INSTRUCTION MANUAL

REF 87061

October 22, 2010

cANCA IFA plus

- 60 determinations -

CE

IVD *In vitro* diagnostic device

Indirect immunofluorescence assay for the determination of IgG antibodies to neutrophil cytoplasmic antigens (ANCA) human serum

Substrate: human granulocytes, ethanol fixed

REF	Catalogue number	LOT	Batch code
	Consult accompanying documents		Manufactured by
	Temperature limitation		Use by
	Consult operating instruction	D	Biological risk

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INTENDED USE

cANCA IFA plus is used for the sensitive qualitative and semi-quantitative determination of IgG antibodies to neutrophil cytoplasmatic antigens (ANCA) in human serum using indirect immunoflorescence assay on ethanol fixed human granulocytes for the differential diagnosis of systemic vasculitis (SV).

Pathogenesis of SV is characterized by inflammatory processes of different blood vessel walls and resulting morphological alterations. Both, arteries and veins can be affected simultaneously. The clinical picture is mainly characterized by general symptoms like exhaustion, fever and weight loss. During the further course of disease symptoms vary dependent on which kind of vessels are affected.

For the serological diagnosis of SV anti-neutrophil cytoplasmic antibodies (ANCA) play an important role. These antibodies are usually determined by indirect immunofluorescence using ethanol fixed human neutrophiles. Depending on the immunofluorescence pattern cytoplasmic ANCA (cANCA) and perinuclear ANCA (pANCA) are distinguished. During fixation ethanol destroys granular membranes in neutrophil cytoplasm and positively charged proteins move to the negatively charged nucleus. Ethanol fixed cells can be used as ANCA screening substrate: cANCA antibodies are demonstrated by a cytoplasmic fluorescence pattern, whereas pANCA antibodies must be differentiated from antibodies to nuclear antigens (ANA) which also show a (peri)nuclear pattern. Confirmation of pANCA pattern is done using formalin fixed granulocytes, on this substrate all ANCA show a cytoplasmic fluorescence pattern:

	Granulocytes ethanol fixed	Granulocytes formalin fixeg
cANCA	cytoplasmic	cytoplasmic
pANCA	perinuclear	cytoplasmic
ANA	(peri)nuclear	(peri)nuclear (reduced fluorescence)

Proteinase 3 (PR3) has been identified as the responsible autoantigen causing the cANCA pattern. PR3 is a serine proteinase with a molecular weight of 29 kDa localized in the primary granule of myeloid cells. PR3 autoantibodies are described as pathognomonic for Wegener's granulomatosis. The pANCA pattern is mainly caused by the reactivity of myeloperoxidase (MPO) which is a cationic protein of a molecular weight of 146 kDa found in azurophilic granules. MPO autoantibodies occur in a variety of vasculitides such as microscopic polyangiitis, Churg-Strauss syndrome and Polyateritis nodosa. Beside these additional antigens are targets for cANCA and pANCA antibodies in patients suffering from primary sclerosing cholangitis and ulcerative colitis.

PRINCIPLE of the TEST

cANCA IFA plus is an indirect immunfluorescence assay for the determination of IgG autoantibodies to neutrophil cytoplasmatic antigens (ANCA) in human serum using ethanol fixed human granulocytes.

The antibodies of the diluted patient samples and controls react specifically with the antigens of the cell culture immobilized on the slides. After an incubation period of 30 min at room temperature (RT), unbound serum components are removed by a wash step.

The bound antibodies react specifically with anti-human IgG conjugated to Fluorescein-isothiocyanat (FITC). After an incubation period of 30 min at RT excessive conjugate is separated from the solid-phase immune complexes by an additional wash step.

Stained slides are read using a fluorescence microscope (excitation wavelength 490 nm, emission wavelength 520 nm). According to the histologic alignment of antigens in ethanol fixed granulocytes the specific cytoplasmic or perinuclear fluorescence staining can be detected.

PATIENT SAMPLES

Specimen collection and storage

Blood is taken by venipuncture. Serum is separated after clotting by centrifugation. The samples may be kept at 2 - 8 °C for up to two days. Long-term storage requires - 20 °C. Repeated freezing and thawing should be avoided. If samples are to be used for several assays, initially aliquot samples and keep at - 20 °C.

Lipaemic samples could bring about a film covering the cell substrate and should not be used. Contaminated samples should be avoided as they may contain proteolytic enzymes which might digest the cell substrate.

Preparation before use

Allow samples to reach room temperature prior to assay. Take care to agitate serum samples gently in order to ensure homogeneity.

Screening: Patient samples have to be diluted **1:20** (v/v) prior to the assay, e.g. **10 µl sample + 190 µl Sample diluent (B)**.

Titration: prepare e.g. a 4-fold serial dilution based on the **1:20** (v/v) dilution in **Sample diluent (B))**, e.g. **50 µl sample dilution + 150 µl Sample diluent (B),** resulting the following dilutions: 1:20, 1:80, 1:320, 1:1280, etc.

TEST COMPONENTS for 60 determinations

A (8706) Ag 6	**Substrate slides** 6 wells coated with human granulocytes, ethanol fixed	10 sealed in a foil pouch
B (8701) DIL	**Sample diluent**	15 ml ready for use capped white
C (9018) BUF PBS	**PBS Buffer** for 2 x 1000 ml PBS solution	2 x 10 g dry substance
D (8023) CONJ	**Conjugate** anti-human IgG (sheep), labeled to FITC	2.5 ml ready for use dropper bottle capped blue
E (8008) MOUNT	**Mounting medium** glycerol solution, PBS buffered, pH 7,4 ± 0,2	3.5 ml ready for use dropper bottle capped white
F (8030) TEMP	**Blotting templates**	10
P (8708) CONTROL	**cANCA Positive control** (diluted human serum) +	0.5 ml ready for use dropper bottle capped pink
N (8709) CONTROL	**Negative control** (diluted human serum) –	0.5 ml ready for use dropper bottle capped green

Materials required

- micropipettes (10, 100, 1000 µl)
- disposable pipette tips
- disposable test tubes and rack
- graduated cylinders, volumetric flasks
- moist chambers
- plastic squeeze wash bottle
- coplin jars or staining dishes with slide racks
- distilled (or de-ionized) water
- fluorescence microscope (excitation wavelength 490 nm, emission wavelength 520 nm)

Size and storage

cANCA IFA plus (87061) has been designed for 60 determinations.

The expiry date of each component is reported on its respective label, that of the complete kit on the box label. Upon receipt, all components of the cANCA IFA plus have to be kept at 2 - 8 °C, preferably in the original kit box.

After opening all kit components are stable for at least 2 months, provided proper storage.

Preparation before use

Allow all components to reach room temperature prior to use in the assay.

The substrate slides are individually covered in a sealed pouch. Allow the slides to reach room temperature before opening.

PBS buffer preparation:
Place content of a one-liter PBS packet into one-liter volumetric flask, add distilled water to the mark. Dissolve dry substance by stirring or shaking. Reconstituted buffer solution should have a pH of 7.4 ± 0.2.

Store the solution in a clean bottle at 25°C or lower. Stable for at least two months. Do not use if pH changes, if the solution turns cloudy, or if a precipitate forms.

Avoid exposure of the conjugate to light.

ASSAY PROCEDURE

- **Dilute patient sera according to test demands (screening, titration) with sample diluent (B)**
- **Do not allow the substrate slides to dry during the test procedure**

1. Bring all reagents to room temperature (18…25°C) before use. Mix gently without causing foam. Remove slides from pouch immediately before use and identify slides using a permanent marking pen.
2. Apply
   1 drop (25 µl) controls (P, N)
   25 µl diluted patient samples
   onto the respective wells. Completely cover the immobilised tissue section. Do not touch antigen surface.
3. Incubate **30 min** at RT (20…25°C) in a moist chamber.
4. Rinse gently with PBS solution (made of C) using a squeeze wash bottle. Do not focus the PBS stream directly onto the wells. To prevent cross contaminations avoid rinsing from one well across other wells. For multi-row slides run PBS stream from the midline of the slide successively along both rows to the edge of the slide.
5. Wash **2 x 5 min** in changing PBS solution in Coplin jars or staining dishes, agitate gently at entry and prior to removal.
6. Remove slides from the wash one at a time, shake off excess PBS tapping the edge of the slide onto absorbent towel, carefully dry around the wells using the template (F). Apply **1 drop (25 µl)** of conjugate (D) to each well of the slides, making sure each well is completely covered.
7. Incubate **30 min** at RT (20-25°C) in a moist chamber, protected from direct light.
8. Rinse gently with PBS solution (made of C) using a squeeze wash bottle as described in 4.
9. Wash **2 x 5 min** in changing PBS solution in Coplin jars or staining dishes, agitate gently at entry and prior to removal.
10. Remove slides from the wash one at a time, shake off excess PBS tapping the edge of the slide onto absorbent towel, carefully dry around the wells using the template (F), apply **2-4 drops** of mounting medium (E) across the slide. Rest the edge of a coverslip against the bottom of the slide allowing the mounting medium to form a continous bead between coverslip and slide. Gently lower the coverslip from the bottom to the top of the slide, avoid air bubbles. Drain excess mounting medium from the edge of the slide with absorbent papaer.
11. Read stained slides using a fluorescence microscope. Avoid longer exposition of one field of vision to minimize bleaching of FITC fluorescence.

Preservation of slides

It is recommended that slides are examined at the same day they are stained. If any delay is anticipated, store slides in a refrigerator (2-8°C) for some days. For long-term preservation, seal edges of slides using nail-varnish, store slides at –20°C.

READING of the RESULTS

Fluorescence intensity

Fluorescence intensity may be semi-quantitated following the guidelines established by the CDC, Atlanta, USA (8):
4+ = maximal fluorescence, brilliant yellow-green
3+ = less brilliant yellow-green fluorescence
2+ = definite but dull yellow-green fluorescence
1+ = very dim subdued fluorescence

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

Negative result

A serum dilution is considered ANCA negative if the fluorescence intensity is less than 1+ and the cells lack the specific fluorescence pattern (cANCA, pANCA).

Positive result

A serum dilution is considered ANCA positive if a fluorescent staining at an intensity of 1+ or greater is seen in the cytoplasm (cANCA) or in perinuclear region (pANCA) of the granulocytes. Cytoplamic pattern demonstrates the presence of cANCA. pANCA antibodies must be confirmed using formalin fixed granulocytes (**pANCA IFA plus, REF 87161**) since perinuclear pattern can also be caused by antinuclear antibodies.

Titration

If semi-quantitative titration is performed, the result should be reported as the reciprocal of the last dilution in which 1+ apple-green fluorescent intensity with a clearly discernable staining pattern is detected.

Using the recommended fourfold serial dilution the endpoint titer can be extrapolated:

1:10 = 3+
1:40 = 2+
1:160 = +/-
1:640 = - The extrapolated titer is 80.

REFERENCE VALUES

ANCA	Titer
negative	**< 20**
positive	**≥ 20**

It is recommended that each laboratory establishes its own normal and pathological ANCA reference ranges for serum levels as usually done for other diagnostic parameters, too.

Test validity

Both the positive and negative control provided in the test kit must be included in each test run. These controls must be examined prior to reading test samples and should demonstrate the following results:

Negative control: The cells should exhibit less than 1+ fluorescence with lack of specific fluorescent staining. The cells could appear reddish-orange due to the counterstain used.

Positive control: cytoplasmic fluorescence of the granulocytes.

If the above mentioned quality criteria are not met, repeat the test and make sure that the test procedure is followed correctly (incubation times and temperatures, sample and wash buffer dilution, wash steps etc.). In case of repeated failure of the quality criteria contact your supplier. A troubleshoting guide is available to check laboratory procedure.

Limitations of Method

cANCA can be demonstrated by indirect immunofluorescence on ethanol fixed granulocytes. A (peri)nuclear fluorescence can also be found in ANA positive sera, pANCA therefore must be differentiated from ANA and confirmed using formalin fixed granulocytes. Confirmation of results using HEp-2 cells is also recommended.

Endpoint titer determination may vary depending on type and condition of the fluorescence microscope used and depending on subjective judgement of different observers.

Samples and wash solutions contaminated with bacteria or fungi could cause unspecific staining of the cell culture substrate.

Proteolytic enzymes in patient samples could result in a damage or loss of the tissue sections fixed on the slide.

Any clinical diagnosis should not be based on the results of in vitro diagnostic methods alone. Physicians are supposed to consider all clinical and laboratory findings possible to state a diagnosis.

CHARACTERISTIC ASSAY DATA

Clinical evaluation

cANCA: 44 sera of patients with Wegener's granulomatosis (WG, clinically confirmed), 398 sera without diagnosis WG

		Wegener's granulomatosis	
		diagnosed +	**diagnosed -**
cANCA IFA plus	**cANCA +**	38	52
	cANCA -	6	346

Sensitivity: 86,4%
Specificity: 86,9%
Agreement: 86,9%

pANCA: 66 sera mit clinically diagnosed idiopatic glomerulonephritis (IGN), Polyarteritis nodosa (PN), pulmorenale syndrome (PRS), 376 sera without clinically diagnosed vasculitis

		IGN, PN, PRS	
		diagnosed +	**diagnosed -**
cANCA IFA plus	**pANCA +**	50	51
	pANCA -	16	325

Sensitivity: 86,4%
Specificity: 75,8%
Agreement: 84,8%

Specificity

A series of 100 normal blood donors were tested and found negative in cANCA IFA plus assay, a specificity of 100% was found.

Precision and Reproducibility

With this immunofluorescence assay, no difference in the interassay and interlot variability by using the controls could be detected.

INCUBATION SCHEME

cANCA IFA plus (87061)

Dilute patient sera: screening dilution / endpoint titration using sample diluent (B)

1	Bring all test reagents and slides to room temperature (20-25°C)		
		Controls	Patient samples
2	Dispense Controls P, N	1 drop (25 µl)	
	Diluted patient samples		25 µl
3	Incubate	30 minutes, room temperature (20-25°C)	
4	Rinse with PBS solution (made of C)		
5	Wash	2 x 5 minutes in changing PBS solution (made of C)	
6	Dispense Conjugate (D)	1 drop (25 µl)	1 drop (25 µl)
7	Incubate	30 minutes, room temperature (20-25°C)	
8	Rinse with PBS solution (made of C)		
9	Wash	2 x 5 minutes in changing PBS solution (made of C)	
10	Place coverslip; 3-4 drops Mounting medium (E) per slide, lower the coverslip (G) gently		
11	Read using a fluorescence microscope		

SAFETY PRECAUTIONS

- **This kit is for in vitro use only**. Follow the working instructions carefully. GA GENERIC ASSAYS GmbH and its authorized distributors shall not be liable for damages indirectly or consequentially brought about by changing or modifying the procedure indicated. The kit should be performed by trained technical staff only.
- The expiration dates stated on the respective labels are to be observed. The same relates to the stability stated for reconstituted reagents.
- The substrate slides are individually covered in a sealed pouch. Do not use if pouch has been punctured.
- Mixing of reagents from different kit lots and from other manufacturers could lead to differences in assay results.
- Avoid time shift during pipetting of reagents.
- All reagents should be kept at 2 - 8 °C before use in the original shipping container.
- Some of the reagents contain small amounts of Sodium azide (< 0.1 %) as preservative. They must not be swallowed or allowed to come into contact with skin or mucosa. Sodium azide may react with lead and copper plumbing building highly explosive metal azides. Flush with sufficient water when disposing of reagents to prevent potential residues in plumbing.
- Source materials derived from human body fluids or organs used in the preparation of this kit were tested and found negative for HBsAg and HIV as well as for HCV antibodies. However, no known test guarantees the absence of such viral agents. Therefore, handle all components and all patient samples as if potentially hazardous.
- Since the kit contains potentially hazardous materials, the following precautions should be observed:
  - Do not smoke, eat or drink while handling kit material,
  - Always use protective gloves,
  - Never pipette material by mouth,
  - Wipe up spills promptly, washing the affected surface thoroughly with a decontaminant.

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