T-SPOT.COVID

Oxford Immunotec

PACKAGE INSERT

For *in vitro* Diagnostic Use

This Package Insert covers use of:

COV.435/300, COV.435/200

Table of contents

Intended Use....3
Summary and Explanation....3
Reagents and Storage....5
Warnings and Precautions....6
Storage & Stability....5
Warnings and Precautions....6
Specimen collection and Handling....7
Instructions for Use....8
Limitations....13
Performance Characteristics....14
Expected values....17
Troubleshooting....18
Abbreviations and Glossary of Symbols....19
References....19
Contact Information....21

1. INTENDED USE

The T-SPOT.*COVID* test is a standardised ELISPOT (Enzyme Linked ImmunoSpot) based technique intended for qualitative detection of a cell mediated (T cell) immune response to SARS-CoV-2 in human whole blood (sodium or lithium heparin). The T-SPOT.*COVID* test is intended for use as an aid in identifying individuals with an adaptive immune response to SARS-CoV-2, specifically the T cell response. This test could be used alongside serology tests to support clinical assessment of individuals, for example, who present with suspected COVID-19 but are SARS-CoV-2 PCR negative and is complementary to serology.

Results are for the detection of a cell mediated (T cell) immune response to SARS-CoV-2. A T cell response to SARS-CoV-2 is generally detectable in blood several days after initial infection, and the duration of time for detectable response post-infection is currently not well characterised.

Reactive results for the T-SPOT.*COVID* test may occur due to infection from other similar viruses and from prior vaccination to SARS-CoV-2.

2. SUMMARY & EXPLANATION

SARS-CoV-2 is a strain of Coronavirus discovered in the Wuhan province of China in 2019. The virus rapidly spread throughout the world during the first few months of 2020 leading to the declaration of a pandemic by the WHO on the 11th March 2020[1]. A number of molecular based tests were rapidly developed and are now widely available throughout the world[2]. These have been, and continue to be widely deployed for confirmation of a current SARS-CoV-2 infection. These tests were initially reported to be highly sensitive and specific, however, systematic reviews of real-world studies suggest that a reasonable estimate for the sensitivity of molecular tests is approximately 70 %[3,4]. Zhao *et al.* reported that out of 173 patients who were hospitalised with acute respiratory symptoms and a chest CT characteristic of COVID-19 disease, just 67 % received positive RT-PCR test results from a respiratory sample during days 1-7 of hospitalisation[5]. These findings suggest that false negatives are common in acute COVID-19 disease if diagnosis relies solely upon molecular testing. Further to this, molecular tests are unable to identify those individuals who have been infected but have since cleared the virus[6]. As such, numerous serology tests have been developed to detect antibodies in the blood or blood products of individuals who have been previously infected with SARS-CoV-2. These tests provide valuable information on the prevalence of exposure to the virus in the general population[2,7], but can also be used as an adjunct to molecular testing when making a clinical diagnosis in acute COVID-19 disease[8].

Studies have shown that an adaptive immune response is usually generated within 2 weeks of SARS-CoV-2 infection, however, a number of studies have shown that an antibody response may not always be present or may be delayed[9,10]. Current literature suggests that some individuals who tested positive for SARS-CoV-2 infection using PCR, may not generate a detectable antibody response[9]. Low levels of SARS-CoV-2-specific antibodies have been frequently observed in individuals who suffered from mild or asymptomatic COVID-19 disease[11,12]. There is also evidence to suggest that in some individuals antibodies decline significantly post infection and at an even faster pace than was observed for MERS and SARS-CoV-1 infection[13,14].

In contrast, several publications have shown that T cell responses to human coronaviruses, including SARS-CoV-1 and SARS-CoV-2 may be robust and long-lasting[15], with some individuals who were infected with SARS-CoV-1 17 years ago still demonstrating T cell responses today[16]. Several studies have shown that SARS-CoV-2 specific T cell immunity is maintained at 6-9 months following primary infection, indicating that T cell responses may outlast transient antibody responses to SARS-CoV-2 infection[15,17]. These findings, together with studies that have demonstrated a critical role for T cells in viral clearance and recovery from SARS-CoV-2[18], suggest that cell-mediated immunity may be an important aspect of the immune response to SARS-CoV-2 infection[19]. Further to this, whilst the dynamics of the SARS-CoV-2 specific T cell response have not yet been fully elucidated, evidence suggests that the majority of individuals infected with SARS-CoV-2 generate functional, IFN-gamma (IFN-γ) producing SARS-CoV-2 T cells that can be detected in the peripheral blood as early as 2-4 days from the onset of symptoms[19]. Tan *et al*. analysed the kinetics of the SARS-CoV-2 specific T cell response during the acute RT-PCR positive phase of the infection, and found that specific T cells were first detected around days 5-7 after symptoms onset with frequencies progressively increasing until around day 15[18]. They also observed a positive correlation between early detection of SARS-CoV-2 specific T cells and early control of infection, leading to milder disease outcomes and rapid viral clearance. Similarly, Weiskopf *et al*. demonstrated that SARS-CoV-2 specific CD4 and CD8 T cells can be detected in the blood of severe COVID-19 patients within the first two weeks post symptom onset[20]. This indicates that although severe SARS-CoV-2 has been reported to lead to lymphopenia[21], SARS-CoV-2 specific T cells are still generated during the early response to infection. Further, a recent study from Rydyznski Moderbacher *et al*. observed that SARS-CoV-2 specific CD4 T cells could be detected as early as 4 days post symptom onset[22]. In agreement with Tan *et al.*, this study also showed that the early emergence of SARS-CoV-2 specific CD4 and CD8 T cells was associated with better disease outcomes. Together these findings not only highlight the importance of T cells in orchestrating the immune response against SARS-CoV-2 in the acute phase of infection, they also suggest that the detection of T cells during acute SARS-CoV-2 infection could provide more

detailed information on an individual's immune response.

A prospective cohort study carried out by Public Health England during the early stages of the COVID-19 pandemic reiterated the importance of T cell monitoring in SARS-CoV-2 infection. In this study 2,826 individuals identified as keyworkers were tested on enrollment for anti-spike IgG (EuroImmun AG) and SARS-CoV-2 reactive T cells using a research use only (RUO) version of the T-SPOT.*COVID* test (Oxford Immunotec)[23] from which this test was developed. Of this keyworker cohort, 154 were recruited on the basis of a previous positive RT-PCR test confirming SARS-CoV-2 infection. 5.8 % of this previously PCR positive population were seronegative, but 88.9 % of these subjects demonstrated robust T cell responses which were detected using the research version of the T-SPOT.*COVID* test. This finding indicates that some infected individuals can generate cell-mediated (T cell) immune responses in the absence of an antibody response. This corresponds with studies performed in household contacts of SARS-CoV-2 infected individuals, which found that contacts were approximately 50 % more likely to develop SARS-CoV-2-specific T cells than antibodies following exposure[24,25]. Together, these findings suggest that T cells may be a more sensitive indicator of past exposure to SARS-CoV-2 than antibody responses.

In the same Public Health England study, the remaining 2,672 study participants were monitored for the subsequent development of PCR confirmed SARS-CoV-2 infection[23]. This follow-up provided the first indication that SARS-CoV-2-specific T cells may be associated with protection against re-infection, as individuals with high numbers of reactive T cells detected using the RUO version of the T-SPOT.*COVID* test were significantly less likely to develop a PCR confirmed SARS-CoV-2 infection during the follow-up period. These preliminary findings correlate with primate studies which demonstrated that the depletion of T cells in monkeys that had recovered from SARS-CoV-2 resulted in re-infection upon re-exposure to the virus, even if SARS-CoV-2 specific antibody responses remained intact. In contrast, monkeys that retained SARS-CoV-2 specific T cells were able to successfully fight off re-infection[26]. In line with previous studies, this animal study also observed a decline in antibody responses following infection, leading the authors to conclude that T cells may be needed for long-term protection from the virus. These findings indicate that T cells play an essential role in immune responses against natural infection with SARS-CoV-2, and as such it is important that robust T cell responses should be induced in response to SARS-CoV-2 vaccines[27]. SARS-CoV-2 specific T cells have been detected in response to many of the current vaccine candidates[28,29,30], and the importance of detecting and monitoring these responses is becoming increasingly well recognised[27]. The research version of the T-SPOT.*COVID* test has been used to demonstrate a specific T cell response following vaccination. Preliminary data shows a significant difference between pre- and post-vaccination T cell spot counts (in-house data).

The T-SPOT.*COVID* assay is a simplified, standardised variant of the ELISPOT assay technique. ELISPOT assays detect and measure T cell responses by enumerating the number of T cells that are secreting cytokine in response to stimulation with antigens. ELISPOT assays are exceptionally sensitive since the target cytokine is captured directly around the secreting cell, before it is diluted in the supernatant, bound by receptors of adjacent cells or degraded. This makes ELISPOT assays much more sensitive than conventional ELISA assays[31,32,33,34]. Sensitivity is important when detecting T cell responses to SARS-CoV-2 since the frequency of T cells may be lower than for other viruses that induce T cell responses[35], and a variety of factors including age[22], severity of disease[24] and immunosuppression[36] have been linked to variability in the magnitude of SARS-CoV-2 specific T cell responses.

The test enumerates effector T cells responding to stimulation using two separate peptide pools derived from the SARS-CoV-2 Spike and Nucleocapsid proteins. The T cell response to each protein is measured in parallel in individual wells. T-SPOT.*COVID* antigen panels are designed as overlapping peptides spanning sequences of the Spike (COV-A) and Nucleocapsid (COV-B) proteins. This peptide design offers maximum epitope coverage for enhanced detection of T cell reactivity and no HLA restrictions. Antigenic formulations of 253 peptides covering the most immunogenic regions of the virus genome allows measurement of the breadth of immunity and ensures the impact of point mutations is minimised. Specificity to SARS-CoV-2 has been enhanced by removing potentially cross-reactive peptide sequences with high homology to other coronaviruses.

PRINCIPLE OF TEST

The immune response to infection with SARS-CoV-2 is mediated through both B cell and T cell activation. As part of the T cell response, T cells are sensitised to SARS-CoV-2 antigens designed to activate both CD4 and CD8 effector T cells, which then produce the cytokine interferon gamma (IFN-γ) when stimulated by these antigens[37,38]. The T-SPOT.*COVID* test uses the enzyme-linked immunospot (ELISPOT) methodology to enumerate SARS-CoV-2-sensitised T cells by capturing interferon-gamma (IFN-γ) in the vicinity of T cells from which it was secreted[39].

Peripheral blood mononuclear cells (PBMCs) are separated from a whole blood sample, washed and then counted before being added into the assay.

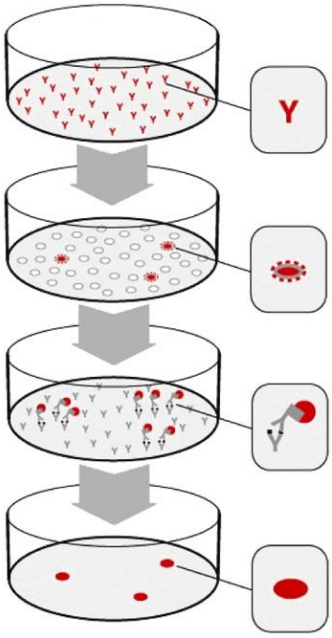
Isolated PBMCs (white blood cells) are placed into microtiter wells where they are exposed to a phytohemagglutinin (PHA) control (a mitogenic stimulator indicating cell functionality), nil control, or two separate panels of SARS-CoV-2 antigens derived from Spike and Nucleocapsid proteins respectively. The PBMCs are incubated with the antigens to

allow stimulation of any sensitised T cells present.

Secreted cytokine is captured by specific antibodies on the surface of the membrane, which forms the base of the well, and the cells and other unwanted materials are removed by washing. A second antibody, conjugated to alkaline phosphatase and directed to a different epitope on the cytokine molecule, is added and binds to the cytokine captured on the membrane surface. Any unbound conjugate is removed by washing. A soluble substrate is added to each well; this is cleaved by bound enzyme to form a (dark blue) spot of insoluble precipitate at the site of the reaction.

Evaluating the number of spots obtained provides a measurement of the abundance of effector T cells in the peripheral blood primed against SARS-CoV-2. These principles behind the T-SPOT test platform are described in Figure 1 below.

***Figure 1**: Principles of the T-SPOT assay system. For illustration only, refer to Section 6, Instructions for Use for detailed procedural instructions.*



First a blood sample is collected from the patient. The white blood cells are separated and then put into the wells of a 96-well microtiter plate. The plates are pre-coated with high affinity antibodies (Y) to Interferon-gamma (IFN-γ), a cytokine released by effector T cells when fighting SARS-CoV-2 infection.

Antigens from the SARS-CoV-2 virus are then added to the wells with the white blood cells to provoke interferon gamma (IFN-γ) secretion from any effector T cells primed against SARS-CoV-2.

Antigen specific responding T cells ( ) release the cytokine ( ), which is captured in the immediate vicinity of the T cells, by the antibodies lining the bottom of the well.

After a short incubation time the wells are washed, removing the antigens and cells from the wells. A conjugated second antibody ( ) is then added which binds to the interferon gamma (IFN-γ) secreted by the T cells (and captured by the primary antibody).

A substrate is then added which produces spots ( ) where the interferon gamma (IFN-γ) was secreted by T cells. The number of spots is counted.

3. REAGENTS & STORAGE

MATERIALS PROVIDED

T-SPOT.*COVID* COV.435/300 (Multi-use 12 x 8-well strip version) and COV.435/200 contains:

1. 1 microtiter plate: 96 wells, supplied as 12x 8-well individual strips in a separate frame (COV.435/300) or 12x8 wells in a single plate (COV.435/200), coated with a mouse monoclonal antibody to the cytokine interferon gamma (IFN-γ).
2. 2 vials (0.8 mL each) Panel A (COV-A): contains Spike antigens, bovine serum albumin and antimicrobial agents.
3. 2 vials (0.8 mL each) Panel B (COV-B): contains Nucleocapsid antigens, bovine serum albumin and antimicrobial agents.
4. 2 vials (0.8 mL each) Positive Control: contains phytohemagglutinin (PHA), for use as a cell functionality control, bovine serum albumin and antimicrobial agents.
5. 1 vial (50 µL) 200x concentrated Conjugate Reagent: mouse monoclonal antibody to the cytokine interferon gamma (IFN-γ) conjugated to alkaline phosphatase.
6. 1 bottle (25 mL) Substrate Solution: ready-to-use BCIP/NBT^{plus} solution.
7. Package insert

Note: Solid 96-well microtitre plates used in the T-SPOT.*COVID* COV.435/200 and 8 well strips used in the COV.435/300 kit are single use items and should be used immediately once opened and not reused. Do not mix components between different kits.

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. If a component has an expiration date later than that on the (outer) kit box, do not retain and do not use that component with another kit; do not use any component in the kit, after the expiration date on the outer kit box.

Store opened kit components at 2-8 °C. Opened components for T-SPOT.*COVID* (COV.435/300) must be used within

8 weeks of opening and for T-SPOT.*COVID* (COV.435/200) within 4 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.**

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® CPT™ or heparinised tubes.
4. T-Cell *Xtend*® reagent - whole blood samples stored at room temperature (18 – 25 ºC) between 0 and 32 hours post venipuncture, can be processed with the use of T-Cell *Xtend* reagent.
5. Ficoll® (if not using CPT tubes).
6. 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.
7. 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 analyser.
8. A humidified incubator capable of 37 ± 1 °C with a 5 % CO_2 supply.
9. An automatic microtiter plate washer or an 8 channel or stepper pipette to manually wash plates.
10. Adjustable pipettes to cover a range of volumes from 1-1000 µL (such as four pipettes capable of delivering volumes of 1-10 µL, 2-20 µL, 20-200 µL and 100-1000 µL) and sterile pipette tips.
11. Sterile PBS solution: such as GIBCO® 1x D-PBS (Life Technologies; catalogue number 14040-133).
12. Distilled or deionised water.
13. 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.
14. Sterile cell culture medium such as GIBCO AIM V® (Life Technologies; 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 the T-SPOT.*COVID* test**. To avoid problems with contaminated media, it can be helpful to dispense bottles of AIM-V or RPMI 1640 into smaller aliquots.

4. WARNINGS & PRECAUTIONS

- For *in vitro* diagnostic use only.
- For professional use only.
- Operators should be trained in the assay procedure and be sure they understand the instructions for use before performing the assay.
- Read the assay instructions carefully before use. Deviations from the instructions for use in this package insert may yield erroneous results.
- Care should be taken when handling material of human origin. All blood samples should be considered potentially infectious. Handling of blood samples and assay components, their use, storage and disposal should be in accordance with procedures defined in appropriate national, state or local biohazard and safety guidelines or regulations.
- Care should be taken when working with chemicals. All chemicals should be considered potentially hazardous. A material safety data sheet for the kit is available from Oxford Immunotec.
- Discard unused reagents and biological samples in accordance with Local, State and Federal regulations.
- The correct number of PBMCs must be added to each well. Failure to do so may lead to an incorrect interpretation of the result.
- Do not mix components from different kit lots.
- Observe aseptic technique to avoid contaminating the reagents, assay wells, cell suspensions and cell culture media.
- Variation to the stated pipetting and washing techniques, incubation times and/or temperatures may influence the actual results obtained and should be avoided.
- Blood should be collected and processed as soon as possible.
- Store and transport blood samples to the laboratory at room temperature (18-25 °C). Do not refrigerate or freeze whole blood samples.
- Failure to adhere to the recommended incubation times and temperatures may lead to an incorrect interpretation of the results.
- Indentations in the membrane caused by pipette or well washer tips may develop as artifacts in the wells which could confuse spot counting.

WARNINGS & PRECAUTIONS SPECIFIC TO THE USE OF T-CELL *XTEND* REAGENT

- The T-Cell *Xtend* reagent has not been evaluated for uses other than with the T-SPOT test platform.

- For *in vitro* diagnostic use only.
- For professional use only.
- Do not use reagent beyond the expiration date.
- Observe aseptic technique when using this product to avoid contamination of the reagent.
- Do not use Cell Preparation Tubes (CPT, Becton Dickinson) or blood collection tubes containing the anticoagulant ethylenediaminetetraacetic acid (EDTA) with the T-Cell *Xtend* reagent.
- Add the T-Cell *Xtend* reagent to the whole blood prior to sample processing.
- Do not dilute or add other components directly to the T-Cell *Xtend* reagent.
- Only use single-use containers for venous blood specimen collection.
- Do not mix different reagent lots.

5. SPECIMEN COLLECTION & HANDLING

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

1. Whole blood samples should be maintained between 18 °C and 25 °C until processed.
2. 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.**
3. Typically, for an immunocompetent patient, sufficient PBMCs to run the assay can be obtained from venous blood samples according to the following guidelines:

   One 8 mL or two 4 mL tubes (CPT) or one lithium heparin 6 mL tube.

   A patient's PBMCs can be pooled, if necessary to obtain sufficient PBMCs from multiple tubes of blood which were collected and processed concurrently.
4. When using the T-SPOT.*COVID* test **without the use of T-Cell *Xtend* reagent** blood samples should be processed within 8 hours of collection. Samples may be collected into either sodium citrate or sodium heparin Vacutainer CPT tubes (Becton Dickinson) with PBMCs separated in the tube using the manufacturer's instructions. Alternatively, blood samples may be collected into lithium heparin tubes with PBMCs being subsequently separated using standard separation techniques such as Ficoll-Paque® or alternative methods to purify the PBMC fraction. Blood collection tubes containing the anticoagulant EDTA should not be used.

a. For CPT blood collection tubes, centrifuge 8 mL CPT tubes at 1600 RCF(g) for 28 minutes or 4 mL CPT tubes at 1800 RCF (g) for 30 minutes at room temperature (18-25 °C).

b. 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).

   ***Note**: Review the manufacturer's instructions before using either CPT tubes or Ficoll-Paque. Ensure the tubes are centrifuged at the correct speed. The speeds given above are in g or Relative Centrifugal Force (RCF). This is not the same as Revolutions Per Minute (RPM). If the centrifuge will only measure speed in RPM, then convert to the recommended RCF value by measuring the rotor radius and using a conversion table. Leucosep tubes (Greiner Bio-One) offer a time-saving approach to density gradient separation. The tubes contain a porous barrier that enables the blood sample to be poured onto the density gradient separation medium, thereby eliminating the need to gently layer on the sample.*

5. When using the T-SPOT.*COVID* test **with T-Cell *Xtend* reagent** blood samples should be collected into lithium heparin tubes. Vacutainer CPT tubes and blood collection tubes containing the anticoagulant EDTA should not be used. The T-Cell *Xtend* reagent should be added prior to PBMC separation using standard separation techniques. Whole blood samples should be stored at room temperature (18-25 ºC) between 0 and 32 hours post venipuncture with the use of T-Cell *Xtend* reagent.

   If the T-Cell *Xtend* reagent is to be used, immediately before cell separation remove the cap from the blood collection tube and add 25 µL of the T-Cell *Xtend* reagent solution per mL of blood sample. Replace the cap and invert blood collection tube gently 8 to 10 times to mix. Incubate for 20 ± 5 minutes at room temperature (18-25 °C) and then proceed to isolate the PBMC layer using Ficoll density gradient centrifugation as presented in sections 4b, and 6 - 9. See the T-Cell *Xtend* reagent package insert for further details.
6. Collect the white, cloudy band of PBMCs using a pipette and transfer to a 15 mL conical centrifuge tube. Bring the volume to 10 mL with cell culture medium. **Cell culture media for the washing steps should be pre-warmed to 37 °C before contact with PBMCs.**

   Circulating factors in whole blood samples are known to interfere with whole blood interferon gamma tests, e.g., rheumatoid factor, heterophilic antibodies, and pre-existing amounts of interferon gamma. The separation and washing of the PBMCs enables removal of these potentially interfering substances prior to performing the assay.

Note: After centrifugation, PBMCs should be extracted using a large bore (e.g. 1 mL) pipette tip, by immersing the pipette tip into the PBMC layer. This cloudy layer should be carefully aspirated and transferred to a sterile conical tube for the wash steps. Ensure that all of the cloudy PBMC layer is collected. It is better to take more 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 tip. If this happens transfer the cells already in the tip into a centrifuge tube and then use a new tip to transfer the remaining PBMCs. A variety of media can be used for washing the cells during these steps 3-5; both AIM V and RPMI 1640 have been used successfully and are recommended.

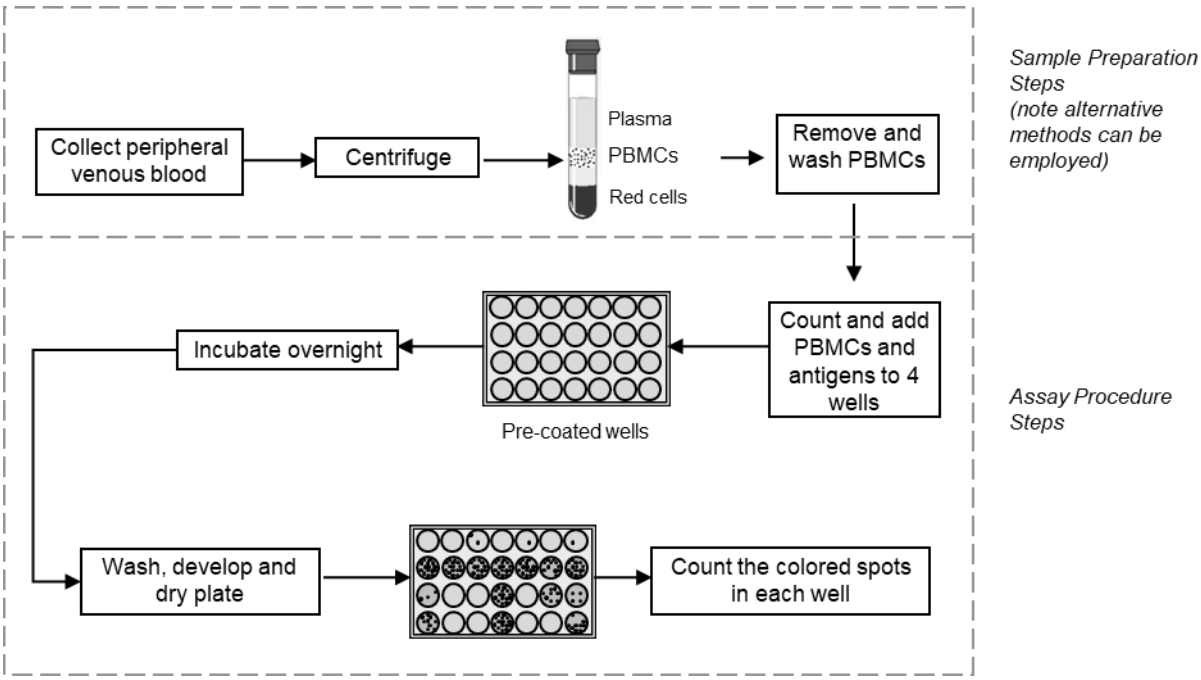
7. Centrifuge at 600 RCF (g) for 7 minutes. Pour off the supernatant and resuspend the pellet in 1 mL medium.
8. Bring the volume to 10 mL with fresh medium and centrifuge at 350 RCF (g) for 7 minutes.
9. Pour off the supernatant and resuspend the pellet in 0.7 mL cell culture medium. **The serum-free medium AIM V has been used successfully and is strongly recommended.**

Note: *Steps 2-7 should be performed in a BLII cabinet to protect the user and prevent contamination of samples.*

6. INSTRUCTIONS FOR USE

A full T-SPOT.*COVID* test plate will process 24 patient samples. The assay is typically carried out on the afternoon of one day and the morning of the following day, to allow the 16-20 hr incubation step to take place overnight. If this timing is used, then on the afternoon of day 1 the blood samples are processed to prepare the PBMCs for the assay and the assay is initiated by adding the PBMCs and antigens to the assay plate and placing the plate into the incubator. On day 2, the plate is removed from the incubator and the development steps are performed and the plate is read. The time to process a full plate is approximately 3 hours of elapsed time (actual hands-on labor time will be less due to the centrifugation steps) on day 1 and 30 minutes of labor time (not including the 1 hour incubation of the secondary antibody and time for plate drying) on day 2. The procedure for conducting the test is summarised in Figure 2 and further described below:

Figure 2*: Diagram illustrating the main steps required to perform the T-SPOT.COVID* test*. Note that not all 96 wells of the plates are shown in the illustration.*



REAGENT PREPARATION

1. The vials of SARS-CoV-2 Spike antigens (Panel A), SARS-CoV-2 Nucleocapsid antigens (Panel B) and the Positive Control are supplied ready to use.
2. Prepare a 1:200 dilution working Conjugate Reagent solution. Calculate the volume of working Conjugate Reagent solution required. Conjugate Reagent can be made to working strength and stored at 2-8 °C up to six weeks before using in the assay.

Note: Each patient sample uses 4 wells. 50 µL diluted Conjugate Reagent will be added to each well. Thus, for one strip (2 samples, 8 wells), prepare 500 µL of working strength solution by adding 2.5 µL of concentrated Conjugate Reagent to 497.5 µL PBS. For one 96-well plate (24 samples), prepare 5 mL of working strength solution by adding 25 µL of concentrated Conjugate Reagent to 497 5µL PBS.

3. The Substrate Solution is supplied ready to use. Prior to removing the plate from the incubator (day 2) remove the substrate solution from storage and allow to equilibrate to room temperature.

CELL COUNTING AND DILUTION

The T-SPOT.*COVID* test requires 250,000 ± 50,000 PBMCs per well. A total of four wells are required for each patient sample; thus 1 x 10^6 PBMCs are required per patient. The number of SARS-CoV-2 T cells in the specimen is normalised 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 analyser.
2. Briefly, for manual counting with a Neubauer hemocytometer using Trypan Blue, add 10 µL of the final cell suspension to 40 µL 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.

   Note: Care should be taken to ensure that the cell suspension is well mixed immediately prior to removal of aliquots 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.
3. Calculate the concentration of PBMCs present in the stock cell suspension.

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

   Note: 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 *test results.*

PLATE SET UP AND INCUBATION

The T-SPOT.*COVID* test 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 (COV-A) (Spike)
- ◯ Panel B (COV-B) (Nucleocapsid)
- ◯ Positive

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. For COV.435/300; each strip will process 2 samples. Use only the numbers of strips that you require. Seal remaining strips in the foil pouch along with the silica gel pouch. The remaining strips must be used within eight weeks of first opening the pouch provided the strips are stored at 2-8 °C during the period.

T-SPOT.*COVID* is a test 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. For COV.435/300 remove the required, 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 ºC.

   Note: Clip the strips to be used into an empty plate frame fitted with an undercover and lid. Frames, covers and lids should be retained and reused.

2. Add in the Panels and the Controls;

   i. Add 50 µL AIM-V cell culture medium to each Nil Control well.
   ii. Add 50 µL Panel A solution to each well required.
   iii. Add 50 µL Panel B solution to each well required.
   iv. Add 50 µL 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.

3. To each of the 4 wells to be used for a patient sample, add 100 µL of the patient's final cell suspension (containing 250,000 PBMCs). Use a new tip for the addition of each individual patient's cells to avoid cross-contamination between wells. Take care not to contaminate adjacent wells, by passing liquid from one well to another if pipette tips are reused for multiple wells.

   Note: Ensure mixing (as in the Cell Counting & Dilution steps) before removal of each 100 µL aliquot.

4. Incubate the plate with the lid on in a humidified incubator at 37 °C with 5 % CO_2 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.

   Note: The CO_2 incubator must be humidified. Check the water dish has sufficient water to ensure a humid atmosphere is achieved.

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 time remove the Substrate Solution from the kit and allow to equilibrate to room temperature.

2. Add 200 µL PBS solution to each well. **Do not use PBS containing Tween® or other detergents, as this causes high background counts**.

   Note: Use freshly prepared or sterile PBS.

3. Discard the PBS solution. Repeat the well washing an additional 3 times with fresh PBS solution for each wash. An automated washer can be used for the washing steps.

   Note: For washing, a multi-channel pipette or a plate washer 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 excess left will further dilute the Conjugate Reagent.

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 µL working strength Conjugate Reagent solution to each well and incubate at 2-8 °C for 1 hour.

   Note: Use of a multi-channel pipette or stepper pipette is recommended. Care should be taken to ensure that the Conjugate Reagent is added to every well as the solution is clear and uncolored - therefore, it may be difficult to see into which wells it has been added.

6. Discard the conjugate and perform the four PBS washes as described in steps 2. and 3. above.

7. Add 50 µL 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 by standing the plate in a well ventilated area or in an oven at up to 37 °C.

   Note: 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.

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 'Reactive' or 'Non-Reactive'. **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 center and a more diffuse periphery. Non-specific artifacts that can occur are smaller, less intense and irregular in shape.**

Note: Spots can be counted directly from the well using a magnifying glass or stereomicroscope or from a digital image captured from a microscope, or plate imager.

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.

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 (see Figures 4a & b for typical results from the US clinical study).

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 ≥ 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[1]. Where the Positive Control spot count is < 20 spots, it should be considered as 'Invalid', unless either Panel A or Panel B are 'reactive 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.

RESULTS INTERPRETATION AND ASSAY CRITERIA

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

Results for the T-SPOT.*COVID* test 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 Reactive if (Panel A-Nil) and/or (Panel B-Nil) ≥ 8 spots.
- The test result is Non-Reactive if both (Panel A-Nil) and (Panel B-Nil) ≤ 4 spots. This includes values 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 the adaptive or cell-mediated immune response to recent or prior infection with SARS-CoV-2.

- **A 'Reactive' result indicates that the sample contains effector T cells sensitised to SARS-CoV-2.**
- **A 'Non-Reactive' result indicates that no effector T cells sensitised to SARS-CoV-2 were detected.**

The interpretation algorithm is described in the following Flow Diagram (Figure 3) and Tables 1-3. This algorithm also includes quality control criteria.

Figure 3 *– Algorithm Flow Diagram*

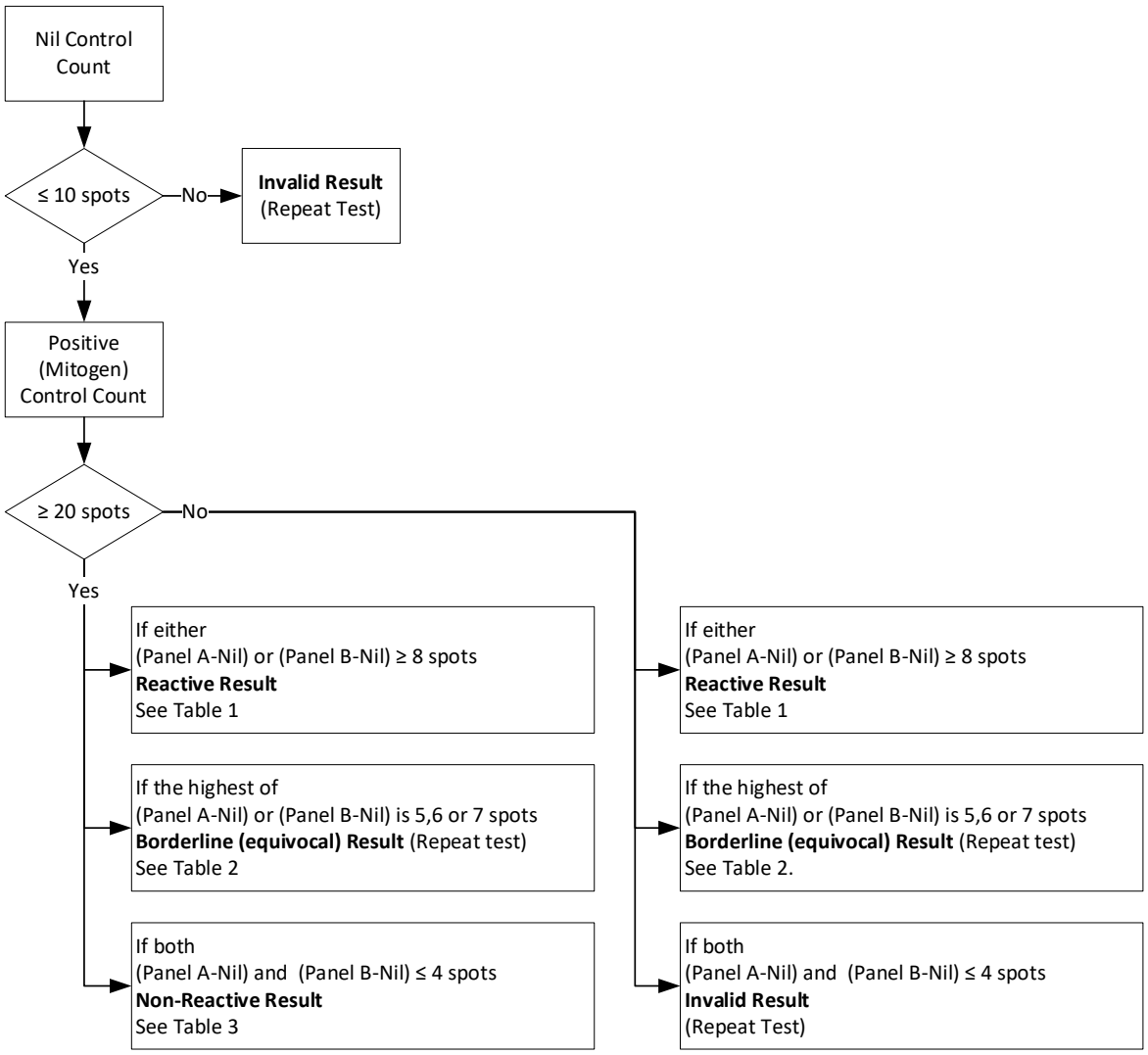


Table 1: *Reactive Interpretation: Either (Panel A minus Nil) or (Panel B minus Nil) ≥8 spots*

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

[†]*Note: The highest Panel-Nil spot count is to be used to determine the test outcome.*

Table 2: *Borderline (equivocal) Interpretation: The highest of (Panel A minus Nil) or (Panel B minus 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	**Borderline (equivocal)***
1	6, 7, or 8	**Borderline (equivocal)***
2	7, 8, or 9	**Borderline (equivocal)***
3	8, 9, or 10	**Borderline (equivocal)***
4	9, 10, or 11	**Borderline (equivocal)***
5	10, 11, or 12	**Borderline (equivocal)***
6	11, 12, or 13	**Borderline (equivocal)***
7	12, 13, or 14	**Borderline (equivocal)***
8	13, 14, or 15	**Borderline (equivocal)***
9	14, 15, or 16	**Borderline (equivocal)***
10	15, 16, or 17	**Borderline (equivocal)***
>10 spots	n/a	**Invalid****

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

Nil Control Well Count	Both Panel A and Panel B has the following number of spots	Result Interpretation
0	≤4	**Non-Reactive**
1	≤5	**Non-Reactive**
2	≤6	**Non-Reactive**
3	≤7	**Non-Reactive**
4	≤8	**Non-Reactive**
5	≤9	**Non-Reactive**
6	≤10	**Non-Reactive**
7	≤11	**Non-Reactive**
8	≤12	**Non-Reactive**
9	≤13	**Non-Reactive**
10	≤14	**Non-Reactive**
>10 spots	n/a	**Invalid****

** 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.*

7. LIMITATIONS

- Deviations from the instructions for use in this package insert may yield erroneous results.
- Incorrect performance of the assay may cause false reactive or false non-reactive responses.
- A false non-reactive result can be caused by incorrect blood sample collection or improper handling of the specimen, affecting lymphocyte function.
- The performance of the T-SPOT.*COVID* test, with or without the use of the T-Cell *Xtend* reagent, has not been adequately evaluated with specimens from individuals younger than age 18 years, in pregnant women, and in patients with hemophilia.
- A false reactive result may be obtained for the T-SPOT.*COVID* test when tested in subjects previously exposed to SARS-CoV-1 and other similar coronaviruses. Alternative tests would be required if these infections are suspected. This kit has been tested using currently available specimens. Performance with new mutations of SARS-CoV-2 have yet to be evaluated.
- Results from T-SPOT.*COVID* testing must be used in conjunction with each individual's epidemiological history, current medical status and results of other diagnostic evaluations.
- A non-reactive test result does not exclude the possibility of exposure to, or infection with, SARS-CoV-2. Patients with recent exposure to SARS-CoV-2 infected individuals exhibiting a non-reactive T-SPOT.*COVID* test result should be considered for retesting within 2 weeks or if other relevant clinical symptoms indicate possible infection.

- A reactive test result does not rule in a SARS-CoV-2 infection or COVID-19 disease and may be as a result of SARS-CoV-2 vaccination; other tests should be performed to confirm the diagnosis of COVID-19 disease such as PCR or antigen testing. A reactive test does not necessarily indicate immunity to SARS-CoV-2.
- Refrigerated and frozen samples are not recommended for use with T-SPOT.*COVID* test.

LIMITATIONS SPECIFIC TO THE USE OF T-CELL *XTEND* REAGENT

1. The T-Cell *Xtend* reagent has not been evaluated for uses other than with T-SPOT tests.
2. Do not refrigerate or freeze whole blood samples. Store and transport blood samples to the laboratory between 18-25 °C.
3. Any deviation from recommended procedures for pipetting, washing techniques, incubation times and/or temperatures may influence test results.

8. PERFORMANCE CHARACTERISTICS

The T-SPOT.*COVID* assay cutoff value was pre-determined during development using Receiver Operating Characteristic (ROC) curve analysis. Maximal discrimination between PCR confirmed positive individuals and those at a low risk of infection was found to be 6 spots. In addition a borderline zone of 5-7 was set to address test variation and uncertainty around the cut off.

Analytical Performance Characteristics

Interference from heterophilic antibodies or intrinsic interferon gamma (IFN-γ) in the blood sample is minimised by the separation and washing of the PBMC fraction from whole blood. This removes background amounts of interferon gamma (IFN-γ), other interfering plasma moieties, hemoglobin and any heterophilic antibodies.

Other cytokines than interferon gamma (IFN-γ) are expected to be produced by leucocytes, including IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, TNFα, IFN-α, and IFN-β. These were examined for cross-reactivity with the antibody pair used in the T-SPOT.*COVID* test. Results demonstrated that the antibody pair used in the T-SPOT.*COVID* test did not show evidence for cross-reactivity with other cytokines.

Intra-assay variability was analysed by comparing the T-SPOT.*COVID* test run on the same plate by the same operator. Experiments were carried out by three operators on nine plates which resulted in a range of % CVs representative of the inherent variation in the test. The range that was obtained for the high spot counts (210.4 ± 11.6) was between 2.2 % - 7.7 % CV (mean % CV = 4.4), mid-range spot counts (71.2 ± 8.5) gave a range of 6.6 % - 16.5 % CV (mean % CV = 11.0 %), whereas spot counts close to the cut-off (mean spot count = 5.7 ± 1.3) gave a mean % CV = 22.0 %.

Inter-assay precision data were collected, where three kit lots were used by three different operators to run the same three samples on six occasions. The coefficient of variation measured across the three samples, three operators and three lots was 3.7 % for samples giving a mean spot count of 210.4. For spot counts close to the T-SPOT.*COVID* test cut-off, the inter-assay variation was 25.0 %. For moderate spot levels, the mean %CV was 13.9 %. The results for the %CV were consistent for each of the batches tested.

Inter-operator reproducibility was assessed using three operators and one plate each from three kit batches. The variation observed between operators was 3.6 %-5.8 % CV.

Clinical Performance characteristics

A study was carried out, using the pre-determined cut off set at 6 spots (data on file), to evaluate the clinical performance of the T-SPOT.*COVID* test in PCR confirmed SARS-CoV-2 infection (using both asymptomatic and symptomatic subjects) to assess test performance in those with acute infection or convalescent subjects. Additionally, the test performance was evaluated in study subjects deemed at a lower relative risk of infection. All samples were tested with an anti-N IgG serology test (Abbott Architect 6R86-32 (COV2-IgG)) as a comparator with the T-SPOT.*COVID* test.

A total of 281 subjects were recruited into the study having met the inclusion criteria. Of these, 169 subjects were recruited into the PCR confirmed SARS-CoV-2 infection group (the positive cohort). Of these, one subject was excluded due to low cell recovery, and 17 due to missing serology results, leaving 151 available for analysis.

A total of 112 subjects were recruited into the Lower Risk cohort, of which confirmatory serology results were unavailable in 4 and 6 further subjects were excluded after a positive confirmatory serology test was returned. That left 102 subjects, of which one sample was excluded for low cell recovery, and one because of technical issues with the T-SPOT.*COVID* test. As a result, 100 lower risk subjects were included in the analysis.

The demographics of the PCR confirmed and lower risk cohorts are summarised below:

Cohort	PCR confirmed SARS-CoV-2 Infection	Lower relative risk of Infection
Number of subjects	168	100
Mean age (years) (sd)	50.5 (15.2) range 19-83	54.7 (15.7) range 18-87
% Male	38 7% (65/168)	36 0% (36/100)
Mean time since first positive PCR test (days) (range)	83.4 (0,249)	Not Applicable
% symptomatic	95 8% (161/168)	Not Applicable

Positive agreement amongst PCR-confirmed individuals

151 patients, identified as previously testing positive for SARS-CoV-2 by PCR were evaluated using the T-SPOT.*COVID* test and the anti-N IgG serology test. The time point from first PCR test result was recorded, which varied from 2 days to 249 days. No T-SPOT.*COVID* results were invalid in this cohort.

Table 4: Percent positive agreement with PCR over time incorporating all results from both T-SPOT.*COVID* and the anti-N IgG serology test using a 6 spot reactivity cut-off and ignoring the borderline zone (borderlines included)

Days since first PCR positive test	T-SPOT.*COVID*		Anti-N IgG	
	Positive agreement	95 % CI	Positive agreement	95 % CI
0-6	100.0 % (1/1)	2.5-100.0 %	0.0 % (0/1)	-
7-13	100.0 % (4/4)	39.8-100.0 %	25.0% (1/4)	6.3-80.6 %
14-30	92.9 % (13/14)	66.1-99.8 %	64.3 % (9/14)	35.1-87.2 %
31-60	92.0 % (69/75)	83.4-97.0 %	80.0 % (60/75)	69.2-88.4 %
Total ≤ 60	**92.6 % (87/94)**	**85.3-97.0 %**	**74.5 % (70/94)**	**64.4-82.9 %**
61-120	84.0 % (21/25)	63.9-95.5 %	76.0 % (19/25)	54.9-90.6 %
121-180	80.0 % (12/15)	51.9-95.7 %	20.0 % (3/15)	4.3-48.1 %
181-240	75.0 % (12/16)	47.6-92.7 %	0.0 % (0/16)	-
>240	100.0 % (1./1)	2.5-100.0 %	0.0 % (0/1)	-
Total >60	**80.7 % (46/57)**	**68.1-90.0 %**	**38.6 % (22/57)**	**26.0-52.4 %**

Table 5: Percent positive agreement with PCR over time incorporating all results from both T-SPOT.*COVID* and the anti-N IgG serology test, using only reactive and non-reactive results for the T-SPOT.*COVID* test (i.e. excluding those within the borderline zone .

Days since first PCR positive test	T-SPOT.*COVID*		Anti-N IgG	
	Positive agreement	95 % CI	Positive agreement	95 % CI
0-6	100.0 % (1/1)	2.5-100.0%	0.0 % (0/1)	-
7-13	100.0 % (4/4)	39.8-100.0 %	25.0 % (1/4)	6.3-80.6 %
14-30	100.0 % (12/12)	73.5-100.0 %	75.0 % (9/12)	42.8-94.5 %
31-60	95.7 % (67/70)	88.0-99.1 %	82.9 % (58/70)	72.0-90.8 %
Total ≤ 60	**96.6 % (84/87)**	**90.3-99.3 %**	**78.2 % (68/87)**	**68.0-86.3 %**
61-120	90.5 % (19/21)	69.6-98.8 %	85.7 % (18/21)	63.7-97.0 %
121-180	83.3 % (10/12)	51.6-97.9 %	16.7 % (2/12)	2.1-48.4 %
181-240	71.4 % (10/14)	41.9-91.6 %	0.0 % (0/14)	-
>240	100.0 % (1./1)	2.5-100.0 %	0.0 % (0/1)	-
Total >60	**83.3 % (40/48)**	**69.8-92.5 %**	**41.7 % (20/48)**	**27.6-56.8 %**

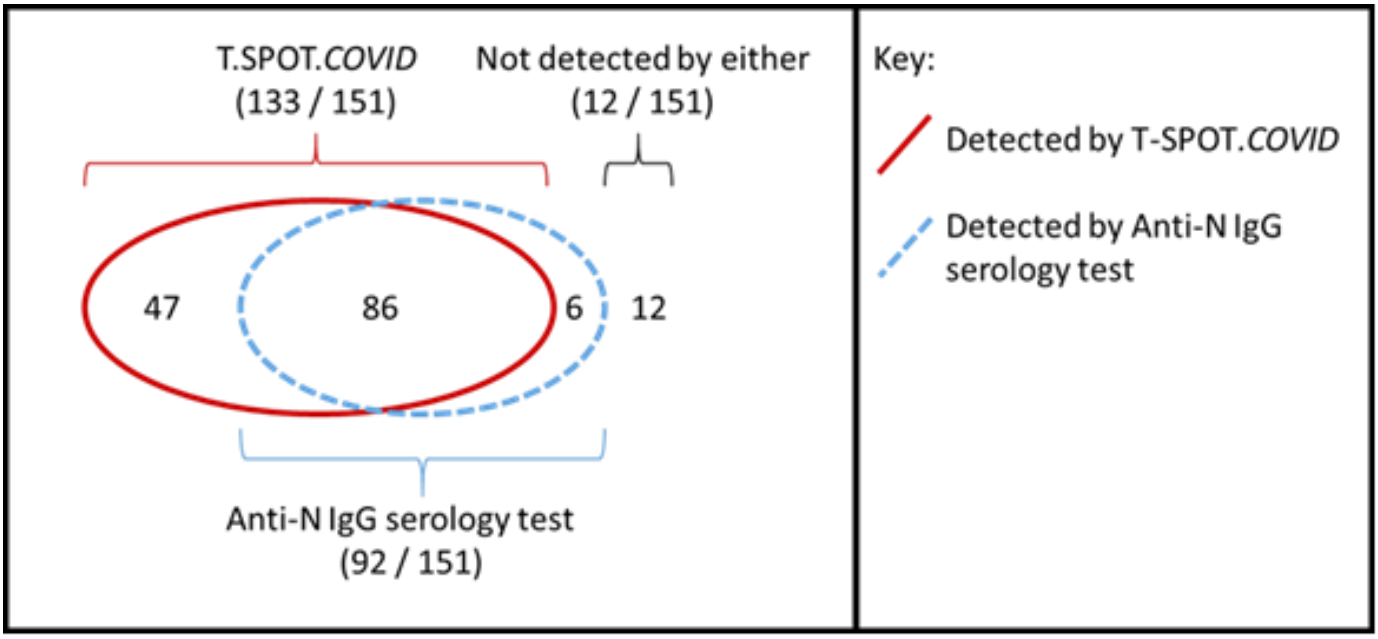
This data shows a percent positive agreement between the T-SPOT.*COVID* test and PCR of 92.6 % (96.6 % excluding borderline results) up to 60 days after a positive PCR test result. After this time point percent agreement falls slightly. For time points beyond 60 days from PCR result, positive agreement was 80.7 % (83.3 % using only determinate results).

Overall this data shows a percent positive agreement between the anti-N IgG serology test and PCR of 74.5 % up to 60 days after a positive PCR test result. After this time point percent agreement falls. For time points beyond 60 days from PCR results, positive agreement for anti-N IgG serology was 38.6 %.

The same data was analysed further to determine whether any patients testing negative with the anti-N IgG serology

test gave a reactive T-SPOT.*COVID* test result and vice versa. The data is represented by a Venn Diagram below (Figure 4) to show how T cell and serology data may complement one another.

Figure 4: Distribution of reactive/positive results between the T-SPOT.*COVID* test and the anti-N IgG serology test in PCR confirmed SARS-CoV-2 infected subjects (n= 151)



Of the 151 SARS-CoV-2 PCR positive samples T-SPOT.*COVID* tested negative in 18 specimens, whereas the anti-N IgG serology test was negative in 59 specimens. The T-SPOT.*COVID* test was positive in 79.7 % (47/59) of the specimens negative by the anti-N IgG serology test. The anti-N serology test was positive in 6 out of the 18 cases where the T-SPOT.*COVID* test was negative. This data shows a combination of both serology and ELISPOT T cell analysis is useful in determining SARS-CoV-2 infection status.

Negative Agreement amongst those at lower relative risk of infection

We enrolled a cohort of participants, in an endemic setting, but who were determined to have a lower relative risk of SARS-CoV-2 infection on the basis of: (i) absence of self-reported symptoms consistent with SARS-CoV-2 infection, (ii) no history of a prior positive SARS-CoV-2 PCR test (iii) no involvement in a vaccine trial and has not received a COVID-19 vaccine and (iv) a negative lateral flow anti-N serology test (Biohit SARS-CoV-2 IgM/IgG Antibody Test Kit) used as a primary screen at the time of enrollment and (iv) confirmation of a negative serology test using a lab serology test (anti-N IgG serology test (Abbott Architect 6R86-32 (COV2-IgG)).

Table 6: Percent negative agreement

	N	Positive	Negative	Negative agreement (%) (95 % CI)
Including borderline zone	100	3	97	97.0 % (91.5-99.4)
Excluding borderline zone	98	2	96	98.0 % (92.8-99.8)

97.0 % of T-SPOT.*COVID* test results (97/100) were below the 6 spot cut off (95 % confidence intervals 91.5-99.4 %). Two results were borderline (5 and 7 spots). When these results were excluded 98.0 % (92.8-99.8 % CI) of T-SPOT.*COVID* test results (96/98) were non-reactive. There were no invalid results.

Although we took all reasonable steps to ensure that this cohort was at low risk of infection, we cannot exclude the possibility that a proportion of this group had, or still have, an asymptomatic infection that was seronegative at the time of testing, but in whom the T-SPOT.*COVID* test was able to detect a T cell response.

9. EXPECTED VALUES

The range of spot counts observed in response to the Nil and positive control antigens and the SARS-CoV-2 antigens that have been observed in our clinical studies (see section 8 for details on clinical study cohorts) is shown in Figures 5a and b.

Figure 5a: Histogram of nil control responses from all study subjects (n = 251).

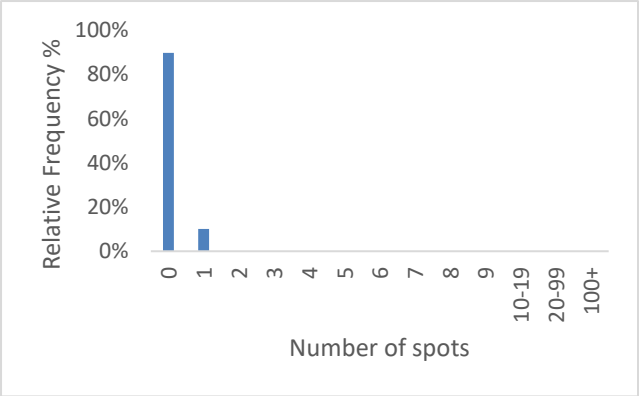
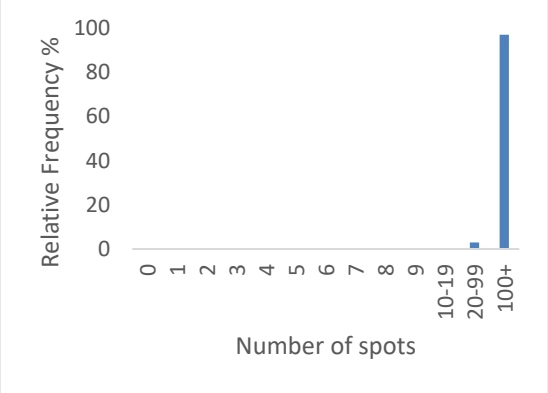


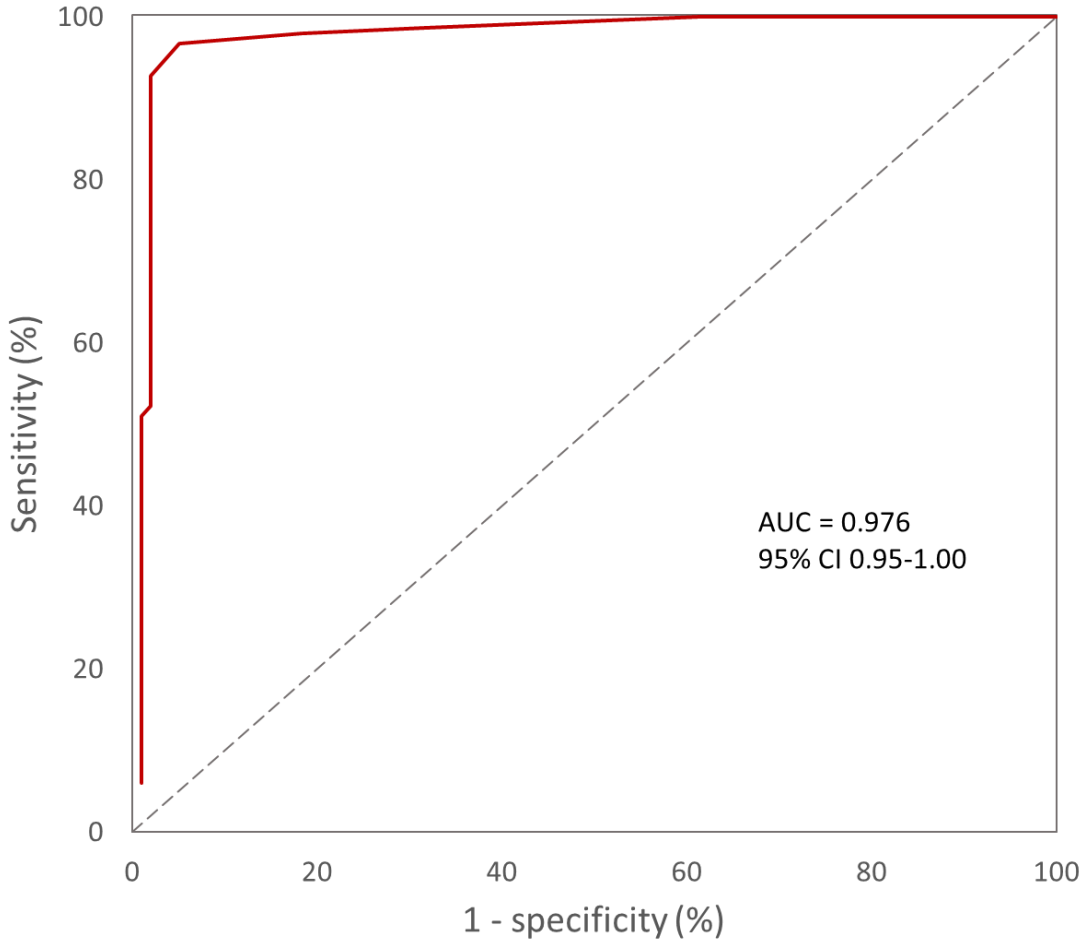
Figure 5b: Histogram of positive control responses from all study subjects (n= 251)



The vast majority of Nil control wells yielded zero spots and no spot counts greater than one were seen in the Nil control. Response to the positive control were robust and no instances of spot counts less than 20 were seen with the positive control.

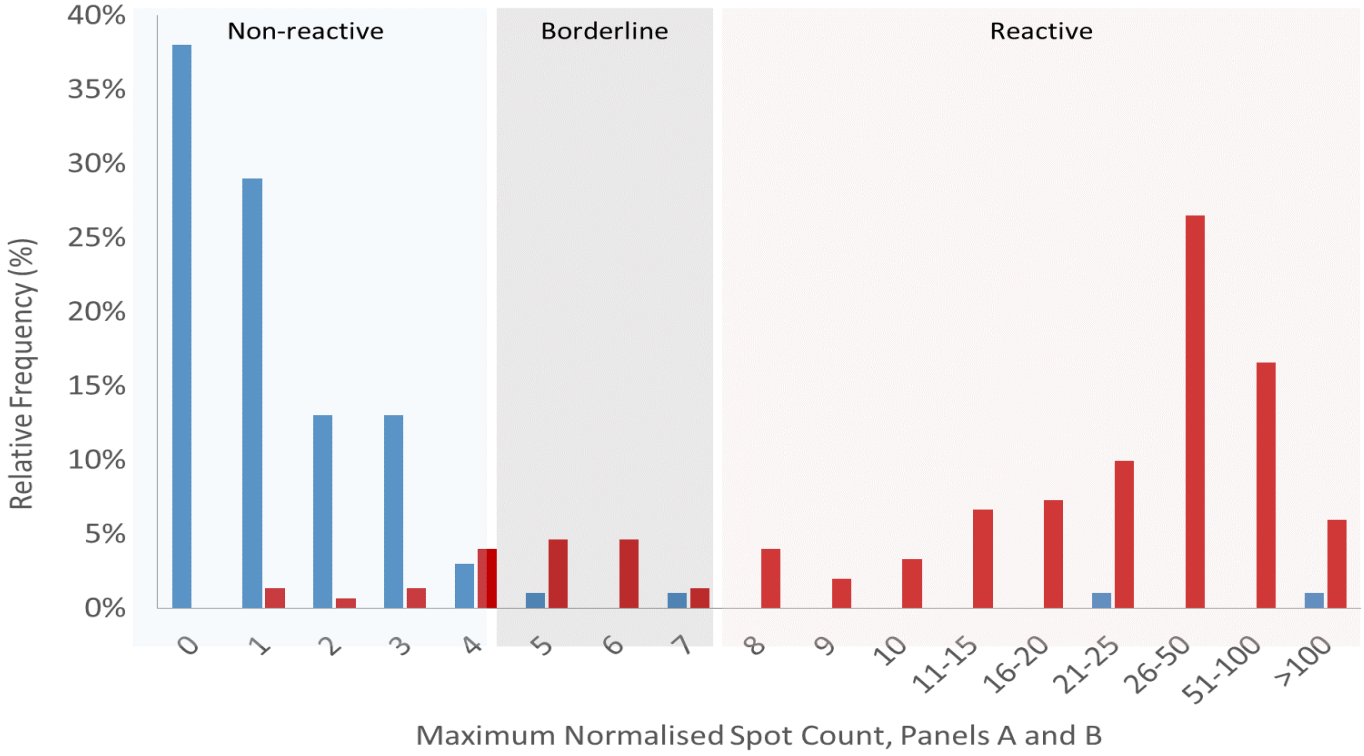
The assay cut off was confirmed during the clinical studies. Figure 6 shows the ROC curve create using data obtaining during the clinical studies. A cut-off of 6 spots provided the maximum separation between the two cohorts, validating the pre-selected level.

Figure 6: Receiver Operating Characteristic Curve created using the validation data generated from 151 PCR confirmed subjects (used to estimated sensitivity) and 100 subjects at lower relative risk of infection (used to estimate specificity).



The same data was also used to confirm the benefit of including a borderline zone as shown in Figure 7.

Figure 7: Graph showing distribution of spot counts observed with the T-SPOT.*COVID* test in US clinical studies with an overlay of the instructed test interpretation criteria. 'Max normalised spot count' is the maximum (panel minus nil) response of either Panel A or Panel B (n = 251). The relative frequency of different spot counts are shown for the lower risk clinical cohort (blue bars) and for the PCR confirmed cohort (red bars)



The majority of subjects in the lower risk cohort (blue bars) showed none to low level of reactivity with 96.0 % within the range of 0-4 spots. PCR confirmed subjects (red bars) showed high levels of reactivity with 23.2 % within 8-20 spots and a majority (58.9 %) >20 spots. The grey shaded region represents the borderline equivocal zone (5, 6 or 7 spots) where, as expected, there is an observed overlap between the spot count distributions of the two study cohorts. Any tests with results within this region should be repeated.

10. 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 ±1 spots from the ROC-determined assay cutoff of ≥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 immune status of the patient.

Invalid Results

Invalid results are uncommon and may be related to the immune status of the individual being tested. 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 without the use of the T-Cell *Xtend* reagent.
- Storage of blood outside the recommended temperature range prior to processing blood samples
- 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.

11. ABBREVIATIONS & GLOSSARY OF SYMBOLS

Abbreviations

AUC	Area Under Curve
BCIP/NBT	5-bromo, 4-chloro, 3-indoylphosphate/nitroblue tetrazolium
CDC	Centers for Disease Control and Prevention
CI	Confidence Interval
CLIA	Clinical Laboratory Improvement Amendments
CPT	Cell Preparation Tubes
CV	Coefficient of Variance
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
ELISPOT	Enzyme-Linked Immnunospot Assay
IFN-γ	Interferon gamma
IL	Interleukin
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PHA	Phytohemagglutinin
RCF	Relative Centrifugal Force
RoC	Receiver Operating Characteristic
RPM	Revolutions per minute
RT-PCR Reverse	Transcriptase PCR
TNF	Tumor Necrosis Factor

Glossary of Symbols

IVD	*In vitro* Diagnostic Medical Device
	Use by/Expiration date (Year-Month-Day)
LOT	Lot number
REF	Catalogue number
⚠	Attention, see instructions for use
	Date of manufacture
	Manufacturer
	Temperature limitation/Store between
	Consult instructions for use
EC REP	EU Authorised Representative

BS EN ISO 15223-1:2016

The symbols used for the T-SPOT.*COVID* test comply with the international standard ISO 15223-1:2016; 'Medical devices – Symbols to be used with medical device labels, labelling and information to be supplied'.

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