T-SPOT®.CMV

Oxford Immunotec

An aid in the assessment of anti-CMV cell-mediated immunity

PACKAGE INSERT

For *In Vitro* Diagnostic Use

This Package Insert covers use of:

The T-SPOT®.*CMV* test kit

Table of contents

Intended Use

The T-SPOT.*CMV* test is an *in vitro* diagnostic test intended to be used to assess a patient's level of anti-CMV cell-mediated immunity. The T-SPOT.*CMV* test is not intended for use in determining CMV infection and should not be used to include or exclude CMV infection.

Introduction

The T-SPOT.*CMV* test is a simplified variant of the ELISPOT assay technique. 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[1]. The T-SPOT.*CMV* test is designed for the detection of effector T cells that respond to stimulation by antigens specific for cytomegalovirus (CMV) by releasing cytokines. The test enumerates individual activated T cells and is suitable for use with patients regardless of age, sex, ethnicity, therapy or immune status.

Principles of the Procedure

Peripheral blood mononuclear cells (PBMCs) are isolated from a whole blood sample and washed to remove any sources of background interfering signal. The PBMCs are then counted so that a standardised cell number is used in the test. This ensures that even with low T cell titres due to weakened immune systems (the immunocompromised and immunosuppressed) there are adequate numbers of PBMCs added to the microtitre wells.

Four wells are required for each sample:

1. Nil Control to identify non-specific cell activation
2. Panel CMV-A: CMV-specific antigen, IE-1
3. Panel CMV-B: CMV-specific antigen, pp65
4. Positive Control: Mitogen solution containing phytohaemagglutinin (PHA, a known polyclonal activator[2]) to confirm PBMC functionality.

The PBMCs are incubated with the antigens to allow stimulation of any sensitised T cells present. Secreted cytokine in this case interferon gamma (IFN-γ), is captured by specific antibodies on the membrane, which forms the base of the well, and the PBMCs 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 spot of insoluble precipitate at the site of the reaction. Each spot represents the footprint of an individual cytokine-secreting T cell, and evaluating the number of spots obtained provides a measurement of the abundance of CMV-sensitive effector T cells in the peripheral blood (Figure 1).

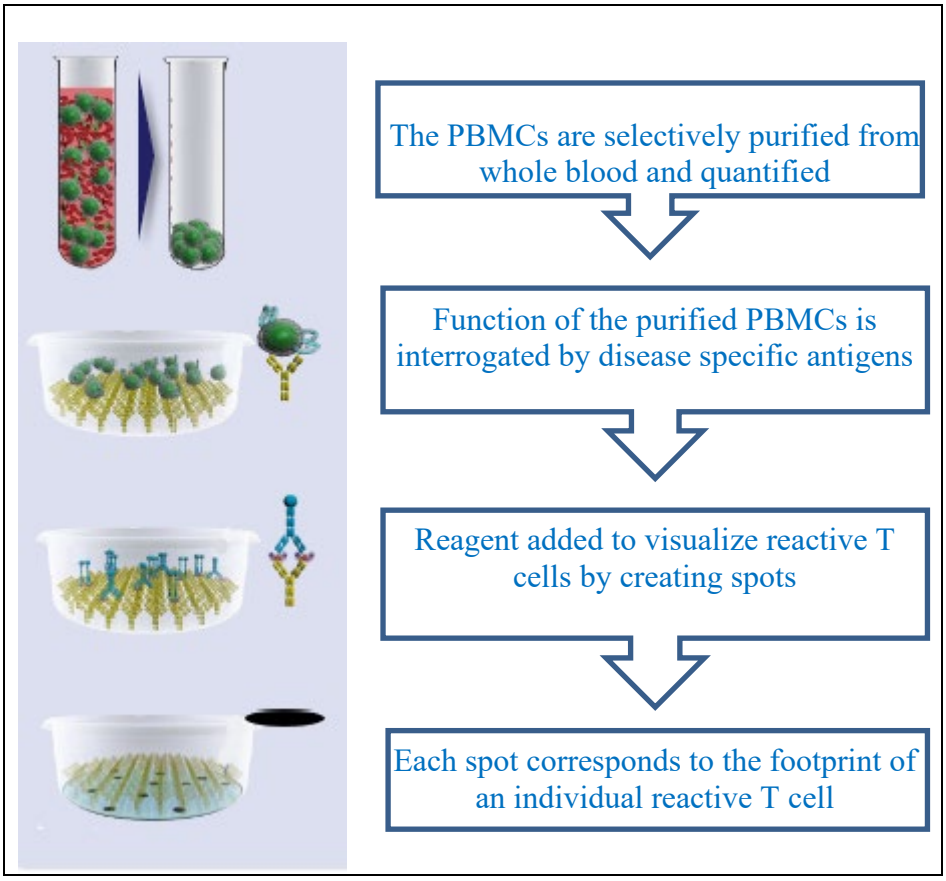


Figure 1. The T-SPOT.CMV test principle.

Limitations

- For *in vitro* diagnostic use only.
- For professional use only.
- Do not mix components from different kit lots.
- Read the test instructions carefully before use.
- Observe aseptic technique to avoid contaminating the reagents, test 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.
- A cell separation method needs to be validated by the laboratory performing the test.
  For the density gradient separation method blood should be collected and progressed into the T-SPOT.*CMV* test within 8 hours. This time limitation may be overcome by using the T-Cell *Xtend*® reagent (available from Oxford Immunotec). When the
  T-Cell *Xtend* reagent is used with the T-SPOT.*CMV* test, the sample storage time is increased to 32 hours.
  Alternative methods of cell isolation can be used such as magnetic bead selection, which allows removal of granulocytes from blood samples, and therefore samples stored for up to 32 hours from collection can be used.
- Store and transport blood samples to the laboratory at 15-25 °C. Do not refrigerate or freeze whole blood samples.
- The T-SPOT.*CMV* test results should be used and interpreted only in the context of the overall clinical picture.

Safety Warnings and Precautions
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 biohazard safety guidelines or regulations.

Care should be taken when working with chemicals. All chemicals should be considered potentially hazardous.

Materials Provided
The T-SPOT.*CMV* kit:

1. 1 microtitre plate: 96 wells, supplied as a solid 96-well plate or 12 x 8-well strips in a frame, coated with a mouse monoclonal antibody to the cytokine, IFN-γ
2. 2 vials (0.8 mL each) Panel CMV-A, IE-1 solution
3. 2 vials (0.8 mL each) Panel CMV-B, pp65 solution
4. 2 vials (0.8 mL each) Positive Control: a mitogen solution, contains phytohaemagglutinin (PHA), for use as a cell functionality control
5. 1 vial (50 µL) 200 x concentrated Conjugate Reagent: mouse monoclonal antibody to the cytokine IFN-γ conjugated to alkaline phosphatase
6. 1 bottle (25 mL) Substrate Solution: ready to use BCIP/NBT^{plus} solution.

Storage
Store all components of the kit at 2-8 °C.
Avoid prolonged exposure of the Substrate Solution to light.

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

Store opened kit components at 2-8 °C. Opened components must be used within 8 weeks of opening.

Equipment and Materials Required but Not Provided

1. 8-well strip plate frame (available from Oxford Immunotec) if a strip format plate used.
2. Class II microbiological cabinet (recommended).
3. Heparinised blood collection tubes.
4. Reagents and equipment required for cell isolation from whole blood.
5. Equipment and reagents to enable counting of PBMCs; such as a haematology analyser for automated counting, Trypan Blue and a haemocytometer for manual counting using a microscope or other methods.
6. A humidified incubator capable of 37 ± 1 °C with a 5 % CO_2 supply.
7. A microtitre plate washer or equipment to manually wash plates.
8. Pipettes and sterile pipette tips.
9. Sterile D-PBS solution: such as GIBCO® 1x D-PBS (Invitrogen; product code 14040-091).
10. Distilled or deionised water.
11. A means of reading the plate such as a microscope, digital microscope, magnifying glass or plate imager.
12. Sterile cell culture medium such as GIBCO AIM V® (available from Oxford Immunotec as 50 mL bottle: product code A18398SA and 500 mL bottle: product

code A18398DJ or Invitrogen; product code 31035-025). The use of this serum free medium for the incubation step is strongly recommended.
RPMI 1640 (Invitrogen; product code: 21875-034) 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.*CMV* test.

Reagent Preparation

1. Microtitre Plate. The T-SPOT.*CMV* microtitre plate is supplied ready to use. Remove the plate from storage and allow to equilibrate to room temperature.
2. The vials of Panel CMV-A are supplied ready to use.
3. The vials of Panel CMV-B are supplied ready to use.
4. The vials of Positive Control are supplied ready to use.
5. Prepare a 1:200 dilution working Conjugate Reagent solution. Calculate the volume of working Conjugate Reagent solution required and prepare immediately prior to use.
6. The Substrate Solution is supplied ready to use. Remove from storage and allow to equilibrate to room temperature.

Procedure

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

Sample Collection and Preparation

Individual users should validate their procedures for collection of PBMCs, enumeration of PBMCs and choice of suitable media to support T cell functionality during the primary incubation stage of the test. Typically sufficient PBMCs to run the test can be obtained from venous blood samples according to the following guidelines:

- Adults and children ≥10 years old: 12 mL of whole blood collected in lithium or sodium heparin tubes (*e.g.* 2 x 6 mL collection tubes)*
- Children ≥2 to <10 years old: 6 mL of whole blood collected in lithium or sodium heparin tube
- Children <2 years old: 2 mL of whole blood collected in lithium or sodium heparin paediatric tube.

**Note: In populations where cell recovery might be problematic (e.g. hematopoietic stem cell transplant patients), an additional tube of blood should be collected.*

If density gradient centrifugation method is used, blood samples must be stored at room temperature and assayed within 8 hours of blood collection or within 32 hours if treated with the T-Cell *Xtend* reagent.

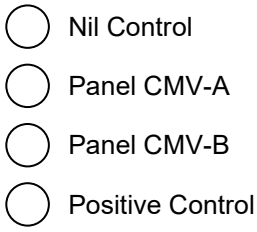
Alternative methods of cell isolation that remove granulocytes from PBMC fraction such as magnetic bead selection can be used for samples stored for up to 32 hours.

PBMCs should be suspended in AIM V medium and counted using a validated method of white blood cell count assessment. Cell suspension should be diluted to 2.5 $x10^6$ PBMCs / mL in AIM V medium. 100 µL of cell suspension containing 250,000 PBMCs will be added in four test wells, as described in the 'Plate Set Up and Incubation' section below.

If PBMC concentration is <2.0 $x10^6$ PBMCs/mL, the suspension should be centrifuged, PBMC pellet re-suspended in 400 µL AIM V medium and counted again. If the number of PBMCs isolated from a patient sample is less than 1,000,000 the highest possible cell concentration should be plated and a correction factor applied to the spot count obtained, as described in the 'Results Interpretation and Test Criteria' section.

Plate Set Up and Incubation

The T-SPOT.*CMV* 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 are arranged vertically on the plate as illustrated below.



Procedure	Notes
1. Remove the plate from the packaging and allow to equilibrate to room temperature.	1. If a strip plate format is used, remove the required number of strips only, return the remainder to storage. Clip the strips to be used into an empty plate frame fitted with an under cover and lid. Frames, covers and lids should be retained and reused.
2. Each patient sample requires the use of 4 individual wells: (i) Add 50 µL AIM V culture medium to each Nil Control well (ii) Add 50 µL Panel CMV-A solution to each well required (iii) Add 50 µL Panel CMV-B solution to each well required (iv) Add 50 µL Positive Control solution to each cell functionality control well.	2. Do not allow the pipette tip to touch the membrane. Indentations in the membrane caused by pipette tips may cause artefacts in the wells. It may be necessary to gently tap the plate to ensure that the solutions cover the membrane at the base of each well. Vigorous agitation should be avoided to minimize cross-contamination of the antigens between 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.	3. Pipette the cell suspension gently up and down to ensure thorough mixing before removal of each 100 µL aliquot. It is recommended that a new tip is used for every addition of each patient's PBMCs to avoid cross-contamination between the 4 wells. If plating 250,000 PBMCs per well is not possible, the cell suspension should be plated undiluted. Plating 75,000 - 250,000 is acceptable and a correction factor should be applied to normalise spot count obtained to 250,000 (see section 'Results Interpretation and Test Criteria').
4. Incubate the plate in a humidified incubator at 37 °C with 5 % CO_2 for 16-20 hours.	4. Avoid disturbing the plate once in the incubator. Do not stack plates as this may lead to uneven temperature distribution and ventilation. Failure to adhere to the recommended incubation time and conditions may lead to an incorrect interpretation of the result. Check the incubator contains sufficient water to maintain humidity for the incubation period.

Spot Development and Counting

During the plate washing and development stages, do not touch the membrane with pipette tips or automated well washer tips. Indentations in the membrane caused by pipette or well washer tips may develop as artefacts in the wells, which could interfere with the spot counting.

Procedure	Notes
1. Remove the plate from the incubator and discard the cell culture medium.	1. At this time remove the Substrate Solution from the kit and allow to equilibrate to room temperature.
2. Add 200 µL D-PBS solution to each well.	
3. Discard the D-PBS solution. Repeat the well washing a further 3 times with fresh D-PBS solution for each wash.	3. Discard all D-PBS from the final wash step by inverting the plate on absorbent paper before proceeding.
4. Dilute concentrated Conjugate Reagent 200 fold in D-PBS to create the working strength solution.	4. Do not use D-PBS containing Tween® or other detergents, as this causes high background counts. Ensure that only a small excess (to allow for wastage) of working strength solution is prepared. For each 8-well strip (each well requiring 50 µL), make up 500 µL of working strength solution by adding 2.5 µL of concentrated Conjugate Reagent to 497.5 µL D-PBS. The conjugate dilution calculator on the CD included with each test kit can be used for this calculation.
5. Add 50 µL working strength Conjugate Reagent solution to each well and incubate at 2-8 °C for 1 hour.	5. Failure to adhere to the recommended incubation time may lead to an incorrect interpretation of the result.
6. Discard the conjugate and perform 4 D-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.	7. Failure to adhere to the recommended incubation time may lead to an incorrect interpretation of the result.
8. Wash the plate thoroughly with distilled or deionised water to stop the detection reaction.	
9. Allow the plate to dry by standing it in a well ventilated area or in an oven at up to 37 °C.	9. Spots become more visible as the plate dries. Allow 4 hours drying time at 37 °C or overnight 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 Test Criteria (see below).	

Results Interpretation and Test Criteria

An optimal number of PBMCs to be added to each of the 4 test wells is 250,000 (1,000,000 PBMCs are required per test).

- If the number of cells added per well was ≥ 75,000 and ≤ 250,000 PBMCs a correction factor must be applied to the spot count obtained in order to normalise spot count to 250,000 PBMCs. Specifically,
    - Factor = 250,000 / number of cells added per well
    - Number of Nil control spots per 250,000 PBMCs = obtained spot count * factor
    - Number of CMV-A spots per 250,000 PBMCs = obtained spot count * factor
    - Number of CMV-B spots per 250,000 PBMCs = obtained spot count * factor
    - Number of PHA spots per 250,000 PBMCs = obtained spot count * factor

- < 75,000 PBMCs added per well: insufficient cell number; a re-test is recommended.
- > 250,000 PMBCs should be diluted to 250,000 PBMCs per 100 µL (in total volume at least 400 µL).

Note, cell suspensions between > 250,000 and ≤ 300,000 can be plated without diluting
Refer to Oxford Immunotec's 'Spot Count Correction Factor Calculator' (CSCF-CMV-UK) for further instructions.

The T-SPOT.*CMV* test results are interpreted by subtracting the spot count in the Nil Control well from the spot count in CMV-A, CMV-B and PHA Panels. The number of spots is indicative of the strength of the cellular immune response to CMV.

Note, PBMC preparation step is described in the 'Sample Collection and Preparation' section.

Quality Control

A typical result would be expected to have few or no spots in the Nil Control. A Nil Control spot count in excess of 10 spots per 250,000 PBMCs should be considered as 'Indeterminate'. Another sample should be collected from the individual and tested.

A typical result would be expected to have greater than 20 spots or show saturation (too many spots to count) in the Positive Control well containing phytohemagglutinin (PHA) that serves as a cell functionality control.

In tests it was demonstrated that 100 % healthy individuals (n=94 tests), 98.6 % kidney transplant patients (n=146 tests) and 99.7 % haematopoietic cell transplant, HCT patients (n=290 tests) gave valid Nil controls (≤10 spots in the Nil control well).

In tests it was demonstrated that 100 % healthy individuals (n=94 tests), 97.3 % kidney transplant patients (n=146 tests) and 93.4 % HCT patients (n=290 tests) gave ≥20 spots in response to PHA.

Test Performance Characteristics

1) Quality Control Test

The T-SPOT.*CMV* test enumerates effector T cells sensitized to CMV antigens. The test performance was demonstrated by testing a cohort of 88 immunocompetent individuals confirmed to be serology-negative or serology-positive based on the CMV-specific immunoglobulin IgG assessment (Table 1).

As an indication of the analytical performance of the test, the overall concordance between serology and T-SPOT.*CMV* test results was calculated: 98.9 %:

- 48 out of 48 serology-negative samples gave low level response in the T-SPOT.*CMV* test: 100 % concordance
  39 out of 40 serology-positive samples gave high level response in the T-SPOT.*CMV* test: 97.5 % concordance.

		T-SPOT.*CMV* test		
		High level	Low level	**Total**
Serology	Positive	39	1	40
	Negative	0	48	48
	Total	39	49	**88**

Table 1. Differentiation between serology-negative (Sero-) and serology-positive (Sero+) individuals based on their cell responses measured in the T-SPOT.CMV test.

2) Reproducibility

The T-SPOT.*CMV* test reproducibility was assessed using samples that give spot counts within three spot count ranges: >70 spots, 20-70 spots and 5-15 spots. Percentage coefficient of variation (% CV) was calculated for the following parameters: intra-assay reproducibility, inter-assay reproducibility, inter-batch reproducibility, inter-operator reproducibility and inter-laboratory reproducibility (Table 2).

	High Spot Count (>70) %CV	**Mid Spot Count (20-70) %CV**	**Low Spot Count (5-15) %CV**
Intra-assay reproducibility	6.93 %	11.21 %	21.48 %
Inter-assay reproducibility	4.01 %	14.38 %	24.48 %
Inter-batch reproducibility	3.99 %	14.33 %	23.14 %
Inter-operator reproducibility	3.98 %	14.36 %	23.86 %
Inter-laboratory reproducibility	3.82 %	14.32 %	24.43 %

Table 2. Reproducibility values obtained for the T-SPOT.CMV test at a low, medium and high spot count ranges.

Clinical Performance

1) Solid Organ Transplantation

The anti-CMV cellular immunity was assessed in 15 Thymoglobulin-treated solid organ CMV-seropositive transplant recipients (renal transplant) at different time-points post-transplantation, between 4 and 22 weeks. The anti-CMV cellular responses were detected in 14/15 patients (93.3 %) using the T-SPOT.*CMV* test.

2) Haematopoietic Stem Cell Transplantation

The reconstitution of the anti-CMV cellular immunity was assessed in 63 CMV-seropositive haematopoietic stem cell transplant recipients using the T-SPOT.*CMV* test at 30, 60, 100 and 180 days post-transplant. The correlation between the T-SPOT.*CMV* test results and CMV reactivation events was assessed. T-SPOT.*CMV* tests were performed no longer than 30 days prior to the occurrence of CMV events.

Patients who demonstrated high level of anti-CMV cellular immunity (high spot count in the T-SPOT.*CMV* test) were at a lower risk of CMV reactivation over the next 30 days (Figure 2). It was demonstrated that the T-SPOT.*CMV* test can be used for assessing the patient's level of anti-CMV cellular immunity and provide an indication for the level of protection against CMV reactivation[3].

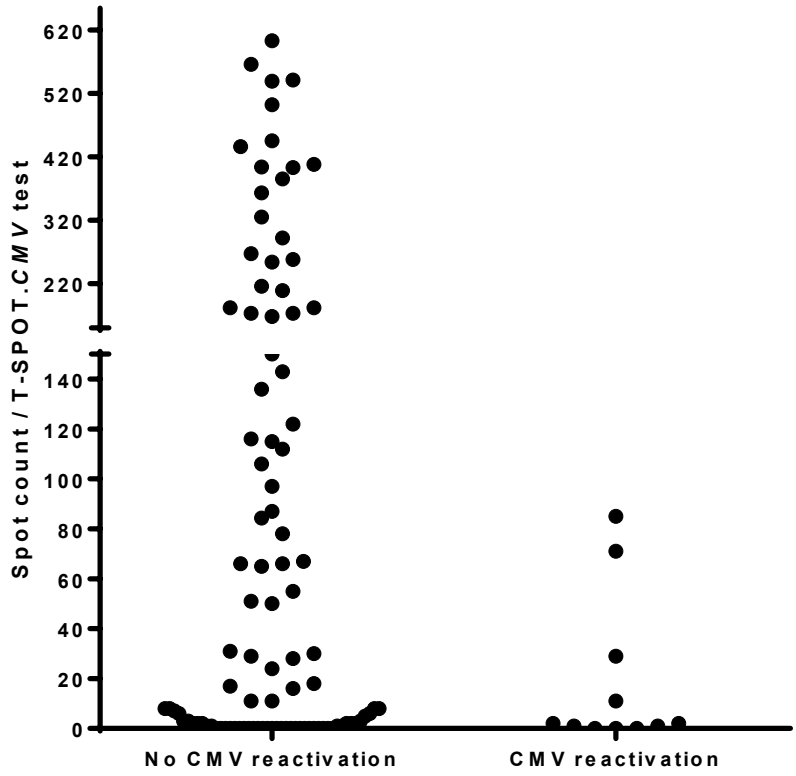


Figure 2. Anti-CMV cellular immunity assessed using the T-SPOT.CMV test in 63 haematopoietic stem cell transplant recipients at baseline, day 30, 60, 100 and 180 post-transplant.

References

1. See www.elispot-analyzers.de/english/science-elispot-assays.html
2. NCCLS Approved Guideline. *Performance of single Cell Immune Response Assays*, I/LA26-A
3. Nesher, Ariza-Heredia, Shah et al. - Immune Monitoring with the T-SPOT®.*CMV* assay of Allogeneic Hematopoietic Cell Transplant (allo-HCT) Recipients: A Proof of Concept in the Clinical Setting. ICAAC 2014. See: https://www.researchgate.net/profile/Lambros-Michailidis/publication/281354868_Immune_Monitoring_with_the_T-SPOTRCMV_assay_of_Allogeneic_Hematopoietic_Cell_Transplant_allo-HCT_Recipients_A_Proof_of_Concept_in_the_Clinical_Setting/links/55e3ba9708ae2fac472134f3/Immune-Monitoring-with-the-T-SPOTRCMV-assay-of-Allogeneic-Hematopoietic-Cell-Transplant-allo-HCT-Recipients-A-Proof-of-Concept-in-the-Clinical-Setting

Glossary of Symbols

	Use by/Expiration date (Year-Month-Day)
LOT	Lot number
REF	Catalogue number
⚠	Attention, see instructions for use
	Manufacturer
▽	Sufficient for “n” tests
IVD	*In vitro* diagnostic device
	Temperature limitation/Store between
	Consult instructions for use

The use of the T-Cell *Xtend* reagent is protected by the following patents and patents pending; US9090871, EP2084508, JP5992393, CN101529221, AU2007-303994, CA2665205, IN2165/DELNP/2009.

The use of the test is protected by the following patents and patents pending; US7575870, EP941478, JP4094674, AU728357, CA2272881; US6207161, US6242567.

Manufacturer:
Oxford Immunotec Ltd
143 Park Drive East, Milton Park,
Abingdon, Oxfordshire, OX14 4SE, UK
www.oxfordimmunotec.com