

T-SPOT[®].TB



An aid in the diagnosis of tuberculosis infection

PACKAGE INSERT

For *In Vitro* Diagnostic Use

This Package Insert covers use of:

T-SPOT.TB 8 (Multi-use 8-Well Strip Plate Format. Catalogue number: TB.300)

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Intended Use

The T-SPOT®.TB test is an *in vitro* diagnostic test for the detection of effector T cells that respond to stimulation by *Mycobacterium tuberculosis* antigens and is intended for use as an aid in the diagnosis of tuberculosis (TB) infection. The T-SPOT.TB test is a simplified enzyme-linked immunospot (ELISPOT) method which enumerates individual TB-specific activated effector T cells.

Introduction

The World Health Organization estimates that one third of the world's population is infected with *M. tuberculosis*. Each person carrying latent TB infection (LTBI) has approximately a 10% chance of progressing to active disease. This rate of progression is elevated among certain groups, including those who have been recently infected and those with a weakened immune system.

The immune response to infection with *M. tuberculosis* is predominantly a Cell Mediated Immune (CMI) response. As part of this response, T cells are sensitised to *M. tuberculosis* antigens. Activated effector T cells, both CD4 and CD8, specifically separated from blood can be enumerated by their ability to be stimulated *in vitro* by these antigens^{1,2}. The use of selected antigens for the *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, *M. canetti*) improves test specificity for these organisms by reducing cross-reactivity to the BCG vaccine and to most environmental mycobacteria^{3,4}. Two separate panels of antigens, which simulate the well characterised proteins ESAT-6 and CFP10, are used to optimise the sensitivity of the test.

The T-SPOT.TB test is a simplified variant of the ELISPOT test technique. ELISPOT tests are exceptionally sensitive since the target cytokine is captured directly around the secreting cell, before it is diluted in the supernatant, captured by receptors of adjacent cells or degraded. This makes ELISPOT tests much more sensitive than conventional ELISA tests⁵. The T-SPOT.TB test is designed for the detection of effector T cells that respond to stimulation by antigens specific for *M. tuberculosis*^{3,4,6-9}. The test enumerates individual activated TB-specific T cells. It is suitable for use with all patients at risk of LTBI or suspected of having TB disease^{10,11}, regardless of age, sex, ethnicity, therapy or immune status.

Principles of the Procedure

Peripheral blood mononuclear cells (PBMCs) are separated 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 those who have low T cell titres due to weakened immune systems (the immunocompromised and immunosuppressed) have adequate numbers of cells added to the microtitre wells. The washing and counting stages as well as the ELISPOT technique provide superior performance for the detection of TB disease and latent TB infection.

Four wells (see Figure 1) are required for each sample:-

1. A Nil Control to identify non-specific cell activation.
2. TB-specific antigens, Panel A (ESAT-6).
3. TB-specific antigens, Panel B (CFP10).
4. A Positive Control containing phytohaemagglutinin (PHA, a known polyclonal activator¹²) to confirm PBMC functionality.

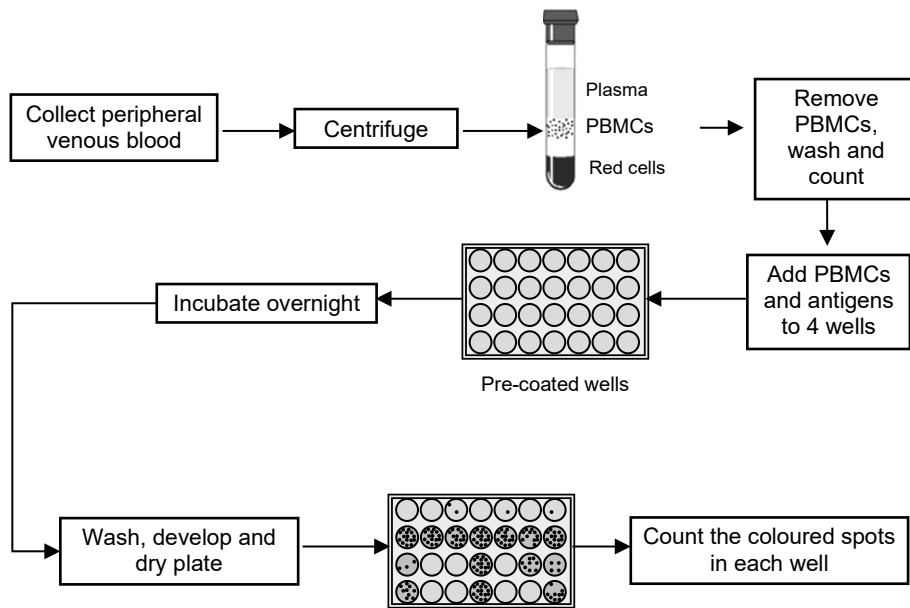


Figure 1: The main steps of the T-SPOT.TB test. Note that each plate contains 96 wells.

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 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 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 *M. tuberculosis* sensitive effector T cells in the peripheral blood.

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.
- Blood should be collected and progressed into the 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, the sample storage time is increased to 32 hours.
- Store and transport blood samples to the laboratory at room temperature (18-25°C). If the T-Cell *Xtend* reagent is to be used, samples can be transported and stored at 10-25°C. Do not refrigerate or freeze whole blood samples.
- The T-SPOT.TB test should be used and interpreted only in the context of the overall clinical picture.
- A negative test result does not exclude the possibility of exposure to or infection with *M. tuberculosis*.
- ESAT-6 and CFP10 antigens are absent from BCG strains and from most environmental mycobacteria, with the exception of *M. kansasii*, *M. szulgai*, *M. marinum*^{3,4} and *M. goodii*.

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 test 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.TB 8 kit contains:

1. 1 microtitre plate: 96 wells, supplied as 12 x 8-well strips in a frame, coated with a mouse monoclonal antibody to the cytokine interferon gamma (IFN- γ).
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 phytohaemagglutinin (PHA), for use as a cell functionality control, bovine serum albumin and antimicrobial agents.
5. 1 vial (50 μ L) 200x concentrated Conjugate Reagent: mouse monoclonal antibody to the cytokine IFN- γ conjugated to alkaline phosphatase.
6. 1 bottle (25mL) Substrate Solution: ready to use BCIP/NBT^{plus} solution.
7. Instructions for Use, which are found on the CD together with the MSDS, Training Guide, T-SPOT cell dilution calculator, conjugate dilution calculator, centrifuge speed calculator and the T-SPOT.AutoReporter programme.

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. 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).
2. Class II microbiological cabinet (recommended).
3. Blood collection tubes, such as Vacutainer® CPT™ (available from Oxford Immunotec) or heparinised tubes.
4. Ficoll®-Paque * Plus or alternative PBMC separation materials.
5. The T-Cell *Xtend* reagent (available from Oxford Immunotec) may be used with samples collected up to 32 hours following venepuncture.
6. Leucosep tubes may be used to simplify the separation of PBMCs using the Ficoll* method.
7. Centrifuge for preparation of PBMCs (capable of at least 1800xg and able to maintain the samples at room temperature (18-25°C)).
8. Equipment and reagents to enable counting of PBMCs; either manually using Trypan Blue and a haemocytometer on a microscope or automatically using a suitable haematology analyzer.
9. A humidified incubator capable of 37 \pm 1°C with a 5% CO₂ supply.
10. A microtitre plate washer or equipment to manually wash plates.
11. Pipettes and sterile pipette tips.
12. Sterile D-PBS solution: such as GIBCO® 1x D-PBS (Invitrogen; catalogue number 14040-091).
13. Distilled or deionised water.
14. A means of reading the plate such as a microscope, digital microscope, magnifying glass or plate imager.
15. Sterile cell culture medium such as GIBCO AIM V® (Invitrogen; catalogue number 31035-025): the use of this serum free medium for the incubation step is strongly recommended. RPMI

1640 (Invitrogen; catalogue number 21875-034) may be used in the initial sample preparation steps only. It is recommended that cell culture media are stored in appropriate aliquots and excess material is discarded after use. Cell culture media should be pre-warmed to 37°C before use with the T-SPOT. *TB* test.

Reagent Preparation

1. Microtitre Plate. The T-SPOT. *TB* 8 microtitre plate is supplied ready to use. Remove the required number of 8-well strips from storage and allow to equilibrate to room temperature. Reseal the remaining strips in the outer foil packaging and include the desiccant pouch.
2. The vials of *M. tuberculosis* ESAT-6 antigens (Panel A) are supplied ready to use.
3. The vials of *M. tuberculosis* CFP10 antigens (Panel 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 (see the T-SPOT conjugate dilution calculator on the CD provided with each test kit). The reagent may be prepared immediately before 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.

Oxford Immunotec Ltd has prepared a Training Guide, which describes the collection and preparation of samples, the selection of cell culture media and methods for counting spots. This is available on the CD supplied with each test kit, by calling +44 (0) 1235 442780 or by downloading from www.oxfordimmunotec.com.

Sample Collection and Preparation

Individual users should validate their procedures for collection of blood samples, separation and enumeration of PBMCs and choice of suitable media to support T cell functionality during the primary incubation stage of the test. Typically, for an immunocompetent patient, sufficient PBMCs to run the test 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 CPT tubes or one 6mL heparin tube
- Children 2-9 years old: one 4mL CPT or heparin tube
- Children up to 2 years old: one 2mL paediatric tube

Blood samples must be stored at room temperature and tested within 8 hours of blood collection, or within 32 hours with storage at 10-25°C if the sample is treated with the T-Cell *Xtend* reagent.

Cell culture media should be pre-warmed to 37°C before use with the T-SPOT. *TB* test.

Procedure	Notes
1. Collect a blood sample according to the instructions supplied with the collection device. Store collected blood at room temperature (18-25°C) or at 10-25°C if the T-Cell <i>Xtend</i> reagent is to be used. Do not refrigerate or freeze.	1. Blood samples can be collected into a variety of tubes. In our laboratories, we have successfully used Vacutainer sodium citrate CPT, sodium heparin CPT and standard lithium heparin tubes. Only lithium heparin tubes can be used with the T-Cell <i>Xtend</i> reagent. EDTA tubes are not recommended.
2. When using CPT blood collection tubes, follow the manufacturer's instructions for separation of PBMCs. For alternative blood collection methods,, separate PBMCs by centrifugation through Ficoll-Paque Plus using published procedures. If Leucosep tubes, or the T-Cell <i>Xtend</i> reagent (available from Oxford Immunotec) are used, follow the protocols provided with these reagents.	2. Centrifuge 8mL CPT tubes at 1600xg for 28min or 4mL CPT tubes at 1800xg for 30min at 18°C where a refrigerated centrifuge is available. Allow the centrifuge to come up to 18°C if lower temperatures have been used previously. If a non-refrigerated centrifuge is used, ensure the temperature does not go above 25°C. Alternatively, dilute the blood with an equal volume of RPMI 1640 medium. Carefully layer the diluted blood (2-3 volumes) onto Ficoll-Paque Plus (1 volume) and centrifuge at 1000xg for 22min while maintaining the temperature between 18 and 25°C. For samples between 8 and 32 hours post venepuncture, use the T-Cell <i>Xtend</i> reagent before layering the sample onto Ficoll-Paque Plus. The centrifuge speed calculator on the CD included with the test kit can help convert speeds from xg to rpm.
3a. Collect the white, cloudy band of PBMCs using a pipette and transfer to a 15mL conical centrifuge tube. Make up the volume to 10mL with cell culture medium.	3a. A variety of media can be used for washing the cells during this process. In our laboratories, both AIM V and RPMI 1640 have been used successfully and are recommended.
4. Centrifuge at 600xg for 7min. Pour off the supernatant and resuspend the pellet in 1mL medium.	4. See 3a. above.
5. Make up the volume to 10mL with fresh medium and centrifuge at 350xg for 7min.	5. See 3a. above.
6. Pour off the supernatant and resuspend the pellet in 0.7mL AIM V culture medium.	6. At this stage, the culture medium for the overnight incubation should be used to resuspend the pellet. In our laboratories, the serum-free medium AIM V has been used successfully and is strongly recommended.

Cell Counting and Dilution

The T-SPOT.*TB* test requires 2.5×10^5 viable PBMCs per well. A total of four wells are required for each patient sample. The correct number of cells must be added to each well. Failure to do so may lead to an incorrect interpretation of the result.

Procedure	Notes
1. Perform a viable cell count.	1. Cells can be counted by a variety of methods, including manual counting using Trypan Blue and a haemocytometer or automated counting using an appropriate instrument.
2. Briefly, for manual counting with a Neubauer haemocytometer, add 10µL of the final cell suspension to 40µL 0.4%(w/v) Trypan Blue solution. Place an appropriate aliquot onto the haemocytometer and count the cells in the grid. For other types of haemocytometer and for automated devices, follow the manufacturers' instructions.	2. Care should be taken to ensure that the cell suspension is thoroughly 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.
3. Calculate the concentration of viable cells present in the stock cell suspension.	3. 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. The T-SPOT cell dilution calculator on the CD provided with each test kit will facilitate this calculation.
4. Prepare 500µL of the final cell suspension at a concentration of 2.5×10^5 cells / 100µL.	4. Ensure cells are thoroughly mixed before removing an aliquot for dilution.

Plate Set Up and Incubation

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

- Nil Control
- Panel A
- Panel B
- Positive Control

Procedure	Notes
1. Remove the pre-coated 8-well strips from the packaging, clip into a plate frame and allow to equilibrate to room temperature.	1. 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 50µL AIM V culture medium to each Nil Control well. (ii) Add 50µL Panel A solution to each well required. (iii) Add 50µL Panel B solution to each well required. (iv) Add 50µL 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 100µL of the patient's final cell suspension (containing 250,000 viable cells).	3. Pipette the cell suspension gently up and down to ensure thorough mixing before removal of each 100µL aliquot. It is recommended that a new tip is used for every addition of each patient's cells to avoid cross-contamination between the 4 wells.

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.
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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.	
2. Discard the cell culture medium and add 200µL D-PBS solution to each well.	2. At this time remove the Substrate Solution from the kit and allow to equilibrate to room temperature.
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 50µL), make up 500µL of working strength solution by adding 2.5µL of concentrated Conjugate Reagent to 497.5µL D-PBS. The conjugate dilution calculator on the CD included with each test kit can be used for this calculation.
5. Add 50µL 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 four D-PBS washes as described in steps 2. and 3. above.	
7. Add 50µL Substrate Solution to each well and incubate at room temperature for 7min.	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) to determine whether a patient sample is 'Positive' or 'Negative' to TB antigens.	10. Spots can be visualised by a number of methods, including manually using a hand held magnifying glass, a suitable microscope, a digital microscope or using a dedicated ELISPOT plate imager. A spot counting training guide (the T-SPOT. <i>Tutor</i> programme) can be obtained via the Oxford Immunotec website.

Quality Control

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

A Nil Control spot count in excess of 10 spots should be considered as 'Indeterminate'. Refer to the T-SPOT.*TB* Training Guide for possible causes (download from www.oxfordimmunotec.com). Another sample should be collected from the individual and tested.

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^{13,14}. Where the Positive Control spot count is < 20 spots, it should be considered as 'Indeterminate', unless either Panel A or Panel B is 'Positive' as described in the Results Interpretation and Test Criteria (see below), in which case the result is valid.

Due to potential biological and systematic variations, where the higher of (Panel A minus Nil Control) and (Panel B minus Nil Control) is 5, 6 or 7 spots, the result may be considered as Borderline (equivocal). 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.

While ESAT-6 and CFP10 antigens are absent from BCG strains of *M. bovis* and from most environmental mycobacteria, it is possible that a 'Positive' T-SPOT.*TB* test result may be due to infection with *M. kansasii*, *M. szulgai*, *M. marinum* or *M. goodii*. Alternative tests are required if these infections are suspected.

Results Interpretation and Test Criteria

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

T-SPOT.*TB* test results 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 minus Nil Control) and / or (Panel B minus Nil Control) ≥ 6 spots.
- The test result is 'Negative' if both (Panel A minus Nil Control) and (Panel B minus Nil Control) ≤ 5 spots. This includes values less than zero.

A 'Positive' result indicates that the sample contains effector T cells reactive to *M. tuberculosis*.

A 'Negative' result indicates that the sample probably does not contain effector T cells reactive to *M. tuberculosis*.

Test Performance Characteristics

Specificity was assessed by testing 93 samples from donors adjudged from medical history and personal information to be at low risk of infection with *M. tuberculosis*. The specificity of the T-SPOT.*TB* test was calculated as 100% (93/93) (95% confidence limits 95.8% - 100%).










Sensitivity was assessed by testing 87 samples from culture confirmed cases of *M. tuberculosis* infection, including immunocompromised groups. The sensitivity of the T-SPOT.*TB* test was calculated as 98.8% (86/87) (95% confidence limits 90.8% - 99.9%).

Reproducibility was assessed, as a surrogate marker of intra-test variation, by analysis of duplicate blood samples run on the same plate. A total of 145 blood samples from 140 individual donors were tested in duplicate (two wells for each of Panel A and Panel B) using the T-SPOT.*TB* test. In 142/145 (97.9%) duplicate analyses, clinical agreement was observed. Two duplicate analyses gave discordant borderline results and only 1/145 samples gave discrepant results.

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Glossary of Symbols

	Use by/Expiration date (Year-Month-Day)
	Lot number
	Catalogue number
	Attention, see instructions for use
	Manufacturer
	Sufficient for "n" tests
	<i>In vitro</i> diagnostic device
	Temperature limitation/Store between
	Consult instructions for use

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Tween is a trademark of Uniqema Americas LLC.

The T-SPOT. *TB* test is protected by the following patents and patents pending:

T-SPOT. *TB* test kit

US7632646, EP1144447, JP4633931, ZA2001-3356; US7901898, US8216795, US8507211, US9005902; US7579141, US8021832, US9238066, US9482671, US9844604, EP1214088, EP2087906, JP4820489, AU773268, CA2372583

T-Cell *Xtend* reagent

EP2084508; US9090871, CN101529221, AU2007-303994, JP5992393, IN289117, CA2665205

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