IFA c-ANCA, p-ANCA - IgG

1. INTENDED USE
Immunofluorescence assay for the semi-quantitative determination of IgG-specific anti-neutrophil cytoplasmic antibodies in human serum and plasma.

2. PRINCIPLE OF THE ASSAY
The detection of antibodies is based on the principle of an Indirect Immunofluorescence Assay (IFA). The slides are coated with human neutrophil granulocytes. Any specific antibodies present in the patient’s sample are bound during the first incubation. After removing unbound material by washing, the presence of specific antibodies is detected using Anti-Human IgG-conjugate during the second incubation. Excess FITC conjugate is then removed. The formation of a stable three-part complex consisting of fluorescein antibody bound to human antibody, which is bound to antigen, can be visualized with the aid of a fluorescence microscope.

3. DIAGNOSTIC RELEVANCE AND INTERPRETATION OF RESULTS
ANCA are present in a variety of vasculitic diseases, these diseases are represented in the tabulation with their characteristic fluorescence pattern and antigen. Both types of ANCA are detectable on ethanol fixed neutrophil granulocytes, cANCA show a granular homogen cytoplasmic fluorescence and no nuclear staining whereas pANCA show a sharply delineated perinuclear staining and no cytoplasmic fluorescence (perinuclear ANCA).

<table>
<thead>
<tr>
<th>Disease pattern</th>
<th>Fluorescence pattern</th>
<th>Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wegener’sche granulomatosis</td>
<td>cANCA, rare pANCA</td>
<td>PR3, rare MPO</td>
</tr>
<tr>
<td>Microscopic polyangiitis</td>
<td>cANCA, pANCA</td>
<td>PR3, MPO</td>
</tr>
<tr>
<td>Churg-Strauss syndrome</td>
<td>ANCA</td>
<td>MPO</td>
</tr>
<tr>
<td>Polymyositis nodosa</td>
<td>ANCA (low prevalence)</td>
<td>rare PR3 or MPO</td>
</tr>
<tr>
<td>Rheumatoid Arthritis</td>
<td>ANCA, atypical ANCA</td>
<td>rare MPO, Laktoteren</td>
</tr>
<tr>
<td>LED</td>
<td>ANCA</td>
<td>rare MPO, Laktoteren</td>
</tr>
<tr>
<td>Colitis ulcerosa (Prävalenz 67%)</td>
<td>pANCA</td>
<td>ANCA, atypical ANCA</td>
</tr>
<tr>
<td>Primär skleros. Cholangitis</td>
<td>ANCA</td>
<td>ANCA, atypical ANCA</td>
</tr>
<tr>
<td>Crohn’s disease (prevalence 7%)</td>
<td>ANCA</td>
<td>ANCA, atypical ANCA</td>
</tr>
<tr>
<td>Autoimmune Hepatitis</td>
<td>ANCA, atypical ANCA</td>
<td></td>
</tr>
</tbody>
</table>

cANCA is associated with a high sensitivity and specificity for Wegener’sche granulomatosis (prevalence 80-95 %), the titre correlates with the course of the disease. In some cases cANCA is detectable at Microscopic Arteritis and Polyarteritis nodosa. The major cANCA target antigen is Proteinase 3.

pANCA is associated with a high sensitivity and specificity for Mikroskopische polyangiiitis und Churg-Strauss syndrom. The major pANCA target antigen is thought to be Myeloperoxidase (MPO).

80% of all patients with a positive pANCA titre show a histological manifested Vasculitis, Colitis Ulcerosa (CU) or PSC.

4. PERFORMANCE CHARACTERISTICS
Specificity / Sensitivity
129 samples were tested parallel in IFA c-ANCA, p-ANCA - IgG and comparison methods (IFA). The specificity and sensitivity are based on the results found.

Specificity ANCA: 91 %
Sensitivity ANCA: 100 %
The results refer to the groups of samples investigated.

Precision and reproducibility
Studies were performed to demonstrate intra and inter-assay variability. Four ANCA positive (two each of c- and pANCA) and one ANCA negative sera were tested starting at a 1:20 dilution to endpoint. They were tested on 5 different lots of slides for four days to determine intra- as well as inter-reproducibility. Negative samples remained negative and positive samples provided the expected titre.

Cross reactivity
Antinuclear antibodies (ANA) may cause positive reactions with the ethanol-fixed neutrophils and might therefore be misinterpreted as positive ANCA reactivity.

5. KIT COMPONENTS
Number and volume of the kit components are indicated on kit label.

1. SLIDES slides coated with neutrophil granulocytes
2. FITC ANTI-HUMAN CONJUGATE ready-to-use anti-human IgG conjugate preservative: <0,1% sodium azide 2,5 ml (IF 190); 2x2,5 ml (IF 191)
3. NEGATIVE CONTROL human plasma (pANCA positive) ready to use preservative: <0,1% sodium azide 0,5 ml (IF 190); 1 ml (IF 191)
4. POSITIVE CONTROL human plasma (cANCA positive) ready to use preservative: <0,1% sodium azide The titre is reported on the label. 0,5 ml (IF 190); 1 ml (IF 191)
5. POSITIVE CONTROL human plasma (cANCA positive) ready to use preservative: <0,1% sodium azide The titre is reported on the label. 0,5 ml (IF 190); 1 ml (IF 191)
6. MOUNTING MEDIUM ready to use 3 ml (all kit size)
7. PBS-BUFFER powder 2x (all kit size)
8. EVANS BLUE ready to use 3 ml (all kit size)
9. Dilution Buffer ready-to-use preservative: <0,1% sodium azide. 20 ml (all kit size)
The safety data sheet (MSDS) is available upon request.

7. STORAGE AND STABILITY
Store all reagents at 1-2°C. Protect them from intense light and do not freeze. The expiration date of each component is indicated on the respective vial label. Do not use reagents beyond the expiration date. The diluted BUF/PBS is stable up to 4 weeks when stored at 1-2°C. Use only SLIDES with an intact vacuum packaging.

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8. MATERIALS AND EQUIPMENT REQUIRED BUT NOT PROVIDED
- distilled water
- graduated cylinder
- coplin jars or staining dishes
- plastic squeeze bottle
- test tubes for sample dilution
- cover slips (24x60 mm)
- volumetric pipette (10 – 1000 µl) with pipette tips
- moist chamber
- timer
- fluorescence microscope with FITC system,
  (excitation wavelength 490 nm, emission wavelength 510 nm)

9. WARNINGS OR PRECAUTIONS
SAFETY PRECAUTIONS
The IFA test is for [IVD] use only.
1. Only qualified and well-trained employees should carry out the assay procedure.
2. The instruction for use describes the applicable test method. In case of modification or applications others than the intended use, or the use of automatic processors, the user has to validate the procedure and take the responsibility for it.
3. Do not mix lot specific reagents, such as [SLIDES], controls and [CONJ|FITC] from different kit lots. [BUF|PBS], [MM], [EVBL] and [SPE|DIL] can be used for all IFA tests.
4. Seal all bottles properly after use in order to avoid bacterial contamination. All samples and kit components should be considered potentially infectious. All control samples have been tested for Hepatitis B antigen, anti-HIV 1 and 2, anti-HCV (CE/IFA) and found to be negative.
5. The coated [SLIDES] are inactivated. However, normal laboratory precautions should be maintained when handling with infectious material. Do not pipette by mouth.
6. Avoid contact with skin and mucous membranes when handling reagents, which contain preservatives (see kit contents). Wash thoroughly with water in case of contact.
7. Controls containing sodium azide may react with lead and copper plumbing, building up explosive metal acids. Flush with sufficient water when disposing of reagents.
8. For disposal the legal regulations have to be followed.

10. SPECIMEN COLLECTION AND STORAGE
1. Microbial contaminated specimen may cause interference.
2. Lipoaemic, haemolytic or icteric samples should only be tested with reservations although in our testing no negative influence has been found.
3. Suitable specimens are serum or plasma (heparinized, EDTA) samples obtained by standard laboratory techniques.
4. The samples should not be heat-inactivated since non-specific results may occur.
5. Patient samples should be stored at 2 – 8°C. For long-term storage -20°C or lower is recommended. Avoid repeated freeze-thaw cycles.
6. Note: Diluted patient samples must be used on the same day.

11. REAGENT PREPARATION
Bring all reagents to room temperature prior to use!
Prepare [BUF|PBS]:
Completely dissolve one vial of [BUF|PBS] in 1 litre of distilled water.
Reconstituted buffer solution should have a pH of 7.3 to 7.6.
Dilution of samples: Dilute patient sample with [SPE|DIL] according to test demands (screening titre).
Controls are ready to use.

12. ASSAY PROCEDURE PIPETTING AND INCUBATION STEPS
Screening Titre: 1:20
Dilute samples with [SPE|DIL] !
Note! Do not allow wells to dry at any time during the test procedure!
1. Take required [SLIDES] out of the foil packets shortly before use and identify slides using a permanent marking pen. Do not touch the wells.
Recommended test for IF 190, IF 191:
well 1 ethanol fixed: [CONTROL|H] [CONJ|FITC] [EVBL]
well 1 formalin fixed: [CONTROL|H] [CONJ|FITC] [EVBL]
well 2 ethanol fixed: [CONTROL|H] [CONJ|FITC] [EVBL]

2. First incubation:
Pipeette 1 drop of each control and 20 – 50 µl of each diluted sample onto the respective wells (covered completely) being careful not to touch cell substrate with pipette tip. The controls should be carried out for every test run. Place [SLIDES] into a well-closed moist chamber to prevent drying.
Incubate [SLIDES] for 30 minutes at room temperature.

3. First wash step:
Rinse the [SLIDES] gently with [BUF|PBS]. Do not focus the buffer stream directly onto the wells. To prevent cross contaminations avoid rinsing from one well across other wells. For ten-wells slides run PBS-stream from the midline of the slides successive along both rows to the edge of the [SLIDES].
Soak the [SLIDES] three times for 5 minutes with [BUF|PBS] in a coplin jar or staining dish with [BUF|PBS].

4. Second incubation:
After the washing procedure, shake off excess [BUF|PBS] (if necessary use blotting paper to draw off excess fluid but do not touch the wells directly) and place [SLIDES] back into moist chamber. Immediately add 1 drop of [CONJ|FITC] to each well.
Incubate [SLIDES] for 30 minutes at room temperature in the dark.

5. Second wash step:
see First wash step.
6. Counter stain:
7. Remove [SLIDES] from the staining dish, rinse briefly with [BUF|PBS], shake off the excess [BUF|PBS] and apply 2-3 drops of [MM] across the slides. Gently lower the cover slip from the bottom to the top of the slides, avoid air bubbles.
8. Read [SLIDES] within 30 minutes at 400-800x total magnification, using a fluorescence microscope (FITC filter combination). Avoid longer exposition of one field of vision to minimize bleaching of FITC fluorescence.

13. SUGGESTIONS FOR TROUBLESHOOTING
In case that the IFA instructions are followed strictly, the reagents are handled with care and the samples and reagents are pipetted carefully, the following kinds of errors can be avoided to a large extend.

<table>
<thead>
<tr>
<th>ERROR</th>
<th>POSSIBLE CAUSES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross contamination</td>
<td>- Too much test material</td>
</tr>
<tr>
<td>- Fluid remaining between the wells, should be carefully dried around outside edges if necessary</td>
<td></td>
</tr>
<tr>
<td>Too few cells or substrate</td>
<td>- Cell lysis following prolonged contact with deionised water (observe the wash procedure)</td>
</tr>
<tr>
<td>- Buffer squirted directly on the substrate in the well (observe the wash procedure)</td>
<td></td>
</tr>
<tr>
<td>- Proteolytic enzymes have attacked the substrate</td>
<td></td>
</tr>
<tr>
<td>- While placing the cover slip on [SLIDES] the monolayer was disturbed</td>
<td></td>
</tr>
<tr>
<td>Inhomogeneous fluorescence</td>
<td>- Sample dried in the well, fluorescence stronger at the edge (moist environment)</td>
</tr>
<tr>
<td>- Sample does not cover the test well (air bubbles/ completely cover well with sample)</td>
<td></td>
</tr>
<tr>
<td>- Buffer crystals on the [SLIDES] (wash)</td>
<td></td>
</tr>
<tr>
<td>- Microscope incorrectly adjusted (check the adjustment of the microscope)</td>
<td></td>
</tr>
<tr>
<td>Unclear picture</td>
<td>- Too much [MM] or air pockets</td>
</tr>
<tr>
<td>- Microscope is dirty (cleaning)</td>
<td></td>
</tr>
<tr>
<td>Little or no fluorescence</td>
<td>- Microbial contamination of the sample or [CONJ</td>
</tr>
<tr>
<td>- Microscope not adjusted</td>
<td></td>
</tr>
<tr>
<td>- pH-value (7.3 – 7.6) of [BUF</td>
<td>PBS] too low</td>
</tr>
<tr>
<td>- [CONJ</td>
<td>FITC] exposed to light (store [CONJ</td>
</tr>
<tr>
<td>Background fluorescence</td>
<td>- [SLIDES] dried in a hot air stream (do not use a hairdryer and do not let the wells dry)</td>
</tr>
<tr>
<td>- Microbial contaminated specimen</td>
<td></td>
</tr>
<tr>
<td>- Marking the [SLIDES] with a wax pencil produces a film on the [SLIDES] (use a waterproof glass marker)</td>
<td></td>
</tr>
</tbody>
</table>
14. VALIDITY OF THE ASSAYS

All controls should be carried out with every test run. The Negative Control shows no specific neutrophil staining. The fluorescence intensity of \text{CONTROL}[+]\text{c} and \text{CONTROL}[+]\text{c} on granulocytes is shown in the Quality Control Certificate. The specific cytoplasmic fluorescence of the \text{CONTROL}[+]\text{c} is visible on the formalin-fixed granulocytes as well, whereas the perinuclear fluorescence pattern of \text{CONTROL}[+]\text{c} changes into a cytoplasmic fluorescence. If controls give no valid levels then results from test samples are not valid too and retesting is required.

15. READING OF THE RESULTS

**Fluorescence intensity:**

The fluorescence intensity may be semi-quantified following our introduction:

- **3+ - 4+** = maximal fluorescence, brilliant yellow-green
- **2+** = less brilliant yellow-green fluorescence
- **1+** = definite but dull yellow-green fluorescence
- **Equivocal – (+)** = very dim yellow-green fluorescence

The degree of intensity is not of clinically relevance and has only limited value as an indicator of titre. Differences in microscope optics, filters and light source may result in differences of +1 or more in intensity.

**Interpretation of results**

A sample is considered negative (titre < 1:20) if no specific neutrophil staining is observed. Positive results should be interpreted regarding the titre and the specific fluorescence pattern of cANCA and pANCA.

**ANCA differentiation**

<table>
<thead>
<tr>
<th>Type of antibodies</th>
<th>Fluorescence pattern on ethanol fixed wells</th>
<th>Fluorescence pattern on formalin fixed wells</th>
</tr>
</thead>
<tbody>
<tr>
<td>cANCA cytoplasmic or “classic” ANCA</td>
<td>Diffuse granular, in part central accentuated cytoplasmic fluorescence, negative staining of nucleus</td>
<td>Diffuse granular, in part central accentuated cytoplasmic fluorescence, negative staining of nucleus</td>
</tr>
<tr>
<td>pANCA perinuclear ANCA</td>
<td>Predominately perinuclear fluorescence, negative staining of Cytoplasm</td>
<td>Diffuse granular, in part central accentuated cytoplasmic fluorescence, negative staining of nucleus</td>
</tr>
<tr>
<td>atypical ANCA Mixtype ANCA/ANA</td>
<td>Cytoplasmic and perinuclear, nuclear fluorescence</td>
<td>Cytoplasmic and perinuclear, nuclear fluorescence</td>
</tr>
<tr>
<td>Antibacterial antibodies</td>
<td>Nuclear fluorescence, negative staining of Cytoplasm</td>
<td>Nuclear fluorescence, negative staining of Cytoplasm</td>
</tr>
</tbody>
</table>

ANA reactions may sometimes be confused with or mimic pANCA staining, it is recommended to differentiate these samples on formalin fixed slides.

A. **SEMIQUANTITATIVE TITRE**

For the semi-quantification of the test results the \text{CONTROL} and the \text{CONTROL}[+]\text{c} and/or \text{CONTROL}[+]\text{c} must be tested with every test run. The fluorescence intensity of the sample can then be compared visually with the fluorescence intensity of the \text{CONTROL} and/or \text{CONTROL}[+]\text{c}.

B. **QUANTITATIVE TITRE**

For quantification of the test results, the samples must be diluted to the endpoint titre. The endpoint titre is determined as the last dilution in which an **equivocal – (+)** reaction is visible.

Sample dilution:

If the fluorescence intensity of the sample is < the fluorescence intensity of the Positive control, please dilute the sample to the endpoint titre of the Positive control (the titre is reported on the label).

If the fluorescence intensity of the sample is ≥ the fluorescence intensity of the Positive control, please dilute the sample at least two dilutions higher than the endpoint titre of the Positive control (the titre is reported on the label).

Regarding diagnostic relevance and interpretation of results see page 1

For further information please visit our website: http://www.viro-immun.de/