INSTRUCTION MANUAL

REF **8063**

20th August 2013

CytoBead® ANCA

- 48 Determinations -

IVD *In-vitro* diagnostic test

CE

Indirect immunfluorescence test for the determination of IgG antibodies against neutrophil cytoplasmic antigens (ANCA) in human serum

Substrate: human granulocytes, ethanol fixated; PR3 and MPO coated beads

REF	Product no.	**LOT**	Lot no.
	See kit insert		Manufacturer
	Storage temp.		Expiry date
	See kit insert	D	Biological hazard

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INSTRUCTIONS FOR USE

CytoBead® ANCA is a reagent set for the determination (qualitative and semi-quantitative) of IgG autoantibodies against antigens in cytoplasmic neutrophil granulocytes (ANCA) in human serum for the diagnosis of systemic vasculitides (SV). This measurement is obtained using indirect immunofluorescence on ethanol fixated human granulocytes and furthermore allows the differentiation of MPO and PR3 specificity by means of multiplex bead technology.

TEST PRINCIPLE

CytoBead® ANCA is an indirect immunofluorescence test for the qualitative and semi-quantitative determination of IgG autoantibodies (AAb) against cytoplasmic antigens of neutrophil granulocytes (ANCA) in human serum. Ethanol fixated human granulocytes allow the determination of the fluorescence pattern (cANCA, pANCA), while antigen coated beads allow the measurement of antibodies against PR3 and MPO.

In the first reaction step AAb in diluted patient samples and controls react specifically with antigens in the cells and on the beads. Unbound components are removed by a wash step following an incubation of 30 minutes at room temperature.

The bound AAb specifically react in the second reaction step with anti-human IgG antibodies, which are coupled with the fluorescence molecule AlexaFluor®488. Excess conjugate molecules are seperated from the immune complexes bound to the solid plase by a further wash step, after 30 minutes incubation at room temperature.

When mounted, the slides are read under a fluorescence microscope (excitation wavelength 490nm, emission wavelength 520nm). According to the histological organization of antigens in the ethanol fixated granulocytes, ANCA positive samples are assigned a specific fluorescence pattern: cytoplasmic or perinuclear.

PATIENT SAMPLES

Separation and storage

Collect blood through venipuncture, allow to clot and isolate the serum by centrifugation. Storage for up to 3 days at 2 - 8 °C is possible, for longer storage samples must be frozen at - 20 °C. Samples should be aliquotted before freezing as repeated freeze-thaw cycles should be avoided.

Lipemic samples must not be used, as a fatty film can obscure the substrate. Contaminated samples can contain proteolytic enzymes which could lead to digestion of the substrate, and therefore may not be used.

Preparation and use

Before use in CytoBead® ANCA, bring sera to room temperature. Mix shortly by vortexing to ensure homogeneity.

Screening: **qualitative**

Dilute patient samples to be tested **1:20** (v/v), eg. **10 µl sample + 190 µl dilution buffer (B)**. 50 µl of diluted patient serum is needed per well.

Titration *(recommended four-fold dilution series)*: **semi-quantitative**

Following a sample dilution of **1:10** (eg. **20 µl sample + 180 µl dilution buffer (B)**) samples are diluted in a 1:4 titration series, eg. **50 µl sample + 150 µl dilution buffer (B),** so that the following titration dilutions are given:
1:10 → 1:40 → 1:160 → 1:640.

TEST COMPONENTS for 48 determinations

A Ag 8	**Slides** 8 wells coated with human granulocytes (ethanol fixated) and beads coated with PR3 or MPO	6 Individually sealed
B DIL	**Dilution buffer** Finished solution	15 ml Ready to use White cap
C BUF PBS	**PBS Puffer** To make 2 x 1000 ml solution	2 x 10 g Solid substance
D CONJ	**Conjugate** Anti-human-IgG (heavy and light chain specific), AlexaFluor®488 labelled	3.0 ml Ready to use Screw-top bottle Blue cap
E MOUNT	**Mounting medium** Glycerin solution, phosphate buffered, pH 7.4 ± 0.2	3.5 ml Ready to use Dropper bottle White cap
F TEMPL	**Blotting paper templates**	6 x
G COVER	**24 x 70 mm coverslips**	6 x
P I CONTROL	**Positive control cANCA** (diluted human serum) +	0.5 ml Ready to use Dropper bottle Red cap
P II CONTROL	**Positive control pANCA** (diluted human serum) +	0.5 ml Ready to use Dropper bottle Yellow cap
N CONTROL	**Negative control** (diluted human serum) –	0.5 ml Ready to use Dropper bottle Green cap

Additional materials and equipment required

- Adjustable micropipettes (10, 100, 1000 µl)
- Pipette tips
- Sample dilution tubes
- Destilled (or deionized) water
- Measuring cylinder or beaker 1 l
- Moist chamber
- Plastic wash bottle
- Staining chamber
- Fluorescence microscope with excitation wavelength 490 nm and emission wavelength 520 nm, magnification 400x (recommended)

Size and storage

Each CytoBead® ANCA kit contains sufficient reagents for 48 determinations.

The expiry date of the complete reagent set is given on the label on the outside of the kit container. The expiry dates of individual reagents can in some cases exceed this, and are marked on each reagent.

Until use, all CytoBead® ANCA reagents can be stored at 2 - 8 °C. The solid substance PBS can be kept at room temperature. All opened test kit components are stable for a minimum of 2 months, provided proper storage at 2 - 8 °C.

Preparation and use

Do not open the packaging of the slides until they have reached room temperature.

Decant the solid PBS buffer (10 g) into a measuring cylinder or beaker (1000 ml) and fill to the mark with distilled water. Dissolve all solids by mixing or shaking. This prepared PBS buffer solution can be stored at room temperature in a sealed glass container. Solutions which are turbid, contaminated or have an altered pH value should be discarded.

Protect the conjugate from light.

TEST PROCEDURE

- **Dilute patient samples as desired with dilution buffer (B)**
- **Do not allow slides/wells to dry out while processing**

1. Bring test reagents to room temperature (RT, 20-25°C), slides should be labelled and only removed from packaging immediately before use
2. Pipette:
   - **50 µl** of each control (PI, PII, N)
   - diluted patient sera

   onto the respective wells. Cover wells completely, do not touch surface with pipette tips.
3. Incubate slides for **30 minutes** at RT in a moist chamber.
4. Use a wash bottle to rinse slides with PBS solution, without positioning the stream directly onto wells. To avoid cross-contamination the fluid should not flow away over other wells, with slides of 2 well rows wash each in turn from the middle outwards.
5. Wash for **3 x 3 min** with fresh PBS solution in a staining chamber, lightly agitating the chamber at the start and when changing PBS.
6. Remove each slide **individually**, shake off PBS, remove any still remaining buffer by carefully blotting with the enclosed paper templates (F) (*→**TIP:** use the edges of the blotting paper to dry the Teflon between wells and the well edges).*
7. Add **50 µl** conjugate (D) onto each well, covering them completely.
8. Incubate slides for **30 minutes** at RT in a moist chamber. Protect from direct light.
9. Wash slides using a bottle of PBS solution, as described in point 4.
10. Wash for **3 x 3 min** with fresh PBS solution in a staining chamber, lightly agitating the chamber at the start and when changing PBS.
11. Firmly shake off PBS buffer, before removing any remnants with the enclosed blotting paper templates (F) (see point 6).
12. Apply **1 drop** of mounting medium per well. Carefully place the coverslip, using tweezers if needed, so that the mounting medium forms a continuous, bubble-free layer. Wipe excess medium from the edge of the slide. ***Pressure on the coverslip, or tapping out of any occurring air bubbles, should be avoided in all cases as there is the danger that this can crush the beads.***
13. Read slides under the fluorescence microscope. Do not remain in one viewing field for too long, to minimise bleaching out of the fluorescence.

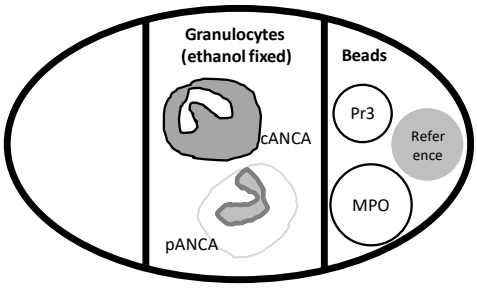
Conservation of the slides

Should immediate reading of the slides not be possible, they can be kept for a few days at 2-8°C. For longer conservation seal the edges of the coverslip with nail polish and store slides at –20°C.

INTERPRETATION of the RESULTS

Fluorescence pattern

The middle section of the wells contains ethanol fixated granulocytes, the right hand section of the wells contains antigen coated beads: PR3 (Ø ca. 10 µm), MPO (Ø ca. 15 µm) and the „Size and Location Reference" (SLR) which, with a diameter of 13 µm lie between the two antigen beads, aid in the differentiation between the two. Due to a co-polymerized dye, the reference always show a green fluorescence. The red base fluorescence of the MPO and PR3 beads can be visualized according to the filter used on the fluorescence microscope.



Fluorescence intensity of the ANCA pattern (cells)

The fluorescence intensity can be classified according to the recommendations of the CDC, Atlanta, USA (10):
4+ = maximal fluorescence, brilliant yellow-green
3+ = less brilliant yellow-green fluorescence
2+ = clear, but matt yellow-green fluorescence
1+ = very weak, subdued fluorescence

Ring fluorescence (beads)

+ = ring fluorescence visible
- = no ring fluorescence visible

Negative result

A sample dilution is classed as ANCA negative if the fluorescence intensity is less than 1+ and there is no characteristic fluorescence pattern (cANCA, pANCA).
Absence of ring fluorescence of the MPO or PR3 beads shows a negative result regarding these antibodies.

Positive result

A sample dilution is classed as ANCA positive if a fluorescence intensity of 1+ or more in the cytoplasm (cANCA) or perinuclear region (pANCA) of the ethanol fixated granulocytes is visible.
Ring fluorescence of the MPO or PR3 beads shows the prescence of the respective antibody.

PR3 Antibody: cytoplasmic staining of the ethanol fixated granulocytes and ring fluorescence of the PR3 beads (smaller than or similar in size to the completely green filled "Size and Location Reference" SLR).

Image: anti-PR3 positive on beads (left) and cells (cANCA, right)

MPO Antibody: perinuclear staining of the ethanol fixated granulocytes and ring fluorescence of the MPO beads (larger in diameter than the completely green filled "Size and Location Reference" SLR).

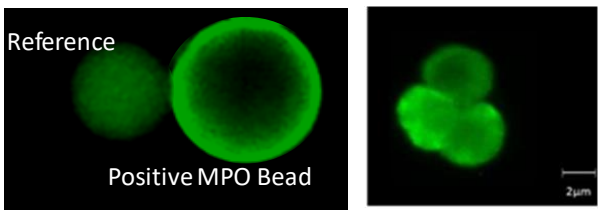


Image: anti-MPO positive on beads (left) and cells (pANCA, right)

Other combinations of fluorescence pattern are possible:

1. Cell pattern is negative, however beads show a clear ring fluorescence. **INTERPRETATION**: Bead result is preferred. With titrations especiallly, positive fluorescence can remain for longer on the beads than on the cells.

2. Cell pattern is positive, however beads show no fluorescence.; **INTERPRETATION**: no ANCAs with PR3 or MPO specificities are present, possible presence of antibodies against other granulocyte antigens (eg. Lactoferrin, elastase, bactericidal/permeability increasing enzyme BPI) or other nuclear components (ANA) must be investigated further.

3. Positive perinuclear pattern on ethanol fixated granulocytes, however no ring fluorescence on MPO beads. **INTERPRETATION**: Presence of antibodies against nuclear components (ANA) is possible, for clarification perform further tests on formalin fixated granulocytes (**pANCA IFA plus, REF 87161**) and/or HEp-2 cells (**ANA HEp-2 Plus, REF 8101**).

4. Strong background signal in the bead region (right side of the well): **INTERPRETATION**: Ring fluorescence of the beads cannot be measured, and so this sample must be tested again **TIP:** Titrate with 1:2 dilution steps from the initial dilution (1:20), eg.100 µl sample + 100 µl dilution buffer.

Titration

A semi-quantitative titration gives the result as the final dilution step in which a 1+ fluorescence is visible on the ethanol fixated granulocytes. The titer is the reciprocal value of this dilution step.

Using the recommended four step titration row the end point of the titration 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

Due to variations between populations, it is recommended that each laboratory establishes its own pathological and normal ANCA reference ranges. Therefore the values given above are for example only.

Test validation

Two positive controls PI (cANCA, anti-PR3) and PII (pANCA, anti-MPO) and one negative control N must be included in every test run. The controls provided in the test kit must show the following results

PI (cANCA): cytoplasmic fluorescence of the granulocytes with an intensity of 1+ to 2+ and ring fluorescence of the smaller bead population (PR3).

PII (pANCA): perinuclear fluorescence of the granulocytes with an intensity of 2+ to 3+ and ring fluorescence of the larger bead population (MPO).

N: no fluorescence of the granulocytes and no ring fluorescence of either bead population.

If the controls do not show the expected results, the test is invalid and must be repeated. Ensure that the instructions given in the kit insert are followed strictly (correct reagent preparation, incubation times and temperatures, careful washing). Upon repeating, if the validation criteria are not met, please contact your supplier.

Limitations of the method

The intensity of the fluorescence does not reflect the antibody concentration, and has no clinical relevance. Differences in lenses, filters and light sources between microscopes can lead to differences in fluorescence intensity.

Titration end-point determination is dependent on type and setup of the fluorescence microscope used, the magnification of the objective as well as the subjective evaluation of the observer.

Samples or wash buffer solutions contaminated with bacteria can lead to unspecific staining of the cell substrate. Proteolytic enzymes in samples can lead to damage or loss of the cell substrate and could also attack the surfaces of the MPO or PR3 coated beads.

A clinical diagnosis should not be made from the results of individual diagnostic methods alone. To make a diagnosis clinicians should consult all possible clinical and laboratory findings.

ASSAY PROTOCOL

CytoBead® ANCA (8063)

Dilute patient samples according to the instructions using dilution buffer (B)

1	Bring test reagents and slides to room temperature		
		Controls	Patient samples
2	Pipette Controls (PI, PII, N)	50 µl	
	Diluted sera		50 µl
3	Incubate	30 minutes, room temperature (20-25°C), moist chamber, protected from direct light	
4	Rinse with PBS solution (from C)		
5	Wash	3 x 3 min in fresh PBS solution (from C)	
6	Pipette conjugate (D)	50 µl	50 µl
7	Incubate	30 minutes, room temperature (20-25°C), moist chamber, protected from direct light	
8	Rinse with PBS solution (from C)		
9	Wash	3 x 3 min in fresh PBS solution (from C)	
10	Mounting; apply 1 drop of mounting medium (E) per well, carefully place coverslip (G), **do not tap down or apply pressure**		
11	Read using a fluorescence microscope		

GENERAL ADVICE and SAFETY PRECAUTIONS

- This test kit is for *in vitro* investigations only, and must be performed by trained laboratory personnel. The instructions must be followed strictly.
- The test kit or its opened reagents are only to be used within the stated stability periods.
- Slides in perforated packages must not be used in the test.
- The mixing of test kit components from different lots, as well as the use of reagents from other manufacturers, can lead to altered results.
- Some reagents contain small quantities of sodium azide (< 0.1%) as a preservative. Do not swallow reagents, and avoid contact with mucus membranes. Sodium azide can form explosive metal azides upon contact with lead and copper pipes, and therefore should therefore be disposed of with copious amounts of water.
- The recommended storage temperature of opened reagents until their next use is 2 - 8 °C.
- All reagents in this test kit of human origin have given negative test results for HbsAg (hepatitis B surface antigen) as well as antibodies against HIV (human immunodeficiency virus) and HCV (hepatitis C virus). However, no test can rule out the presence of infectious agents with absolute security. Reagents should therefore always be treated as potentially infectious material.
- When handling the components of this test kit, as well as patient samples and controls, regulations for health and safety and for handling potentially infectious materials and hazardous chemicals must be observed. In particular the following rules:
  - Do not eat, drink or smoke!
  - Do not pipette by mouth!
  - Wear gloves to avoid contact with reagents and sera!
  - Observe safety measures given on individual test components!
- We recommend that laboratories establish their own quality controls through the inclusion of internal control sera (see regulations of the German Federal Law gazette I, p. 1657, 1988 as well as the guidelines of the German Medical Association for the quality control of quantitative laboratory tests, Dt. Ärzteblatt 98, Volume 42, p. A2747-59, 2001).

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