and Visual Aids



Figure 16: Aspirate 200µL of PBS from a reagent tray

*Note:*

*For washing, an 8 channel or stepper pipette and a plastic reservoir to hold PBS may be used shown in Figure 16.*

**Notes:**

*With force, dispense 200µL in to each well to wash thoroughly (Figure 17).*

*If using a plate washer, ensure the manifold is adjusted so that the tips do not touch the membrane. After the final wash, tap the plate on lint-free towel to ensure all PBS is removed – any excess left will further dilute the Conjugate Reagent.*

*When washing the plate, ensure that all the wells are full in between washes.*



Figure 17: Dispense 200µL of PBS into each well

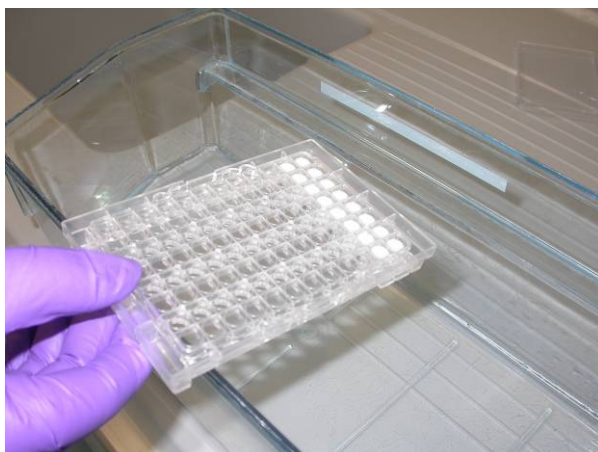


Figure 18: Invert plate into a container

**Note:**

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

*Discard PBS by inverting the plate over a suitable container after each wash (Figure 18).*

## Package Insert Instructions For Use

4. If not already prepared during the reagent preparation step, dilute concentrated Conjugate Reagent 200X in PBS to create the working strength solution.
5. Add 50 $\mu$ L working strength Conjugate Reagent solution to each well and incubate at 2-8°C for 1 hour.
6. Discard the conjugate and perform the four PBS washes as described in steps 2 and 3 above.
7. Add 50 $\mu$ L Substrate Solution to each well and incubate at room temperature for 7 minutes.
8. Wash the plate thoroughly with distilled or deionised water to stop the detection reaction.
9. 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 4 hours drying time at 37°C or at least 16 hours at room temperature.

### Tips:

*In order to load all wells quickly, a multi-channel pipette and a plastic reservoir is recommended.*

*Ensure all residual 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 19: Perform Conjugate 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 PBS. Mix by inverting 5-6 times.*

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

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



Figure 20: Add substrate and incubate at 18-25°C for 7 mins

### Note:

*Use of an 8 channel or stepper pipette is recommended for pipetting the Substrate (Figure 20)*

*The substrate solution should be used at room temperature and is supplied ready to use.*

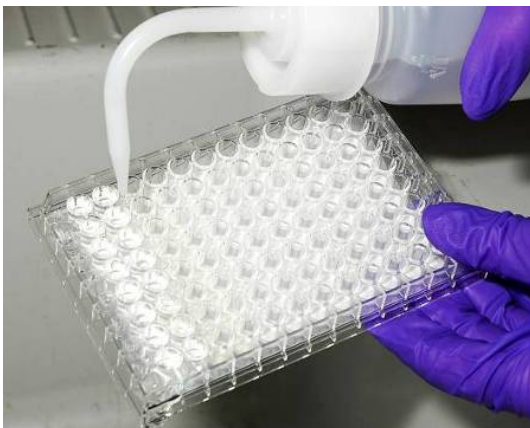


Figure 21: Wash wells with distilled or deionised water

*After washing the plate with distilled or deionised water (Figure 21), 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.*

## Package Insert Instructions For Use

10. Count and record the number of distinct, dark blue spots on the membrane of each well. Apply the Results Interpretation and Assay Criteria (see below) to determine whether a patient sample is 'Positive' or 'Negative'. **The spots produced as a result of antigen-stimulation should appear as large, round and dark spots. Often a gradient effect can be observed with a darker centre and a more diffuse periphery. Non specific artifacts that can occur are smaller, less intense and irregular in shape.**

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.

## Training Notes and Visual Aids

### Example Results

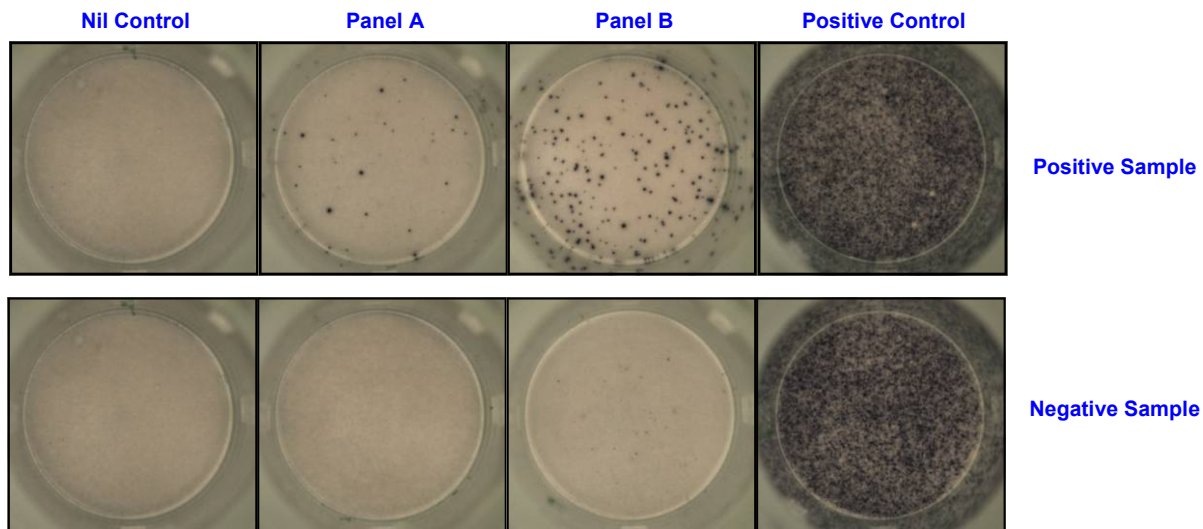


Figure 22: Two typical T-SPOT.TB results. Top = Positive. Bottom = Negative.

Spots can be counted directly from the well using a magnifying glass, a stereomicroscope, or from a digital image captured from a low powered microscope.



# QUALITY CONTROL

## Package Insert 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.

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 results should be considered invalid. These results are usually due to operator issues such as: suboptimal 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 'Invalid'.

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>3</sup>. Where the Positive Control spot count is  $< 20$  spots, it should be considered as 'Invalid', unless either Panel A or Panel B are 'Positive' or 'Borderline (equivocal)' as described in the Results Interpretation and Assay Criteria (see below), in which case the result is valid.

In the case of Invalid results, these should be reported as "Invalid" and it is recommended to collect a further sample and re-test the individual.

### Training Notes and Visual Aids

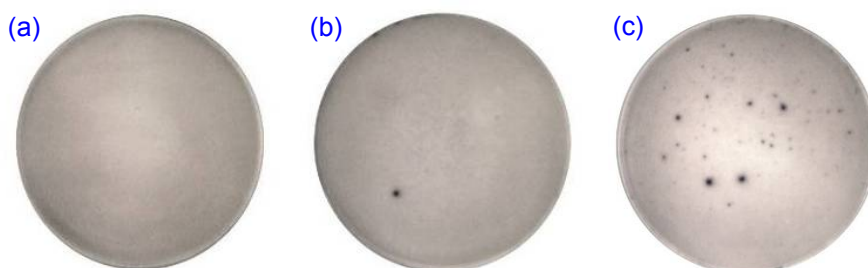


Figure 23: Typical images from Nil control wells: (a) shows a well with 0 spots (b) shows a well with 1 spot and (c) shows an invalid Nil control well of  $>10$  spots

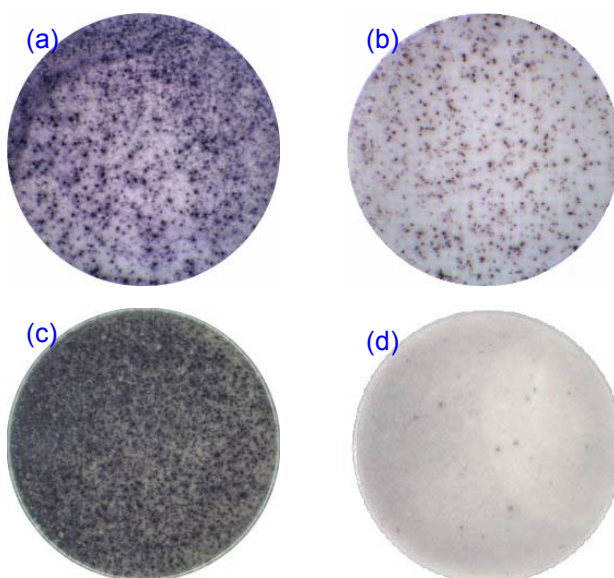


Figure 24: Typical images from Positive control wells (a) (b) and (c) all shown acceptable responses for patient samples. (d) shows an invalid Positive control well due to  $<20$  spots.

# RESULT INTERPRETATION



Harnessing the power of T cell measurement

**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 LTBI, requires a combination of epidemiological, historical, medical and diagnostic findings that should be taken into account when interpreting T-SPOT. *TB*. Refer to the most recent CDC guidance (<http://www.cdc.gov/nchstp/tb>) for detailed recommendations about diagnosing TB infection (including disease) and selecting persons for testing.

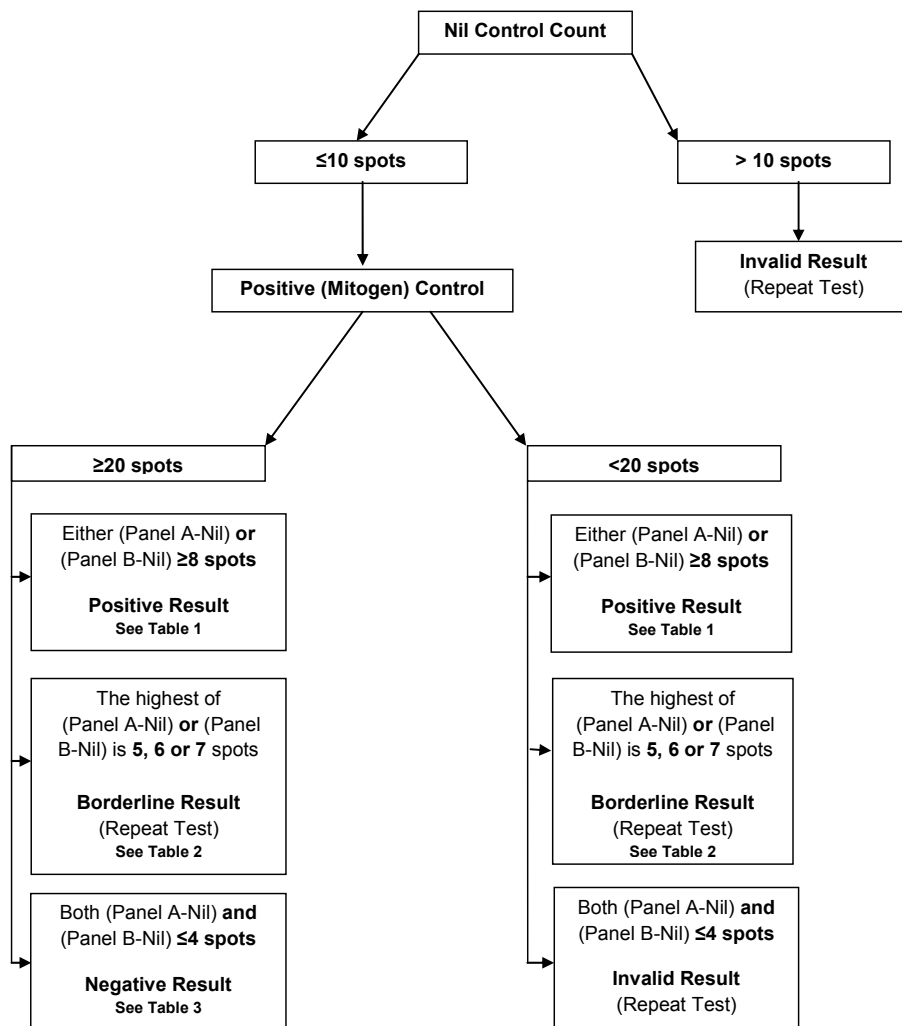
Results for T-SPOT. *TB* are interpreted by subtracting the spot count in the Nil control well from the spot count in each of the Panels, according to the following algorithm:

The test result is Positive if (Panel A-Nil) and/or (Panel B-Nil)  $\geq 8$  spots

The test result is Negative if both (Panel A-Nil) and (Panel B-Nil)  $\leq 4$  spots. This include results less than zero.

**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 retesting by collecting another patient specimen is recommended.**

If the result is still Borderline (equivocal) on retesting with another specimen, then other diagnostic tests and/or epidemiologic information should be used to help determine TB infection status of the patient.



**Package Insert Instructions For Use**

**Table 1: Positive Interpretation: Either (Panel A-Nil) or (Panel B-Nil) ≥8 spots**

Nil Control Well Count	Either Panel A or Panel B has the following number of spots <sup>†</sup>	Result Interpretation
0	≥8	<b>Positive</b>
1	≥9	<b>Positive</b>
2	≥10	<b>Positive</b>
3	≥11	<b>Positive</b>
4	≥12	<b>Positive</b>
5	≥13	<b>Positive</b>
6	≥14	<b>Positive</b>
7	≥15	<b>Positive</b>
8	≥16	<b>Positive</b>
9	≥17	<b>Positive</b>
10	≥18	<b>Positive</b>
>10 spots	n/a	<b>Invalid</b>

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

**Table 2: 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>Invalid**</b>

**Table 3: Negative Interpretation: Both (Panel A-Nil) and (Panel B-Nil) ≤4 spots**

Nil Control Well Count	Both Panel A and Panel B has the following number of spots	Result Interpretation
0	≤4	<b>Negative</b>
1	≤5	<b>Negative</b>
2	≤6	<b>Negative</b>
3	≤7	<b>Negative</b>
4	≤8	<b>Negative</b>
5	≤9	<b>Negative</b>
6	≤10	<b>Negative</b>
7	≤11	<b>Negative</b>
8	≤12	<b>Negative</b>
9	≤13	<b>Negative</b>
10	≤14	<b>Negative</b>
>10 spots	n/a	<b>Invalid**</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 retesting by collecting another patient specimen is recommended.

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

## 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 cutoff of  $\geq 6$  spots. Borderline (equivocal) results, although valid, are less reliable than results where the spot count is further from the cut-off. Retesting of the patient, using a new sample, is therefore recommended. If the result is still Borderline (equivocal) on retesting, 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>12</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 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 Technical Support in the United States contact: 1 – 877 – 20-TSPOT (87768).

## 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 26.

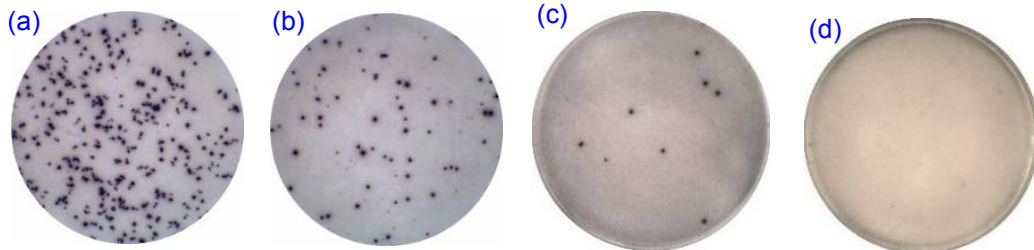


Figure 26: Typical images from Panel A and B antigen wells, (a) Positive sample (>20 spots); (b) Positive sample (>20 spots); (c) Positive sample (8 spots) and (d) Negative sample (0 spots)

### Example patient interpretations:

	<u>Well</u>	<u>Spot count</u>	<u>Valid?</u>
<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 ≥ 8) 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 ≤ 4) 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 = Invalid (Repeat Test)**

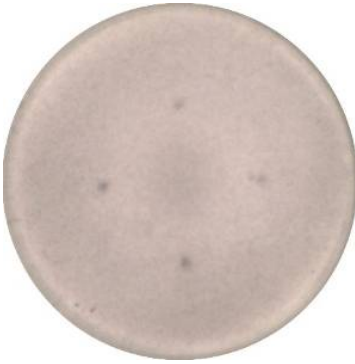
<b>Patient 4.</b>	Nil Control	= 0	✓
	Panel A	= 0	✓
	Panel B	= 7	✓
	Positive Control	= <20	X

Positive control value <20 however, Panel B value subtract the Ni control value = 7 therefore:

**Result = Borderline (equivocal) (Repeat Test)**

## SPOT INTERPRETATION — TROUBLESHOOTING

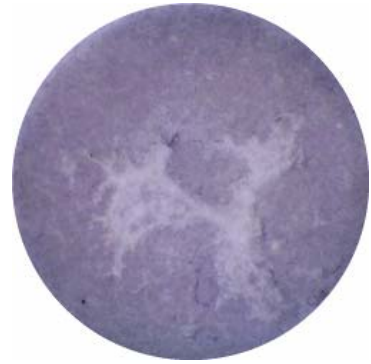
If wells contain debris or have a high background care should be taken when counting spots. See the examples below:



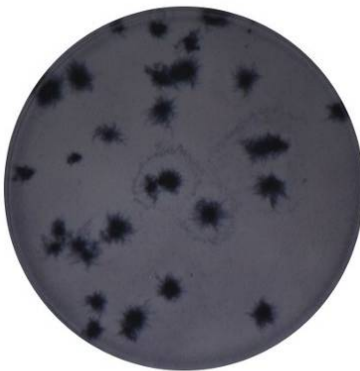
**Example 1:** 4 Mark Effect. These marks can be attributed to over excessive pressure on the back of the solid 96 well plate (TB.200) caused when tapping solutions out of the plate during the plate washing steps. [In this case the spot count = 0].



**Example 2:** High background in panel well. Although a high background is uncommon with T-SPOT.TB, spots can still be seen over background. [In this case the spot count = >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]



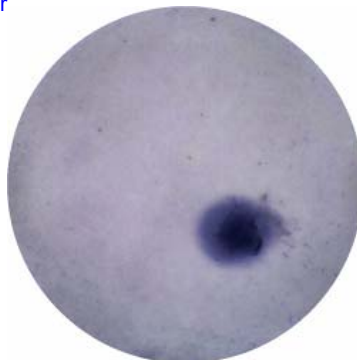
**Example 4:** Fungal contamination in the well. These growths are not round in shape. Rather they appear as not uniform and have fibrous edges. They are also much larger than ELISPOT spots. [In this case spot Count = 0]. Care should be taken not to allow a contamination to occur. Check incubator, medium and assay reagents for possible contamination.



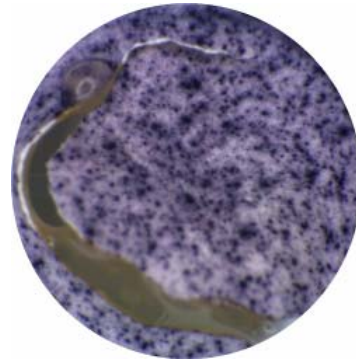
**Example 5:** Pipette tips can cause dark marks on the membrane. However spots will still be evident. [In this case the spot count = 0]. Care should be taken when pipetting reagents and cells to not touch the membrane with pipette tips.



**Example 6:** 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 7:** 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 8:** Broken membrane. Spots are still evident in this positive control well. [In this case spot count = >20]. Care should be taken when pipetting into the wells to ensure that the membranes are not damaged.



## References

1. NCCLS. Performance of single cell immune response assays; approved guideline. NCCLS document I/LA26-A.
2. 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.
3. 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.

T-SPOT and the Oxford Immunotec logo are trademarks of Oxford Immunotec Limited  
AIM-V, RPMI 1640 and GIBCO are trademarks of Invitrogen  
BD, CPT and Vacutainer are trademarks of Becton Dickinson  
Ficoll-Paque is a trademark of GE Healthcare Technologies  
Tween is a trademark of ICI Americas

Fast-Read Disposable Counting Slides are supplied by Immune Systems Limited

T-SPOT. *TB* is protected by the following patents and patents pending:  
EP 0941478, US 09/308,725, AU 728357, CA 2272881, JP 524410/98, EP 1152012, AU 765013, EP1144447, US 09/830,839, JP 2000-579635, US 09/916,201, WO 02/054072, WO 9709428, US 6290969, US 6338852, US 09/724,685, AU 727602, BR 9610262, CA 2230885, CN 1200147, CZ 9800628, HU 9900902, IL 123506, NO 9800883, PL 325373, TR 9800411, ZA 9607394, WO 9709429, AU 9671587, JP 11514217, BR 9610268, CA 2230927, CN 1200146, WO 9501441, US 5955077, EP 706571, AU 682879, CA 2165949, NZ267984

T-SPOT<sup>®</sup>. *TB* incorporates patented technology under license from the Statens Serum Institut, Copenhagen, Denmark and Isis Innovation Limited, Oxford, UK.

T-SPOT. *TB* is sold under license from the Public Health Research Institute and may be used under PHRI patent rights only for human *in vitro* diagnostics.

© Oxford Immunotec Limited, 2008. All rights reserved.

Oxford Immunotec, Inc.  
2 Mount Royal Ave, Ste. 100  
Marlborough, MA 01752 USA  
Toll Free: 877-20 TSPOT  
www.oxfordimmunotec.com

Oxford Immunotec, Ltd.  
94C Milton Park, Abingdon  
Oxfordshire, OX14 4RY, UK  
Tel: +44 (0) 1235 442796

