1. Intended use

- The CINAkit allows the indirect immunofluorescence detection of internal matrix phosphoprotein (protein kinase) 65-68 kD (pp65) of Human Cytomegalovirus (HCMV) in peripheral blood leukocytes. The pp65 detection in peripheral blood leukocytes allows the diagnostic of acute or reactivated infection.

2. Summary and explanations

- Recital:
  - Initially described in 1988-1989 (7, 10, 42, 43), the antigenemia was introduced as based on immediate early antigen (I.E.A) detection. It has been now demonstrated that it is based on the staining of the internal matrix phosphoprotein (protein kinase) 65-68 kD (pp65) (14). In a positive specimen the antibodies specific for I.E.A are staining an average of 10% of the number of cells stained by anti pp65 antibodies (14, 15, 23).
  - Advantages:
    - The HCMV pp65 antigenemia is a rapid (8, 9, 14, 15, 16, 18, 20, 21, 22, 23, 25, 27, 28, 29, 34), quantitative (20, 22, 27), easy to read (5, 9), method for the diagnostic of active Cytomegalovirus infections (18, 24).
    - Antigenemia is more sensitive than viral isolation (8, 16, 22, 29, 40), rapid culture (29), serology (22, 28). In all cases the sensitivity is 100% in symptomatic patients (16).
    - Antigenemia can be detected from several days to one week before the onset of symptoms (24, 28) and from 2 to 25 days before rapid culture (10, 12, 16). Antigenemia is also positive for a longer period than viremia demonstrated by co-culture (4).
    - This test can be even performed by laboratories where the culture are not available, and only requires as specific apparatus a simple cytocentrifuge.
    - The Argen anti pp65 pool is of interest since it stains a higher number of cells in positive samples, and is giving a brighter fluorescence when compared to clone 1C3 alone and others (13, 35, 36, 45, 55, 56, 57, 58, 59).
    - As opposed to some other antigenemia diagnostic reagents, no confirmed false negative has been reported with the use of CINAkit (16).
    - The new direct Erythrocytes lysis procedure (80, 80) allowing to perform rapid antigenemia is described after.
  - Category of patients:
    - Antigenemia is successfully used in the diagnostic and follow-up of HCMV infections in heart (4, 8, 9, 11, 12, 13, 15, 16), heart-lung (28), liver (16, 22, 40), kidney (14, 16, 18, 17, 21, 28, 24, 46, 47, 50, 51), bone-marrow (14, 29, 48, 54), transplant recipients and in AIDS patients (5, 8, 9, 11, 25, 28, 30, 39).
    - The HCMV is responsible of a high mortality rate in AIDS patients (25, 37, 38) and tissue or organ graft recipients (23, 29, 39).
3. Principle of the test

- HCMV antigenemia IF kit is an indirect immunofluorescent test that allows rapid detection of Human Cytomegalovirus antigen in leukocytes from peripheral blood.
- This method uses a monoclonal antibodies pool which recognizes the lower matrix structural phosphoprotein 65-68 kDa (protein kinase, pp65), present in the nucleus of infected cells within one hour post infection.
- The antibodies pool recognizes two epitopes of the protein expressed in the nucleus of peripheral blood HCMV infected leukocytes.
- The follow-up of graft recipients by antigenemia can allow to modulate the immunosuppressive or antiviral treatments or to initiate the antiviral treatment in the early steps of infection.
- Antigenemia is particularly recommended for follow-up of graft recipients since it can be detected when HCMV isolation by rapid or conventional method is inhibited by antiviral prophylactic administering.

Follow-up of graft:
- The rapid HCMV infection diagnosis in graft recipients allows to distinguish between the graft rejection and infection symptoms.
- Antigenemia is often positive between 3rd to 6th week after transplantation. The infection pick is around the 40th day after transplantation.
- Even very low antigenemia positivity is significant after bone marrow transplantation.
- The follow-up of graft recipients by antigenemia can allow to modulate the immunosuppressive or antiviral treatments or to initiate the antiviral treatment in the early steps of infection.
- Antigenemia is particularly recommended for follow-up of graft recipients since it can be detected when HCMV isolation by rapid or conventional method is inhibited by antiviral prophylactic administering.

Follow-up of AIDS:
- During AIDS the HCMV infections are very frequent when the number of CD4 cells is < 100 / µL.
- HCMV infection in AIDS patients should be carefully and regularly monitored by antigenemia for initiation and monitoring of treatment.
- A kinetic study is always preferable: a significant increasing in 2 successive specimens is predictive for HCMV disease although a low and stable (< 10 positive leukocytes / 10^6 cells) should not be taken into account.
- The monitoring of patients by using antigenemia can allow to initiate a treatment in the early steps of infection and to follow-up the treatment efficacy.
- Discrepant results negative culture (viremia) / positive antigenemia observed with patients under antiviral treatment are rarely associated with an efficient therapy.
- The continuance of antigenemia with a negative viremia indicates the needs to continue the treatment.
- A negative antigenemia can be considered as a good sign of therapy efficacy.

Interpretation:
- The HCMV isolation from leukocytes of blood (viremia) and/or pp65 antigenemia are evidence for active systemic infection.
- A positive pp65 antigenemia demonstrate a disseminated infection that can stay asymptomatic or evolve to a visceral attack.
- A single stained cell indicates a positive antigenemia.
- The appearing and the severity of clinical signs is correlated with the number of positive cells / spot. Generally these signs are appearing from 50 positives cells / spot of 2 x 10^5 cells.
- The antigenemia positivity level is reciprocaly proportional to patient immunocompetency.
- The ratios of stained cells observed on positive specimens are the following:
  - 1/100 to 1/100 000 for monocytes
  - 1/100 000 to 1/100 000 for polymorphonuclear cells

Technical choice:
- Although some authors describe that a delay between sample collection and staining does not change the antigenemia results, most of them are describing a significant decreasing of the signal during the time.
- An increasing number of users are considering that the fact to delay the test by storing the blood specimen for 24h at +2/+8°C is not altering the patients follow-up quality.
- Antigenemia can be performed on peripheral blood specimens collected with heparine or EDTA.
- The use of methanol or ethanol leads to a loss of signal.
- It has been demonstrated that the indirect immunofluorescence revelation is more sensitive than immunoperoxidase activity neutralization.
- The ARGENE pool of monoclonal antibodies (1C3 + AYM-1) labels different epitopes expressed in the nuclei of infected peripheral blood polymorphonuclear leukocytes and monocytes, and thus ensures avoiding any false negative that can theoretically be caused by mutation of one of the epitope recognized, since a double mutation in two specific parts of the antigen is at very low risk.

### 3. Principle of the test

- HCMV antigenemia IF kit is an indirect immunofluorescent test that allows rapid detection of Human Cytomegalovirus antigen in leukocytes from peripheral blood.
- This method uses a monoclonal antibodies pool which recognizes the lower matrix structural phosphoprotein 65-68 kDa (protein kinase, pp65), present in the nucleus of infected cells within one hour post infection.
- The antibodies pool recognizes two epitopes of the protein expressed in the nucleus of peripheral blood HCMV infected polymorphonuclear and mononuclear cells, during blood dissemination.

**The Antigenaemia procedure is performed in 2 h:**
- Direct Erythrocytes lysis.
- Cytocentrifuge 2 x 10^5 cells.
- Formaldehyde fixation and membrane permeabilization.
- Mouse monoclonal antibodies anti-HCMV pp65.
- Anti-Mouse IgG-HqM F(ab’); FITC “human adsorbed”.
- Read under fluorescent microscope.
4. Composition of the kit

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>PBS</td>
<td>5 vials</td>
<td>powder for 5 L</td>
</tr>
<tr>
<td>B</td>
<td>Erythrocytes lysing solution</td>
<td>1 vial</td>
<td>45 mL (20x) for 900 mL</td>
</tr>
<tr>
<td>C</td>
<td>Fixative solution</td>
<td>1 vial</td>
<td>110 mL (5x) for 550 mL</td>
</tr>
<tr>
<td>D</td>
<td>Foetal calf serum</td>
<td>1 vial</td>
<td>30 mL for washing solution</td>
</tr>
<tr>
<td>E</td>
<td>Permeabilization solution</td>
<td>1 vial</td>
<td>110 mL (5x) for 550 mL</td>
</tr>
<tr>
<td>F</td>
<td>Anti HCMV ppUL83 (pp65) blend (yellow cap)</td>
<td>1 vial</td>
<td>3 mL ready to use</td>
</tr>
<tr>
<td>G</td>
<td>Secondary antibody F(ab')2 FITC (green cap)</td>
<td>1 vial</td>
<td>3 mL ready to use</td>
</tr>
<tr>
<td>H</td>
<td>Mounting fluid</td>
<td>1 vial</td>
<td>4 mL ready to use</td>
</tr>
<tr>
<td>K</td>
<td>Dextran 6%</td>
<td>1 vial</td>
<td>60 mL ready to use</td>
</tr>
</tbody>
</table>

Note: This kit contains sufficient reagents for 10 series of 5 determinations, using a 50 mL capacity recipient "HELLENDAHL" for fixation, permeabilization and washing steps.

5. Storage of reagents

- All reagents must be stored at +2/+8°C, in the dark, until expiration date mentioned on labels, except the reagent J (mounting medium) that can be stored at room temperature.
- Keep the antibodies and the reagent D (foetal calf serum) in the fridge until use.
- Reconstituted reagents A, C, E can be stored for one day at room temperature, other reagents are reconstituted just before use.

6. Reconstitution of reagents

- All reagents except PBS are reconstituted just before use.
- Allow reagents to reach room temperature and homogenize before use. Check particularly the perfect homogeneity of reagents B and E before to dilute them.
- "PBS" (A) : each vial is reconstituted with 1 liter demineralized water.
- "Erythrocytes lysing solution" (B) is perfectly homogenized and diluted just before use 1/20 in demineralized water. Note: reconstitution of the reagent B with PBS instead of distilled water inactivate the reagent.
- "Fixative solution" (C) is diluted 1/5 in demineralized water.
- "Permeabilization solution" (E) is diluted 1/5 in demineralized water.
- "Washing Solution": 1% FCS (D) in PBS (A) for washing steps during fixation and permeabilization.

7. Warning and precautions

This kit is intended for in vitro use only

7.1. Warning and precautions for immunology:
- Periodically control visible light or UV microscope (clean the lenses and objectives, alignment and wear of the bulb)
- Use immunofluorescence slides cleaned with alcohol.

7.2. General warning and precautions
- Handle and dispose all specimens, materials, reagents and effluents potentially infectious (in accordance with the regulation in force).
- The use of this product is reserved for a qualified and trained staff.
- The use of this product is reserved for a qualified and trained staff.
- Read all instructions carefully before performing this assay
- Respect times, temperatures, centrifugation speed as prescribed.
- Do not use reagents after expiry date printed on labels
- Do not interchange reagents from kits with different batch numbers or from other suppliers
- The control and the maintenance of the equipment being used should be carried out in accordance with GLP standards (or equivalent).
- Wear single use gloves at the time of the handling of the reagents and samples. Carefully wash the hands after handling.
- Equilibrate the reagents at room temperature.
- Do not invert the caps of the bottles
- Never pipet by mouth
- Do not smoke, drink or eat in dedicated work areas
7.3. Reagent specific warning and precautions

<table>
<thead>
<tr>
<th>Reagent (C)</th>
<th>Toxic (Contains formaldehyde, thimerosal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R34</td>
<td>Toxic by inhalation, in contact with skin and if swallowed.</td>
</tr>
<tr>
<td>R34</td>
<td>Causes burns.</td>
</tr>
<tr>
<td>R40</td>
<td>Limited evidence of a carcinogenic effect</td>
</tr>
<tr>
<td>R43</td>
<td>May cause sensitisation by skin contact.</td>
</tr>
<tr>
<td>S26</td>
<td>In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.</td>
</tr>
<tr>
<td>S36/37/39</td>
<td>Wear suitable protective clothing, gloves and eye/face protection.</td>
</tr>
<tr>
<td>S45</td>
<td>In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).</td>
</tr>
<tr>
<td>S51</td>
<td>Use only in well-ventilated areas.</td>
</tr>
<tr>
<td>S60</td>
<td>This material and its container must be disposed of as hazardous waste.</td>
</tr>
<tr>
<td>S61</td>
<td>Avoid release to the environment. Refer to special instructions/safety data sheets.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reagent (D, E)</th>
<th>Harmful (Contains thimerosal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R20/21/22</td>
<td>Harmful by inhalation, in contact with skin and if swallowed.</td>
</tr>
<tr>
<td>R33</td>
<td>Danger of cumulative effects.</td>
</tr>
<tr>
<td>S28</td>
<td>After contact with skin, wash immediately with plenty of soap suds</td>
</tr>
<tr>
<td>S36</td>
<td>Wear suitable protective clothing.</td>
</tr>
<tr>
<td>S45</td>
<td>In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).</td>
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</tr>
</tbody>
</table>

8. Procedure conditions

- Allow reagents to reach room temperature before use.
- Verify pipette exactness and precision.
- Once the test begins, all the steps must be performed without interruption by following the handling time indicated.
- Do not allow antibodies and foetal calf serum (reagents D, F and H) to stay at room temperature for a long period.

9. Blood samples collection

- Take in an aspetic way 7 mL of venous blood using a heparinized vacutainer (green cap). It is also possible to collect on EDTA.
  
  *Note:* For patients with deep leucopenia (neutrophils <200/µm³) a minimum volume of 10 mL can be required if dextran separation is used.

  - Shake the sample gently by overturning.
  - Send the sample to the laboratory at +2/+8°C without delay, following local regulations for transport of infectious samples.
  
  *Note:* Although some authors describe that a delay between sample collection and staining does not change the antigenemia results (41, 14, 24, 27), an increasing number of users are nevertheless considering that the fact to delay the test by storing the blood specimen for 24h at +2/+8°C is not altering the patients follow-up quality.

  *Note:* For the diagnosis of neonatal or congenital infection the volume of blood sample to collect could be 2 or 3 m.L. The volume of Dextran solution to use for the separation of polymorphonuclear cells must be modified proportionally. All the other parts of procedure remain exactly the same.

10. Test procedure

**Rapid Antigenemia**

- The HCMV antigenemia procedure was initially including a leukocytes separation step (Dextran or natural sedimentation) followed by an erythrocytes lysis step. The new rapid antigenemia described hereafter allows to perform directly the lysis step on 2 mL of total blood, then to follow with cell density adjustment/centrifugation. The total handling time goes from 4 hours to 2 hours.

- As the direct lysis procedure allows collection of all white cell populations (including lymphocytes), it may be of interest in cases of important lymphocytosis to use the dextran separation. Dextran sedimentation allows the collection of the preferred polymorphonuclear cells, the main target cell population for the antigenemia test. In this case please refer to Dextran separation & post-separation lysis procedures at the end of package insert.
10.1. Direct lysis:
- Gently homogenize blood sample by overturning. Do not use vortex.
- In a 15 mL conical bottomed centrifuge tube, distribute 2 mL blood + 8 mL 1x reconstituted lysis solution.
- Homogenize gently with pipette.
- Incubate for 5 minutes at room temperature.
- Centrifuge at 160 g for 10 minutes at +2/+8°C.
- Eliminate the supernatant by overturning the tube over a disinfectant solution such as 0.5 or 1.0% sodium hypochlorite. Take care not to eliminate the cell pellet.
- Resuspend the pellet in 8 mL of 1x reconstituted lysis solution. Do not incubate, perform immediately the next step.
- Centrifuge at 160 g for 10 minutes at +2/+8°C.
- Eliminate the supernatant by overturning as in previous step and by taking care not to eliminate the cell pellet.
- Resuspend the pellet in 1 mL exactly of PBS (A) and homogenize gently with pipette.
- Perform cell density adjustment (see "Counting cell suspension").

10.2. Counting cell suspension:
- Note: A skill user could estimate the most adapted PBS volume to use (from 0.5 to 2 mL) to resuspend the pellet, depending on its size, in order to reach the appropriate cell density. The calculation formula proposed in "Cell density adjustment" will be modified consequently.
- Count the leucocytes using preferably a "Kova glassstic slide" (ARGENE code 31-030) or if this material is not available: a Malassez cell / haemocytometer.
- Note: Single use "Kova glassstic slides" avoid the numerous and hazardous steps of washing required with normal Malassez cell or other re-usable systems.

10.3. Cell density adjustment
- Adjust the leucocytes concentration to $2 \times 10^6$ cells/mL in PBS (allowing for a loss of about 20% during cytocentrifugation) as follows:
  - A = Cell concentration previously found.
  - $A \text{ cells/mL} \times 1 \text{ mL} \times 0.8$ (loss of 20%) = B mL
  - If B = 1: The cell density is optimal for cytocentrifugation do not change anything.
  - If B > 1: The cell density is too high for cytocentrifugation. To dilute: $(B - 1) = \text{volume of PBS to add to the cell suspension to obtain the right density.}$
  - If B < 1: The cell density is too low. The cell suspension must be concentrated using centrifugation: Add 5 mL of PBS to the cell suspension, centrifuge at 220 g for 10 mn at +18/22°C in a temperature controlled centrifuge. Eliminate the supernatant by overturning and resuspend the cell pellet in PBS in B mL exactly.
- If cytocentrifugation cannot be performed immediately, place the cell suspension at +2/+8°C in the fridge, in a recipient containing ice. Use this suspension as soon as possible.

10.4. Cytocentrifugation:
- Prepare 3 slides for each patient tested:
  - 2 slides for diagnostic.
  - 1 security back-up slide to freeze at -78/-82°C in case of problem during revelation or to use as a control for secondary antibody (see reading - interpretation).
- Cytocentrifuge 100 µL (2 x $10^5$ cells/spot) at 900 rpm for 3 min under low acceleration rate (Cytospin 3, Shandon).
- During the cytocentrifugation, prepare the washing solution required for washing steps during fixation and permeabilization (see "Reconstitution of reagents").
- Just allow the spots to dry, do not wait more than needed.

10.5. Fixation and permeabilization:
- Use only the fixative provided in the kit.
- Do not use acetone as more and more authors agree that the sensitivity with formaldehyde as fixative is superior to that of acetone [8, 13, 27, 44].
- Immerse the slides in the reconstituted fixative solution (C) (see "Reconstitution of reagents") for 10 min at room temperature (under the hood).
- Immerse immediately (do not allow to dry) the slides 5 min in the washing solution (see "Reconstitution of reagents").
- Immerse immediately (do not allow to dry) the slides in the reconstituted permeabilization solution (E) (see "Reconstitution of reagents") for 5 min at room temperature.
- Immerse immediately (do not allow to dry) the slides in the washing solution (see "Reconstitution of reagents") minimum 5 min - maximum 1 h.
- Note: This step allows a short lunch or coffee break.
- Wipe carefully the excess of liquid around the spots taking care not to damage the spots. Do not allow the spots to dry (except for the slides intended to be immediately frozen at -78/-82°C).
- Stain two slides per patient (freeze a third slide immediately at -78/-82°C).
10.6. Staining:

- A positive control slide stored at -18/-22°C will be used as positive control.
  
  Note: The control slides frozen intended to be stained are immersed with the slides of the day in the washing solution after permeabilization (see "Reconstitution of reagents"). Do not thaw the frozen slides in contact of air. Do not refreeze thawed slides.

- Circle the spots with a pen for immunochemistry to avoid spreading of reagents.
- Deposit 1 drop of anti-HCMV pp65 1C3+AYM-1 monoclonal antibody (F).
- Incubate for 30 min at +37°C in a humidified chamber.
- Wash 3 x 1 min by immersion in PBS (A) using a new solution for each bath.
- Wipe carefully the excess of liquid around the spots taking care not to damage the spots. Do not allow the spots to dry.
- Deposit 1 drop of FITC secondary antibody (H).

  Note: Antibodies must stay at +2/+8°C until use.
- Incubate for 30 min at +37°C in a humidified dark chamber.
- After incubation check that no spot has dried if yes, put a mark on the corresponding slide and keep in mind that trouble in reading could come from this step.
- Wash 3 x 1 min by immersion in PBS (A) using a new solution for each bath.
- Quickly immerse in tap water to eliminate crystals.
- Perfectly dry the slides.
- Mount the slides in reagent J.

11. Reading - interpretation

- Read under the fluorescence microscope by using dry lens 40x and / or oil immersion 100x lens. For a rapid scanning of the spot it is also possible to use a 25x lens but the characteristic staining pattern will be confirmed by using 40x lens.
- Read two spots per patient.
- The whole surface of the spots is scanned, looking for fluorescent nuclei polylolate or not.
- A background or possible non specific staining will be verified by reading the security slide stained by incubating the secondary FITC antibody only.
- A positive slide from the bank at -78/-82°C will be stained with the slides of the day and used as a positive control.
- If the separation technic used is the simple sedimentation it will be possible to find stained polymorphonuclear cells and monocytes. The staining ratios are the following:
  - 1/100 to 1/100 000 for polymorphonuclear cells
  - 1/10 000 to 1/100 000 for monocytes
- If the separation is performed with Dextran only polymorphonuclear cells will be observed.
- As the direct lysis procedure allows collection of all white cell populations (including lymphocytes), it may be of interest in cases of important lymphocytosis to use the Dextran separation. Dextran sedimentation allows the collection of the preferred polymorphonuclear cells, the main target cell population for the antigenemia test. In this case please refer to Dextran separation & post-separation lysis procedures at the end of package insert.
- A single stained cell is sufficient to indicate antigenemia.
- Results are reported as number of antigen positive cells for 2x10^5 cells.
- Reading difficulties may be encountered:
  - At the periphery of the spot, some cells may appear slightly greenish:
    - These should not be considered as stained.
  - All the cells appear greenish:
    - This is also an artifactual fluorescence.
  - Peri-nuclear staining of cells:
    - Indicates a problem associated with the fixation-permeabilization steps or drying during revelation.
    - It has been reported in one case of auto-immune disease (lupus erythematosus) an almost unreadable very weak nuclear staining (monocytes only). To discriminate this non specific "staining" read a second slide from the same patient, but incubated with secondary FITC antibody only. If the image obtained is the same then the staining is non specific. In the case of patient with auto-immune disease and infected with HCMV the positive staining intensity cannot be confused with the very weak non specific staining.
  - The microscope focusing is difficult or impossible:
    - The light alignment and lamp should be verified. All dirty optical parts should be cleaned.
    - It may also be caused by an incomplete drying before mounting
  - Cells are looking damaged:
    - This can be due to the use of vortex, or by a too long delay between sample collection and technic, or because operating conditions were not followed. The use of single use plastic aspiration system intended for molecular biology use (very small orifice) may also damage the cells.
12. Material and reagents required but not supplied

12.1. Reagents:
- Positives control slides
- Demineralized water.
- Sodium Hypochloride.

12.2. Small material:
- Heparinized vacutainer or equivalent.
- 5 mL & 1 mL single use pipets.
- Adjustable 100 μL pipette and single use tips.
- Adjustable 10 μL pipette and single use tips.
- 15 mL conical bottomed centrifuge tube.
- Slides for cytocentrifuge cleaned with alcohol.
- Slides washing bath "Hellendahl" of 50 mL capacity for 16 slides maximum.
- Kova glassitic slides Argene ref: 31-030 or Malassez cell
- Pen with water repellent ink for immunochemistry.
- Humidified chamber.
- Coverslips.
- Single use gloves

12.3. Heavy material:
- Refrigerator +2/+8°C
- +37°C incubator.
- Hood.
- Temperature controlled centrifuge.
- Cytocentrifuge (Cytospin 2 or 3, Shandon).
- Immunofluorescence microscope equipped with a filter for FITC (maintained regularly).
- -78/-82°C Freezer (positive control slides storage).

13. Cross reactivity

- No labelling was detected with the range of viruses studied. Then, no cross reaction of the monoclonal antiCMV antibodies of the CINAnit was observed with the following viruses:
  - Herpesviridae :
    - Herpes simplex type 1 (HSV-1) strain Mc Intyre.
    - Herpes simplex type 2 (HSV-2) strain M.S.
    - Varicella Zoster Virus (VZV) strain ATCC VR586.
    - Epstein-Barr virus (EBV) strain B95.8.
    - Herpesvirus type 6 (HHV-6) A variant strain TAN.
    - Herpesvirus type 6 (HHV-6) B variant strain HST.
  - Other viruses :
    - Immunodeficiency virus (HIV) (virion Switzerland).
    - Adenovirus 5 (ADV) strain ATCC VR1082
    - Influenza A (IA) strain A/Texas/1/77
    - Influenza B (IB) strain B/Ann A/1/86
    - Mumps strain ATCC VR365
    - Measles strain ATCC VR24
    - Respiratory Virus Syncytial group A (RSV-A) strain LONG
- The slides used were controled as positive with the specific monoclonal antibodies for each tested virus.
14. Performances

14.1. Expected values:
- CINAkit was evaluated at four sites in a combined clinical study in United States.
- A total of 507 samples corresponding to 295 solid organ transplant patients, 9 bone marrow transplant patients, 142 HIV+/AIDS patients, 18 cancer patients and 43 with other disorders. 83 samples (16.4%) were determined to be positive using CINAkit.

14.2. CINAkit performance characteristics:

14.2.1. Culture comparison
a) Study 1
- CINAkit was compared to CMV virus detection by culture from human peripheral blood leukocytes. 507 clinical specimens were evaluated at four sites.

<table>
<thead>
<tr>
<th>CINAkit</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>29 6 35</td>
</tr>
<tr>
<td>-</td>
<td>54 418 472</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>83 424 507</td>
</tr>
</tbody>
</table>

- Sensitivity = 29/35 = 82.9%
- Specificity = 418/472 = 88.5%

- The imprecision of culture compared to CMV Antigenemia, has been documented and the discrepant results appear to be a consequence of antiviral therapy or sample variability. (ref 26,23)
- Discrepant results were resolved by looking for evidence of CMV infection on other kind of patient samples (urine or broncho alveolar lavage samples) or by using another test and by reviewing patient clinical profile for other related findings as evidence of CMV infection. In addition, evidence of CMV infection was obtained from CMV serology.

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>74 18 92</td>
</tr>
<tr>
<td>-</td>
<td>9 406 415</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>83 424 507</td>
</tr>
</tbody>
</table>

- Resolved sensitivity = 74/92 = 80.4% (Confidence Interval 95% = 72.3-88.5%)
- Resolved specificity = 406/415 = 97.8% (Confidence Interval 95% = 96.4-99.2%)
- Positive predictive value 74/83 = 89.2% (Confidence Interval 95% = 82.5-95.9%)
- Negative predictive value 406/424 = 95.8% (Confidence Interval 95% = 93.8-97.7%) 

b) Study 2
- CINAkit was evaluated in a clinical study performed in one French site.
- 103 blood samples corresponding to solid organ transplant patients or HIV+ patients were evaluated in comparison to culture.

<table>
<thead>
<tr>
<th>CINAkit</th>
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<tbody>
<tr>
<td>+</td>
<td>27 1 28</td>
</tr>
<tr>
<td>-</td>
<td>8 67 75</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>35 68 103</td>
</tr>
</tbody>
</table>

- Sensitivity = 96.4 %
- Specificity = 89.3 %

- The worst specificity of the antigenemia compared to the culture is appearance and due to false negative results in culture, detected by the antigenemia and confirmed as true positive by other methods.

14.2.2. Plasma PCR comparison
- CINAkit was evaluated in a clinical study performed in one French site.
- 114 blood samples corresponding to HIV+ patients were evaluated in comparison with a plasmatic CMV PCR (Freymuth et al. J. Clin.Microbiol 1994, 32, 1614-18)

<table>
<thead>
<tr>
<th>CINAkit</th>
<th>TOTAL</th>
</tr>
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<tbody>
<tr>
<td>+</td>
<td>37 8 45</td>
</tr>
<tr>
<td>-</td>
<td>14 55 69</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>51 63 114</td>
</tr>
</tbody>
</table>

- Sensitivity = 82.22 %
- Specificity = 79.7 %

- The sensitivity compared with the PCR is 82.2 % and the specificity 79.7%. Again the lower specificity of the antigenemia is only apparent and due to a better detection sensitivity of the technique.
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16. “Classical” separation procedures

16.1. Peripheral blood leukocytes separation:
- Sedimentation separation:
  - Gently homogenize blood sample by overturning. Do not use vortex.
  - Keep the heparinized blood in an upright position for about 20 min (30 min maximum) at +37°C, to allow sedimentation (Do not completely close the cap during incubation).
  - Handle the tube gently after incubation to avoid any erythrocytes remixing.
  - Using a 1 or 2 mL pipette (+ pipet aid), gently transfer the leucocytes containing buffy coat into a 15 mL conical bottomed centrifuge tube. Collect as close as possible (1 mm) from the erythrocytes, but avoid to aspirate them.
  - Add 8 mL PBS (A).
  - Centrifuge at 220 g for 10 min at +18/22°C in a temperature controlled centrifuge.
  - Prepare the lysis solution during centrifugation (see "Reconstitution of reagents").
  - Discard the supernatant by overturning.
  - Start immediately erythrocytes lysis described hereafter.
- Dextran separation:
  - As the direct lysis procedure allows collection of all white cell populations (including lymphocytes), it may be of interest in cases of important lymphocytosis to use the Dextran separation. Dextran sedimentation allows the collection of the preferred polymorphonuclear cells, the main target cell population for the antigenemia test.
  - Gently homogenize blood sample by overturning. Do not use vortex.
  - Add 1 mL of Dextran solution (K) in a 10 mL conical bottomed centrifuge tube to 5 mL of blood.
  - Mix gently with pipet.
  - Keep the tube in an upright position for 20 min at +37°C, to allow sedimentation (Do not completely close the cap during incubation).
  - Handle the tube gently after incubation to avoid any erythrocytes remixing.
  - Using a pipet, gently transfer the polymorphonuclear cells containing supernatant (2-4 mL) into another conical bottomed centrifuge tube. Avoid to collect erythrocytes.
  - Add 8 mL of PBS (A).
  - Centrifuge at 220 g for 10 min at +18/22°C in a temperature controlled centrifuge.
  - Prepare the lysis solution during centrifugation (see "Reconstitution of reagents").
  - Discard the supernatant by overturning.
  - Start immediately erythrocytes lysis described hereafter.

16.2. Erythrocytes lysis:
- Note: The Lysis solution provided is suitable for use either in direct lysis procedure or after leukocytes separation (natural sedimentation or Dextran).
- Resuspend the pellet in 2 mL of reconstituted erythrocytes lysing solution (B).
- Mix well with pipet (avoid air bubbles) and incubate for 2 mn at room temperature.
- After incubation time add 8 mL PBS (A).
- Centrifuge at 220 g for 10 mn at +18/22°C in a temperature controlled centrifuge.
- Repeat the red cell lysis step if necessary (no more than 2 lysis in total).