INTENDED USE

Act-T4 Cell™ Kit allows developing a functional assay for measuring of immune response mediated by activated CD4+ T cells. This kit is based on a patented method (OX40 assay) for the identification of a subpopulation of CD4 effector T cells; these cells present the activation markers CD134 and CD25 on their surface, which can be detected and quantified by flow cytometry after incubating blood with mitogens and/or specific antigens of pathogens to study. The increase in the percentage of this subpopulation of activated CD4+ T cells is indicative of immune responsiveness of the organism to the applied stimulus. This reagent must be used by flow cytometry qualified personal.

PRINCIPLES OF THE PROCEDURE

Flow cytometry is a technique which provides analysis of multiple characteristics of single cells. This is achieved through hydrodynamic focusing of cells which are passed one by one by a laser beam. The interaction of the cells with the light source originates signals of two different types: those generated by scattered light (FSC/SSC), which are proportional to the relative size and complexity of the cell, and those produced by the emission of light by the fluorochromes present in/on the cell. These light signals collected by detectors, are transformed into electric impulses, amplified and registered as digital signals to be processed by a computer.

When the reagents are added to the sample, the mixture of fluorochrome-labelled antibodies bind specifically to the antigens they are directed against, allowing the detection by flow cytometry of the different cell subsets. The erythrocyte population, which could hinder the detection of the target population, is eliminated by the use of a red blood cell lysing solution previous to the acquisition of the sample on the cytometer.

SUMMARY AND EXPLANATION

Clinical investigations into the presence and extent of antigen-specific T cell immunity are becoming more relevant at present. Therefore the necessity for antigen-specific functional CD4 T cell assays in routine diagnostic clinical laboratories is real. Recently, a whole blood technique involving flow cytometry and detection CD25 and CD134 (OX40) expression on the surface of activated CD4+ T cells (OX40 assay) was demonstrated to be highly accurate. The results obtained were concordant with those obtained from more traditional methods of antigen-specific T cell detection. Several studies have validated the preclinical use of this method against virus (e.g., CMV, EBV, HCV, HIV) or bacteria (e.g., *Mycobacterium tuberculosis*) (1-4), which can also be used to evaluate general immunocompetence against mitogens.

CD134 is a secondary costimulatory molecule. Genetic studies in mice have shown that CD134-CD134L (OX40-OX40L) interactions are crucial for the generation of memory T cells, promoting the survival of antigen-specific effector T cells (5). Expression of CD134 in CD4+ T cells reaches its maximum level 24-48 hours following the activation by antigen or mitogen; unstimulated CD4+ T cells do not express CD134 (6). After stimulation, T cells express the high-affinity receptor IL2R (CD25) (7). Coexpression of CD134 and CD25 surface molecules allows specific detection of activated antigen-specific CD4+ T cells following antigen exposure by flow cytometry. Combining whole-blood culture with detection of this induced coexpression of CD25 and CD134 affords an extremely simple assay that allows detecting responses to a diverse range of antigens. Results are consistent with serology test, cell proliferation studies and other techniques based on IFN-gamma production levels, but this whole-blood assay (OX40 assay) detects much higher levels of antigen-specific cells than intracellular cytokine assays, and also the cells retain viability and can be sorted for in vitro expansion for molecular studies and potentially for therapeutic studies (8).

Act-T4 Cell™ kit is intended to be used after incubating whole blood during 44-48 hours in a culture medium with the antigens/mitogens focusing of cells which are passed one by one by a laser beam. The interaction of the cells with the light source originates signals of two different types: those generated by scattered light (FSC/SSC), which are proportional to the relative size and complexity of the cell, and those produced by the emission of light by the fluorochromes present in/on the cell. These light signals collected by detectors, are transformed into electric impulses, amplified and registered as digital signals to be processed by a computer.

When the reagents are added to the sample, the mixture of fluorochrome-labelled antibodies bind specifically to the antigens they are directed against, allowing the detection by flow cytometry of the different cell subsets. The erythrocyte population, which could hinder the detection of the target population, is eliminated by the use of a red blood cell lysing solution previous to the acquisition of the sample on the cytometer.

REAGENT COMPOSITION

Sample type

Heparinized whole blood

Material included

Act-T4 Cell™ kit includes two components with enough volume for 100 tests:

- One vial with the following 4-antibody combination:

<table>
<thead>
<tr>
<th>Fluorochrome</th>
<th>FITC</th>
<th>PE</th>
<th>PerCP-Cyanine5.5</th>
<th>APC</th>
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</thead>
<tbody>
<tr>
<td>Antibody</td>
<td>CD4</td>
<td>CD134</td>
<td>CD3</td>
<td>CD25</td>
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<tr>
<td>Clone</td>
<td>Edu-2</td>
<td>134-1</td>
<td>UCHT1</td>
<td>M-A251</td>
</tr>
</tbody>
</table>

- Erythrocyte lyse-non-wash solution (Quicklysis™ (CYT-QL-1)).

All components contains sodium azide (NaN₃) ≤0.09% (m/v). Reagents are not considered sterile.

Material required but not included

- Complete culture medium IMDM or RPMI-1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.
- Phytohaemagglutinin (PHA; Sigma Aldrich) or staphylococcal enterotoxin B (SEB; Sigma Aldrich) to be used as positive control at a final concentration of 5 μg/ml or 1 μg/ml respectively.
• Generic or specific antigens for sample activation.
• Polystyrene cell culture plates.
• CO₂ incubator.
• Flow cytometer equipped with 488 nm ion argon laser and 633 nm red diode laser, and appropriate computer hardware and software associated.
• FACS tubes: test tubes suitable for obtaining samples in the flow cytometer used. Usually tubes with a rounded bottom for 6 ml, 12x 75 mm are used.
• Automatic pipette and tips
• Chronometer
• Vortex Mixer

STORAGE CONDITIONS
Act-T4 Cell™ kit is stable until the expiration date shown on the label, when stored at 2-8º C. The reagents should not be frozen or exposed to direct light during storage or during incubation with sample. Keep all reagent vials in a dry place. Once opened, the vials must be stored in a vertical position to avoid any possible spillage. The pH of the erythrocyte lysing solution may increase during storage, which may affect the position of cells in an FSC/SSC dot plot. The pH of the reagent should be between 7 and 7.4. If the pH is out this range, it should be adjusted by adding diluted solutions of HCl or NaOH.

WARNINGS AND RECOMMENDATIONS
1. For research use only.
2. Alteration in the appearance of the reagent, such as the precipitation or discoloration indicates instability or deterioration. In such cases, the reagent should not be used.
3. It contains ≤0.09% (m/v) sodium azide (CAS-Nr. 26628-22-8) as a preservative, but even so care should be taken to avoid microbial contamination of reagent or incorrect results may occur.
   - Sodium azide (Na₃N₃) is harmful if swallowed (R22). If swallowed, seek medical advice immediately and show this container or label (S46).
   - Wear suitable protective clothing (S36).
   - Contact with acids liberates very toxic gas (R32).
   - Azide compounds should be disposed of as hazardous waste (S60).
4. All patient specimens and materials with which they come into contact are considered biohazards and should be handled as if capable of transmitting infection (R16), and disposed according to the legal precautions established for this type of product. Also recommended is handling of the product with appropriate protective gloves and clothing, and its use by personnel sufficiently qualified for the procedures described. Avoid contact of samples with skin and mucous membranes. After contact with skin, wash immediately with plenty of water.
5. Use of the reagent with dilutions, incubation times or temperatures different from those recommended may cause erroneous results. Any such changes must be validated by the user.
6. Any serious incident relating to the product must be reported to Cytognos S.L. as well as the competent professional authority of the Member State in which the user is established.

PROCEDURE
Whole blood culture
Whole blood sample must be collected in commercially available sodium heparin-treated tubes (R, 10). It is recommended to process whole sample within 6 hours after venepuncture to obtain the maximum levels of antigen-specific response (R).  
1. Mix 0.25 ml of sodium heparin-anticoagulated whole blood with 0.25 ml of culture medium (IMDM or RPMI-1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin) in sterile well cell culture plate (dilution 1:1). For each sample to study prepare:
   a. Negative control culture: 0.25 ml of heparinized whole blood mixed with 0.2 ml of complete culture medium.
   b. Positive control culture: 0.25 ml of heparinized whole blood mixed with complete culture medium and PHA at 5 μg/ml (PHA tube), and/or 0.25 ml of heparinized whole blood mixed with 0.25 ml of complete culture medium and SEB at 1 μg/ml (SEB tube).
   c. Antigen-specific culture: 0.25 ml of heparinized whole blood mixed with complete culture medium and peptides or lysates of antigens to study at final concentration recommended by manufacturers.
2. Incubate all cultures at 37°C for 44-48 hours in a humidified atmosphere of 5% CO₂ in air.

Whole blood staining and cell acquisition by flow cytometry
3. After incubation, pipette 100 μl of each culture (negative control, positive control and antigen-specific culture) into separated FACS tubes and add 20 μl of the 4-antibody mixture provided in the Act-T4 Cell™ kit to each tube.
4. Mix well and incubate for 15 min at room temperature protected from light.
5. Add 1 ml of erythrocyte lysis-non-wash solution provided in the Act-T4 Cell™ kit to each tube.
6. Mix well and incubate for 10 min at room temperature protected from light.
7. Mix on a vortex (low speed) to reduce cell aggregation. Acquire a minimum of 50,000 events immediately after staining or store at 4°C for 1 hour maximum. Instrument calibration must be performed according to manufacturers’ advice. Before acquiring samples, adjust threshold or discriminator to minimise debris and ensure that populations of interest are included.

Flow cytometry analysis
Cytognos recommends the use of the analysis software Infinicyt™ (11), which provides a revolutionary approach for data integration and multidimensional analysis of flow cytometry data. Its innovative features make the analysis and interpretation of the results easier, faster and more accurate. Infinicyt™ comprises exclusive tools that allow a better identification and description of the different cell populations. Complete information about Infinicyt™ can be found on the website: www.infinicyt.com.

To analyze the results obtained after processing a sample with Act-T4 Cell™ kit, we recommend to follow these indications:
1. Select apoptotic/dead cells using a forward scatter vs side scatter dot plot to exclude them from the analysis.
2. Select T cells (CD3+) performing a CD3-PerCP-Cyanine5.5 vs side scatter gate followed by a second gate on lymphoid area on a forward scatter vs side scatter dot plot. Note that PHA file shows increased granularity and size in CD3+ lymphocytes and requires a wider gate to capture the whole lymphocyte population [4].

3. Select CD4+ T cells based on their CD3 and CD4 positive expression.

4. Finally activated CD4+ T cells can be identified by the coexpression of CD25 and CD134 surface receptors. Gate for CD4+ T CD25+CD134+ cells is fixed on basis of negative control (no Ag) and positive control (PHA and/or SEB tube) cultures. Afterwards, this gate is applied to subsequent antigen tubes.
4. Fix gate for CD4+CD25+CD134+ T cells on basis of negative control and positive control tubes

Results of activated CD4+ T cells are usually expressed as percentage of CD4+CD25+CD134+ T cells respect to CD4+ T cells.

LIMITATIONS
- It is advisable to acquire stained samples as soon as possible to obtain optimal results. Non-viable cells may show unspecific staining. Prolonged exposure of samples to lytic reagents may cause white cell destruction and targeted population cell loss.
- When using whole blood lysing procedures some red blood cells may not be lysed, for instance if there are nucleated red blood cells or if abnormal protein concentration and haemoglobinopathies are observed. This may cause misleading results since unlysed red blood cells are counted as leucocytes.
- Results obtained by flow cytometry may be erroneous if cytometer laser is misaligned or if gates are incorrectly set.
- Knowledge of antigen normal expression pattern and its relation to other relevant antigens is paramount to carry out an adequate analysis.

QUALITY CONTROL
- Pipettes precision and cytometer calibration should be verified to obtain optimal results.
- In multicolour panels, fluorochromes emit in wavelengths that can show certain spectral overlap which must be corrected by electronic compensation. Optimal compensation levels can be established by analysing cells from healthy individuals stained with mutually exclusive monoclonal antibodies conjugated with appropriate fluorochromes.
- This product has been manufactured in accordance with standards of production and quality system of the ISO 13485:2012 standard.

REFERENCES

WARRANTY
This product is warranted only to conform to the quantity and contents stated on the label. There are no warranties that extend beyond the description on the label of the product. Cytognos’s sole liability is limited to either replacement of the product or refund of the purchase price.
# EXPLANATION OF SYMBOLS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>![Symbol 1]</td>
<td>Use by (YYYY-MM)</td>
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<tr>
<td>![Symbol 2]</td>
<td>Storage temperature limitation</td>
</tr>
<tr>
<td>![Symbol 3]</td>
<td>Keep out of sunlight</td>
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<td>![Symbol 4]</td>
<td>Consult instructions for use</td>
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<tr>
<td>![Symbol 5]</td>
<td>For research use only</td>
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<td>Batch code</td>
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<td>![Symbol 7]</td>
<td>Catalogue number</td>
</tr>
<tr>
<td>![Symbol 8]</td>
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