Instruction manual

1. TITLE:
Complement fixation antigen Influenza A/H1N1 (CF Ag influenza A/H1N1)

2. INTENDED USE:
CF antigen of Influenza type A is intended for the detection and titration of IgG and IgM antibodies against the specific, structurally conservative antigens of Influenza virus type A H1/N1 and H3/N2 in serum samples by complement fixation reaction.
Antibodies, if present in the tested sample, reacting with the Influenza type A CF antigen indicate a previous contact with the virus or with the Influenza type A antigen.
Seroconversion or the four-fold increase of antibody titre, when comparing two separate samples (paired samples), indicates a recent infection or the post-vaccination antibody response. The latter of the paired samples is usually withdrawn 2 – 3 weeks after the first one, i.e. these samples are from the acute and the convalescent phase of infection. CF antigen of Influenza A contains the currently circulating subtype of Influenza A.

3. DESCRIPTION:
A suspension of formaldehyde inactivated Influenza virus type A particles in allantoid liquid of chicken embryos, partially purified, preserved with glycerol.

Warning: Although the infectivity of virus particles contained within the vials is greatly reduced, adhere to the health and safety instructions for working with infectious (biologically hazardous) material during handling the material (see paragraph 7).

4. PACKAGE CONTENT:
CF Ag Influenza A/H1N1, 1 ml                                           5 vials
Working dilution: min 1:32
Note: The recommended working dilution of the antigen is printed on the vial label.

5. MATERIALS REQUIRED BUT NOT PROVIDED
Microtitrate plates type „U“ (96 x 300 μl) and a suitable plate cover
Micropipettes 10 – 100 μl, 100-1000 μl
Pipette tips
Multichannel (8 or 12) pipette 20 – 200 μl
Reagent reservoirs for the multichannel pipette
Microtubes 1,5 ml, rack
Microplate shaker
Centrifuge with a swing-out rotor (1500 x g)
Graduated centrifuge tubes 15 ml
Biohazard box
Ice cubes/cooled rack
Thermal incubator 37 °C
Refrigerator +2 to +10 °C
Veronal buffer pH 7.3-7.4
Haemolytic system (sheep erythrocytes in sterile Alsever solution + haemolytic amboceptor)
Guinea-pig complement (alexin)
Controls (Influenza A antibody positive serum samples)
Distilled water
6. WORKING PROCEDURE
Warning: Validity and reproducibility of the results can only be achieved when adhering to the described procedure accurately!

6.1. Reagent preparation

6.1.1. CF antigen
- Dilute the CF antigen in Veronal buffer (VB) according to the recommended dilution ratio printed on the vial label. (recommended ratio 1:16 means to mix, for example, 10 µl of CF antigen with 160 µl of Veronal buffer).
- Store the diluted antigen in a dark place at +2 to +10 °C.
- The diluted antigen is stable for 24 hours.

6.1.2. Preparation of 2% (V/V) suspension of sheep erythrocytes
Note: Prepare only if you do not use the haemolytic system ready-to-use.

Work under sterile conditions, pipette within a biohazard box.
- Mix sheep blood with sterile Alsever’s solution 1:1 (V/V).
- Make aliquots for a single use and store them at +2 to +10 °C, up to 1 month.
- Before use aspirate plasma above the erythrocyte sediment.
- Resuspend the erythrocytes in Veronal buffer. Use twice the volume of the original blood suspension in Alsever’s solution.
- Spin the content of the tube at 1000xg/10 min at 4°C.
- Aspirate the supernatant and the buffy coat and discard.
- Repeat the steps of erythrocyte resuspension and centrifugation two more times.
- It is recommended to use a centrifuge tube with graduation since the next step is to dilute the erythrocyte sediment to form 10% erythrocyte suspension (V/V) in Veronal buffer.
- Prepare 10% (V/V) stock suspension by resuspending the sediment in nine volumes of Veronal buffer (e.g. 1 ml of sediment + 9 ml of Veronal buffer).
- Prepare 2% (V/V) suspension by dilution of the 10% (V/V) stock suspension in Veronal buffer.
- It is recommended to measure the concentration of erythrocytes by an appropriate colorimetric assay and standardize the dilution according to the measurement.
- Store at +2 to +10°C. Both, the stock and the working dilution of erythrocytes are stable for 24 hours. For longer storage of the stock suspension add antibiotics.

6.1.3. Ambroceptor stock solution
Ambroceptor solution will be used to prepare the haemolytic system, see 6.2.2 d.
- Work under sterile conditions, pipette within a biohazard box.
- Reconstitute the haemolytic amboceptor also called hemolysine (antibodies against erythrocytes), e.g. Sevapharma Hemolytic amboceptor, according to the manufacturer instructions.
- Dilute the reconstituted amboceptor 1:100 in 100 ml of sterile Veronal buffer to prepare a stock solution of amboceptor.
- Store in dark place at +2 to +10°C.
- Stability of the stock solution of amboceptor: 6 month.
- Ambroceptor dilution before the assay—see Preparation of haemolytic system 6.2.2 d)

6.2. Complement titration in the presence of antigen
Complement titration has to be done for each new lot of complement, a new lot of CF antigen or amboceptor. The aim of the titration is to determine the titre of complement in relation to the amboceptor solution.
Note: Complement is a thermo-labile complex. It is necessary to use ice cold reagents and keep all the tubes and solutions on ice during reconstitution of the freeze-dried complement and during dilutions!

Day 1
6.2.1. Complement reconstitution and its dilution
- Reconstitute freeze-dried guinea-pig complement (e.g. ALEXIN Sevapharma) in ice-cold deionized water.
- Just before use prepare a set of complement dilutions ranging from 1:10 to 1:120 in ice-cold Veronal buffer (see table below), use 1.5 ml PP microtubes placed on ice.
6.2.2. Titration of complement in the 96-well U-shape microplate in the presence of antigen

a) Pipetting scheme

<table>
<thead>
<tr>
<th>complement dilution</th>
<th>1:10</th>
<th>1:20</th>
<th>1:30</th>
<th>1:40</th>
<th>1:50</th>
<th>1:60</th>
<th>1:70</th>
<th>1:80</th>
<th>1:90</th>
<th>1:100</th>
<th>1:110</th>
<th>1:120</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALEXIN (µl)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Veronal buffer (µl)</td>
<td>100</td>
<td>200</td>
<td>300</td>
<td>400</td>
<td>500</td>
<td>600</td>
<td>700</td>
<td>800</td>
<td>900</td>
<td>1000</td>
<td>1100</td>
<td>1200</td>
</tr>
</tbody>
</table>

b) Pipetting of reagents:
- Place the microtitre plate (U-well) on ice or a cooled workbench.
- Pipette 25 µl of Veronal buffer in each well in the rows A and B.
- Pipette 50 µl of Veronal buffer in each well in row C.
- Pipette 75 µl of Veronal buffer in the wells of the first 4 columns of row D.
- Pipette 25 µl of the diluted CF antigen (see 6.1.1) to each well in rows A and B.
- Pipette 25 µl of complement dilutions to rows A, B, C (1:10 to 1:120).
  The dilution 1:10 into wells A1, B1, C1, the dilution 1:20 into A2, B2, C2, etc.
- Cover the plate with a cover

c) Incubation
- Incubate for 16 – 20 hours (overnight) at +2°C to +10°C.

Day 2
d) Dilution of Haemolytic system (HS)
  Prepare fresh HS dilution just before use.
  Dilute the stock solution of amboceptor (1:100) (see 6.1.3) to the final concentration of 2 minimal hemolytic doses per 25 µl of amboceptor. Dilute the original amboceptor according to titre declared by the producer.

Example:
Amboceptor titre declared by the producer ……1 : 6000
Stock solution: 100x diluted
Formula:
Dilution for the assay = original titre x dilution to make the stock solution x final concentration in MHD per 25 µl
Dilution for the assay = 1:6000 MHD (original titre) x 100 (dilution to make the stock solution) x 2 MHD per 25 µl (final concentration) = 1/30
- Prepare amboceptor solution for the assay by dilution of the stock amboceptor solutions (e.g. 1 ml of stock amboceptor solution + 30 ml of Veronal buffer).
- Pipette equal volume of 2% suspension of sheep erythrocytes and of amboceptor solution (2 MHD per 25 µl).

**Prepare sufficient volume of the haemolytic system: the minimal volume is calculated as the number of wells x 50 µl.**
- Vortex the suspension and let it stand for 30 min at 37°C.
- Vortex again the suspension and use it immediately.

e) **Adding the haemolytic system (HS) to the wells with complement:**
- Allow the microplate with the complement reach room temperature (~10 minutes).
- Pipette 50 µl of the freshly prepared HS to all wells.
- Mix the content of the wells in a microplate shaker.
- Cover the plate with a cover.

f) **Incubation of HS with complement**
- Incubate the plate for 30 min at 37°C. Mix the content of the plate during incubation (after 15 minutes) and after the incubation in the microplate shaker.
- Erythrocytes sedimentation
- Either 1) spin the plate in a centrifuge for 5 min/200xg or 2) let the erythrocytes sediment by gravity by incubation at +2°C to +10°C for at least 2 hours.
- Read the results immediately. Do not postpone the reading for more than 2 additional hours and in the meantime keep the plate at +2°C to +10°C.

6.2.3. **Reading and interpretation of the complement titration results**
a) **Reading the results**
- Evaluate the level of haemolysis in relation to the complement dilution, mark with symbols the presence and the amount of the erythrocyte sediment and the haemolysis level:

  **Evaluation chart**
  0 or - : without sediment, total haemolysis/ transparent, red-brownish haemoglobin solution.
  1 or + : approximately 25% of erythrocytes found in a small distinct sediment/ transparent, bright red haemoglobin solution.
  2 or ++ : approximately 50% of erythrocytes found in the sediment/ transparent, pink haemoglobin solution.
  3 or +++ : approximately 75% of erythrocytes found in the sediment/ transparent, light pink haemoglobin solution.
  4 or ++++ : 100% of erythrocytes in massive sediment/ transparent, colourless solution

b) **Interpretation:**
- **The complement titre in the presence of CF antigen (rows A, B)**
  The optimal complement titre is the highest dilution, which causes the total haemolysis (score 0/-)
  One minimal hemolytic dose (MHD) of complement is equal to the amount of complement contained within 25 µl of the complement solution diluted according to complement titre (1:6000). The optimal complement dilution is equal to 2 units of complement in 25 µl.
  **Use 2 MHD of complement in CF reactions used for serum testing (i.e. use complement in dilution 2 times lower than the original titre)**
  - The complement titre without CF antigen (row C)
  - The difference between the complement titre in rows A, B (in the presence of antigen) and in row C (without antigen) is the degree of antigen mediated inhibition of complement activity.
  - **Haemolytic system control(row D):**
    HS control wells should contain erythrocyte sediment without any traces of haemolysis (score 4)

**6.3. Serum titration by the complement fixation reaction**
Note: The procedure takes 23 – 30 hours.

Day 1

Required reagents:
- CF antigen in dilution used during the complement titration
- Complement diluted to the working dilution (2 MHD per 25 µl)
- Haemolytic system prepared according to 6.2.2.d
- Diluted serum samples and controls (positive and negative serum samples)

6.3.1. Collection and storage of serum samples
- Human blood and its derivate should be considered as infectious material.
- Haemolytic, lipaemic, icteric and microbially contaminated sera are not suitable for testing.
- Serum samples can be stored at +2 to +10°C for the short term storage (no longer than 48 h), otherwise freeze samples and keep them at −20 °C.
- Repeated freezing a thawing is not recommended.
- Paired serum samples, one from the acute and the other from the convalescent phase of the infection, are usually withdrawn two weeks apart from each other. The first sample (acute phase) have to be taken during the early acute phase. The pair sera should be tested in the same time (during the same experiment)

6.3.2. Inactivation of the complement within the tested serum samples and in controls
- We recommend including a suitable control (positive sample) in each experiment. The control is prepared from serum sample with known antibody titre.
- Inactivate samples by incubation at 56°C for 30 min in a water bath.

6.3.3. Dilution of samples
- Dilute the inactivated serum samples in Veronal buffer 1: 8 (V/V).
- The 1:8 dilutions are the primary dilution used in serum titration. This dilution is usually used in sample screening experiments and also for the test of serum complement inhibitory activity (test without presence of CF antigen in the reaction).
- Dilute sera further, reciprocally. Make the dilutions directly within the microplate wells.

6.3.4. Recommended scheme of titration for eleven (11) serum samples (including controls)
Note: We recommend including suitable controls (positive sample) in each experiment.

<table>
<thead>
<tr>
<th>Sample</th>
<th>1</th>
<th>2</th>
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<th>5</th>
<th>6</th>
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<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
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<tbody>
<tr>
<td>Serum dilution</td>
<td>1:512</td>
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<tr>
<td>Serum dilution</td>
<td>1:256</td>
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<tr>
<td>Serum dilution</td>
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<td>Serum dilution</td>
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<tr>
<td>Serum dilution</td>
<td>1:32</td>
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<tr>
<td>Serum dilution</td>
<td>1:16</td>
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<tr>
<td>Serum dilution</td>
<td>1:8</td>
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<tr>
<td>Serum dilution</td>
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</table>

- Pipette 25 µl of Veronal buffer into the columns 1 to 11 of rows A to F. The two bottom rows (G, H) remain empty.

6.3.5. Pipetting of serum samples
Note! The described protocol is related to the scheme above

- Pipette 25 µl of Veronal buffer into the columns 1 to 11 of rows A to F. The two bottom rows (G, H) remain empty.
- Pipette 25 μl of serum samples (diluted 1:8) into bottom three rows in the respective columns. Mix and dilute the samples further by multichannel pipette and sequentially transfer 25 μl from row F (1:16 dilution) up to row A (1:512 dilution). Discard the redundant 25 μl.
- Pipette 25 μl of Veronal buffer into the complement control wells in column 12/row C-H (0.5-2 MHD of complement) and 75 μl into the haemolytic system control wells column 12/row A-B.

6.3.6. Pipetting of antigen
Use the diluted CF antigen (see 6.1.1.)
- Pipette 25 μl of the diluted antigen into wells in column 1-11/rows A-G and into all the complement control wells (column 12/row C-H).
- Do not pipette the antigen into the haemolytic system control wells (column 12/row A, B).
- Do not pipette the antigen into the row H/column 1-11. Those wells serve as a control of serum mediated complement inhibition activity.
- Tap the plate to ensure the proper mixing of the reagents.

6.3.7. Complement dilution and pipetting
Warning: use ice cold reagents, keep them on ice during work, use cooling tube racks and if possible a cooled working bench.

Use the optimal complement dilution. The optimal complement dilution is determined during the complement titration in the presence of the antigen (see 6.2.). Dilute the complement in Veronal buffer to 2 MHD per 25 μl just before use.

Dilute complement solution (2 MHD per 25 μl) further in Veronal buffer in order to prepare the complement dilutions to test its haemolytic ability (complement controls 0.5-2 MHD per 25 μl).
- Mix 0.1 ml of complement (2 MHD per 25 μl) with 0.1 ml of Veronal buffer = 1 MHD per 25 μl
- Mix 0.1 ml of complement (1 MHD per 25 μl) with 0.1 ml of Veronal buffer = 0.5 MHD per 25 μl
- Pipette the complement solution (2 MHD per 25 μl) into all wells in column 1-11.
- Pipette the complement solution (2 MHD per 25 μl) into the 2 MHD complement control wells in column 12/row G, H.
- Pipette the complement solution (1 MHD per 25 μl) into the 1 MHD complement control wells in column 12/row E, F.
- Pipette the complement solution (0.5 MHD per 25 μl) into the 0.5 MHD complement control wells in column 12/row C, D.

Note: Do not pipette any complement into the haemolytic control wells (column 12/row A,B).
- Tap the plate to ensure the proper mixing of the reagents and cover the plate with a cover.

6.3.8. Incubation
- Incubate the plate for 16 – 20 hours (overnight) at +2°C to +10°C.

Day 2

6.3.9. Dilution of Haemolytic system (HS)
See the protocol in 6.2.2. d).

6.3.10. Pipetting of Haemolytic system (HS)
See the protocol in 6.2.2. e).
- Let the microplate reach room temperature (~ 10 min).
- Pipette 50 μl of HS into all wells.
- Cover the plate.
- Mix the content in a microplate shaker.

6.3.11. Incubation and reading of results
See the protocol in 6.2.2. f).
- Incubate the plate for 30 min at 37°C. Mix the content of the plate during incubation (after 15 minutes) and after the incubation in the microplate shaker.
- **Erythrocytes sedimentation**
- Either 1) spin the plate in a centrifuge for 5 min/200xg or 2) let the erythrocytes sediment by gravity by incubation at +2°C to +10°C for at least 2 hours.
- Read the results immediately. Do not postpone the reading for more than 2 additional hours and in the meantime keep the plate at +2°C to +10°C.

6.3.12. **Reading the haemolysis scale, interpretation of results and test validation criteria.**

   a) **Hemolysis scale:**
   Report the results for each serum according to the evaluation chart mentioned in 6.2.3. a).

   b) **Interpretation of results:**
   The serum titre, i.e. the titre of the specific anti-antigen complement fixating antibodies, is defined as the highest serum dilution which preserves most of the erythrocytes from lysis:
   - score +++ (approximately 75 % of erythrocytes are found in the sediment/ the solution is transparent and of light pink colour).
   - A sample is considered positive (+) if its titre is higher than 1:8.
   - A sample is considered weakly positive (+/-) if its titre is 1:8.
   - A sample is considered negative (-) if its dilution 1:8 does not bind complement (score 0, total haemolysis).

   c) **Validation of the test:**
   - **Serum inhibitory activity** (column 1-11/row H) has to be negative (score 0, total haemolysis).
     The presence of erythrocyte sediment indicates the lack of lysis due to the serum mediated and antigen independent complement binding and the probability of false positive results. Therefore it is not possible to draw any conclusion about the presence of specific antibodies in the serum sample.
   - **Haemolytic system control** (column 12/row A, B) (no serum, no antigen and no complement) has to be positive (score ++++, massive sediment without haemolysis).
     Amboceptor (hemolysin) should not caused haemolys in the absence of complement.
   - **Complement control reactions** (no serum) have to results in:
     Complement dilution: score:
     - 2 MHD 0
     - 1 MHD +
     - 0.5 MHD ++++/++++
     Excess or the lack of adequate function of complement in the optimal dilution (2 MHD per 25 µl) causes underestimation or overestimation of the serum titres.
   - **Positive control serum** (internal control, serum with known specific antibody titre, tested simultaneously with the other samples):
     Serum inhibitory activity has to be negative (row H), the titre estimated during the test should not differ from the one expected.

6.3.13. **Interpretation of results in sample screening experiments/serum titre estimation experiments**

1. The reaction detects both the IgG and IgM class of antibodies.
2. Serum samples reported as „0“ in dilution 1:8 are considered “negative”.
3. Serum samples reported as + or ++ in dilution 1:8 are considered “weakly positive” and described as serum with the titre 1:8 in the serum titration experiments (+/-).
4. Serum samples with titres 1:8 and higher are considered to be “positive” in screening experiments and positive with antibody titre 1:8 or higher in the serum titration experiments.

6.3.14. **Interpretation of results from paired serum samples**
<table>
<thead>
<tr>
<th>Result / serum titre</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. sample</td>
<td>2. sample</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1:8 +/-</td>
<td>1:8 +/-</td>
</tr>
<tr>
<td>-</td>
<td>1:8 +/-</td>
</tr>
<tr>
<td>-</td>
<td>Serum titre higher than 1:8 +</td>
</tr>
<tr>
<td>Serum titre higher than 1:8 +</td>
<td>The same titre as in the first sample</td>
</tr>
<tr>
<td>Serum titre higher than 1:8 +</td>
<td>A higher titre than that of the first sample</td>
</tr>
</tbody>
</table>

7. DIAGNOSTIC VALUE OF THE TEST
Single serum measurement can not be conclusive in determining the etiology of the disease.

It is necessary to use a wide spectrum of laboratory diagnostic methods to be certain in confirming or excluding the disease etiology.

The detection limit of the CF reaction may not be sufficient for the detection of low levels of a vaccine induced antibody response.

8. SAFETY PRECAUTIONS
Influenza A CF antigen is intended for laboratory use only
The product should be regarded as contagious and handled and disposed of according to the appropriate regulations.
Serum samples should be handled as potentially infectious material.
Do not smoke, eat or drink during work.
Do not pipette by mouth.
Wear disposable gloves while handling reagents or samples and wash your hands thoroughly afterwards.
Avoid spilling or producing aerosol.
Clean the working place with disinfectant afterwards, submerge or wash all laboratory glass and equipment that went into contact with appropriate disinfectant solution.
Dispose waste according to paragraph 9.

9. WASTE DISPOSAL

9.1. Dispose of the waste produced during the test
Waste disposal is governed by decree no. 41/2005 of Ministry of the Environment of the Czech Republic.

<table>
<thead>
<tr>
<th>Waste type</th>
<th>Code and category of the waste</th>
<th>Recommended way of decontamination and disposal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Content of microtitre wells</td>
<td>180103 N</td>
<td>Disinfection (Incidur 2%/2 hours) and autoclaving (min. 170 kPa/30 min).</td>
</tr>
<tr>
<td>Empty microtitre plate (after use) and pipette tips</td>
<td>180103 N</td>
<td>Stored in a separate container intended for storage of infectious waste. Disposed of by a specialized company (accreditation required).</td>
</tr>
</tbody>
</table>

9.2. Dispose of the CF antigen containers
According to ČSN 770052-1.-2 and the decree no. 41/2005 of Ministry of the Environment of the Czech Republic are the vials considered as the infectious waste. Decontaminate by submerging into 2% Incidur solution or autoclave at 120°C for 30 minutes. Store in a separate container intended for storage of infectious waste.

10. STORAGE AND EXPIRATION
Store the product at +2 to +10°C.
Under proper storage the product expiration is the date printed on the vial label. Sediment, which may form at the bottom of the vial, has no influence on the function of the complement fixation antigen. Shake gently the bottle before use to resuspend the sediment. Product is shipped in cooling bags. Transport duration up to 72 hours have no influence on expiration.

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English translation version 3/2011

Next recommended revision: 03/2011