INSTRUCTION MANUAL

REF 8063

December 22, 2015

CytoBead® ANCA

- 48 determinations -

IVD *In-vitro* diagnostic test

CE

Indirect immunfluorescence test for the determination of IgG antibodies against neutrophil cytoplasmic antigens (ANCA) and the glomerular basal membrane (GBM) in human serum

Substrate: human granulocytes, ethanol fixed; proteinase 3 (PR3), myeloperoxidase (MPO) and glomerular basement membrane (GBM) coated beads

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

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INTRODUCTION

CytoBead® ANCA is a reagent set for the determination (qualitative and semi-quantitative) of IgG autoantibodies against antigens in cytoplasmic neutrophil granulocytes (ANCA) and antibodies against antigenic structures of the glomerular basement membrane (GBM) in human serum for the differential diagnosis of ANCA associated vasculitis and systemic vasculitis (Granulomatosis with Polyangiitis, Microscopic Polyangiitis, Eosinophilic Granulomatosis with Polyangiitis) as well as autoimmune renal disorders (Goodpasture syndrome).

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 fixed human granulocytes allow the determination of the fluorescence pattern, cytoplasmic (cANCA) or perinuclear (pANCA). The antigen coated beads allow the measurement of antibodies specific for PR3, MPO and GBM.

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 fixed granulocytes, ANCA positive samples are assigned a specific fluorescence pattern: cytoplasmic or perinuclear and furthermore a specific fluorescence halo of the PR3 and MPO beads. An anti-GBM positive sample (without MPO-ANCA) assigned no cellular fluorescence pattern, but a specific fluorescence halo of the GBM beads.

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, hemolytic and/ or contaminated samples must not be used.

Preparation and use

Before use in CytoBead® ANCA, bring sera and reagents to room temperature.

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 (8408) Ag 8	**Slides** 8 wells coated with human granulocytes (ethanol fixed) and beads coated with PR3, MPO and GBM		6 individually sealed
B (8620) DIL	**Dilution buffer**		20 ml ready to use, capped white
C (9018) BUF PBS	**PBS Puffer** for 3 x 1000 ml PBS solution		3 x 10 g solid substance
D (8510) CONJ	**Conjugate** anti-human-IgG (heavy and light chain specific), AlexaFluor®488 labelled with DAPI		10 ml ready to use, capped blue
E (8008) MOUNT	**Mounting Medium** glycerol solution, PBS buffered, pH 7,4 ± 0,2		3.0 ml Ready for use Dropper bottle White cap
F (8075) TEMPL	**Blotting paper templates**		6 x
G (9318) COVER	**70 x 22 mm coverslips**		12 x
P I (8702) CONTROL	**Positive control** (pooled diluted human serum, bead control)	+	2.0 ml ready to use, dropper bottle, capped red
P II (8112) CONTROL	**Positive control** (diluted human serum, cell control)	+	2.0 ml ready to use, dropper bottle, capped red
N (8302) CONTROL	**Negative control** (diluted human serum)	–	2.0 ml ready to use, dropper bottle, capped green

Required equipment (not supplied)

- Adjustable micropipettes (10, 100, 1000 µl)
- Pipette tips
- Sample dilution tubes
- Destilled (or deionized) water
- Measuring cylinder or beaker 1 l
- Moist chamber
- Vacuum pump or water pump jet
- Staining dishes or Coplin jar
- 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 should 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 for 4 weeks. Solutions which are turbid, contaminated or have an altered pH value should be discarded.

Protect the conjugate from light.

TEST PROCEDURE

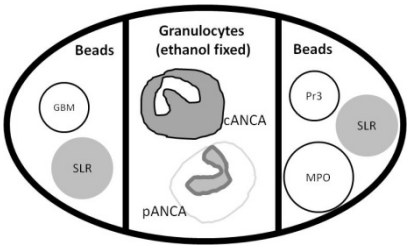
- **Dilute patient samples as desired with dilution buffer (B)**
  10 µl sample + 190 µl dilution buffer (B) → 50 µl / well
- **Do not allow slides/wells to dry out while processing**
- **Read the slides within 3 hours after finishing the staining !**

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:
   - **1-2 drops** (50µl) of each control (P I, P II, N)
   - **1-2 drops** (50µl) diluted patient sera
   onto the respective wells. Cover wells completely, do not touch surface of well.
3. Incubate slides for **30 minutes** at RT in a moist chamber.
4. Aspirate the liquid from each well using a vacuum pump.
5. Wash for **5 x 2 min** with prepared PBS solution in a staining dish, 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 **1-2 drops** (50µl) 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. Aspirate the liquid from each well using a vacuum pump
10. Wash for **5 x 2 min** with fresh PBS solution in a staining dish, lightly agitating the chamber at the start and when changing PBS.
11. Remove slides from the wash one at a time, shake off excess PBS by tapping the edge of the slide onto absorbent towel, carefully dry around the wells using a blotting template (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 stained slides using a fluorescence microscope. Avoid longer exposition of one viewing field to minimize bleaching of fluorescence

INTERPRETATION of the RESULTS

Fluorescence pattern

The middle section of the wells contains ethanol fixed granulocytes, the right hand section of the wells contains different sized antigen coated beads: PR3 (Ø ca. 9 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. The left hand section contains GBM coated beads (Ø ca. 10 µm) and SLR. Due to a co-polymerized dye, the reference always show a green fluorescence. The red base fluorescence of the MPO, PR3 and GBM 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 of the cells is less than 1+ and there is no characteristic fluorescence pattern (cANCA, pANCA).
Absence of ring fluorescence of the MPO, PR3 or GBM beads shows a negative result regarding these antibodies.

Positive result

A sample dilution is classed as ANCA positive if a fluorescence intensity of ≥ 1+ in the cytoplasm (cANCA) or perinuclear region (pANCA) of the ethanol fixed granulocytes is visible as well as the positivity of PR3 and MPO beads on the right side of the well:

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

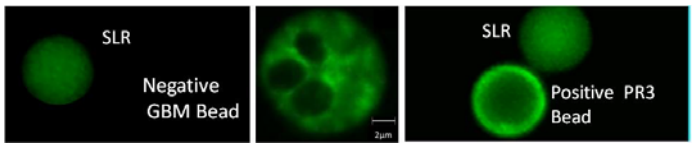


Image: anti-PR3 positive on beads (right) and cells (cANCA, middle)

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

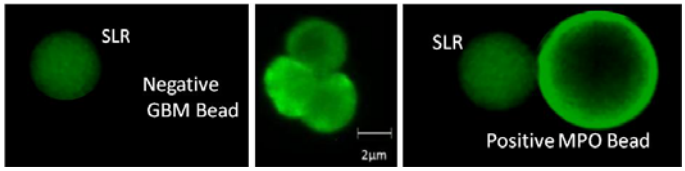


Image: anti-MPO positive on beads (right) and cells (pANCA, middle)

GBM antibody: A sample is classed as anti-GBM positive if a positivity of the GBM beads is present (left side of the well); in 20% of the cases a pANCA pattern on the cells and a positivity of the MPO beads can be seen(smaller than or similar in size to the completely green filled "Size and Location Reference" SLR).

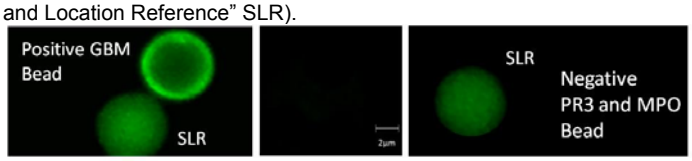


Image: anti-GBM positive on beads (left) and negative cells (middle)

Other combinations of fluorescence pattern are possible:

1. Cell pattern is negative, however beads show a clear ring fluorescence. **INTERPRETATION**: Test should be repeated with lower dilution. With titrations especiallly, positive fluorescence can remain for longer on the beads than on the cells. **TIP:** Obtaining this result with the 1:20 screen, repeat with a 1:10 dilution to detect weak positive ANCA 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 fixed 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 fixed granulocytes (**pANCA IFA plus, REF 87161**) and/or HEp-2 cells (**ANA HEp-2 Plus, REF 8101**).
4. Strong background signal in the bead regions (right and left sides 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 positivity is visible and expressed with the reciprocal value of this dilution step.
Using the recommended serial dilution the end point of the titration can be extrapolated:

1:10	=	3+	
1:40	=	2+	
1:160	=	+/-	
1:640	=	-	The end point 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.

Test validation

Two positive controls 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 (bead control): mixed pattern fluorescence of the granulocytes with an intensity ≥ 2+ and ring fluorescence of the MPO and PR3 beads (right side) and GBM beads (left side).

PII (cell control): cytoplasmic fluorescence of the granulocytes with an intensity of ≥ 1+ and ring fluorescence of the PR3 beads (right side).

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 but thorough 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 always reflect the antibody concentration. Differences in the microscope equipments (lenses, filters and light sources) and maintenance status can lead different results.

The end point determination is dependent on type and setup of the fluorescence microscope used.

Samples or 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, PR3 or GBM coated beads.

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

Intra- and inter-assay Precision

For the determination of intra-assay precision of Cyto-Bead ANCA three serum dilutions were analyzed eight times testing each parameter (PR3/MPO/GBM) by measuring fluorescence intensities (AKLIDES). The intra-assay values do not exceed 15 % of CV.
Inter-assay precision was determined according to the intra-assay precision study of three batches of CytoBead ANCA assay and found lower than 15 % of CV.

Sensitivity and specificity

Sensitivity and specificity of the Bead populations were detected by measurement of 59 ELISA positive patient samples (Institute of Immunology, Technical University Dresden), including reactivities against PR3 (n= 37), MPO (n= 10) and GBM (n= 12). Samples from healthy donors were used as controls (n=34):

	Anti-PR3	Anti-MPO	Anti-GBM
Sensitivity (%)	91.2	100	100
Specificity (%)	100	100	100

ASSAY PROTOCOL

CytoBead® ANCA (8063)

Dilute patient samples according to the instructions using dilution buffer (B)
→ 10 µl sample + 190 µl dilution buffer (1:20)

1	Bring test reagents and slides to room temperature		
		Controls	Patient samples
2	Pipette Controls (P I, P II, N)	**1-2 drops** (50 µl)	
	Diluted sera		**1-2 drops** (50 µl)
3	Incubate	30 minutes, room temperature (20-25°C), moist chamber	
4	Aspirate each well using a vacuum pump		
5	Wash	**5 x 2 min** in PBS solution (from C)	
6	Pipette conjugate (D)	**1-2 drops** (50 µl)	**1-2 drops** (50 µl)
7	Incubate	30 minutes, room temperature (20-25°C), moist chamber, protected from direct light	
8	Aspirate each well using a vacuum pump		
9	Wash	**5 x 2 min** in 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 **WITHIN 3 HOURS!**		

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!

LITERATURE

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