INSTRUCTION MANUAL

REF **8220**

December 22, 2015

CytoBead® ANA 2

- 80 Determinations -

IVD *In-vitro* diagnostic device

CE

European Patent EP 2 362 222

Indirect immunfluorescence test for the determination of antibodies against nuclear and cytoplasmic antigens in human serum

Substrate: HEp-2 cells, completed with ANA antigens (dsDNA, Scl-70, SS-A/Ro60, SS-A/Ro52, SS-B, Jo-1, Sm, Sm/RNP) coated beads

REF	Product no.	**LOT**	Lot no.
	See kit insert		Manufacturer
	Storage temperature		Expiry date
	See instruction manual		Biological risk

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INTRODUCTION

CytoBead® ANA 2 is a reagent set for the determination of IgG antibodies against nuclear and cytoplasmic antigens in human serum for the diagnosis of systemic autoimmune diseases. This determination is using indirect immunofluorescence on fixated HEp-2 cells and allows furthermore the ANA differentiation by the use of antigen coated beads (dsDNA, Scl-70, SS-A/Ro60, SS-A/Ro52, SS-B, Jo-1, Sm, Sm/RNP).

Systemic rheumatic inflammatory diseases, e.g. systemic lupus erythematosus (SLE), progressive systemic sclerosis (PSS), primary Sjögren's syndrome, dermatomyositis or Sharp syndrome (mixed connective tissue disease – MCTD) are characterized by the presence of specific antibodies directed against components of the nucleus and cytoplasm. These can function as markers for various disease spectra as well as activity parameters (1, 2).

The immunofluorescence test on HEp-2 cells is a sensitive screening test for the determination of anti-nuclear antibodies (ANA). By the evaluation of different fluorescence patterns the underlying antigens and associated diseases can be determined (3, 4). The use of ANA/ENA antigen coated beads allows the differentiation of the ANA antibodies from the positive HEp-2 cells.

TEST PRINCIPLE

CytoBead® ANA 2 is an indirect immunofluorescence test for the determination of anti-nuclear antibodies (ANA). Through the recognition of fluorescence patterns on HEp-2 cells, conclusions on underlying antigens and associated diseases can be made (3, 4). Furthermore, the antibody differentiation is possible using beads coated with ANA antigens (dsDNA, Scl-70, SS-A/Ro60, SS-A/Ro52, SS-B, Jo-1, Sm, Sm/RNP).

Antibodies in diluted patient samples and controls react specifically in the first step with antigens on the HEp-2 cells and beads fixed onto slides. Unbound components are removed through a wash step, following 30 minute incubation at room temperature.

Bound antibodies react specifically in a second reaction step with anti-human antibodies coupled with fluorescein-isothiocyanate (FITC). Excess conjugate molecules are removed from immune complexes bound to the solid phase through a further wash step, following 30 minute incubation at room temperature.

After covering, slides are read under a fluorescence microscope (excitation wavelength 490nm, emission wavelength 520nm). Specific fluorescence patterns are given according to the histological arrangement of antigens in the HEp-2 cells. Furthermore according to the antibody in the patient serum ring fluorescence around the antigen coated beads can be found.

PATIENT SAMPLES

Separation and Storage

Blood is collected through venipuncture, allowed to clot, and the serum isolated through centrifugation. Storage for up to 3 days is possible at 2 - 8 °C, for longer storage samples must be frozen at - 20 °C. Samples should be aliquoted before freezing, as repeated freeze-thaw cycles are to be avoided.

Lipemic samples should not be used, as a fatty film can obscure the substrate. Contaminated samples can contain proteolytic enzymes, which can digest the substrate, and must therefore not be used.

Preparation and Use

Bring sera to room temperature before use in CytoBead® ANA 2. Mix shortly by vortexing to ensure homogenity.

Dilute patient samples to be tested **1:80** (v/v), e.g. **5 µl sample + 400 µl sample buffer (B)**. 80 µl of diluted patient sample is needed per well.

TEST COMPONENTS for 80 Determinations

A (9453) Ag 8	**Slides** 8 wells coated with HEp-2 cells, as well as beads coated with ANA antigens (dsDNA, Scl-70, SS-A/ Ro60, SS-A/Ro52, SS-B, Jo-1, Sm, Sm/RNP)	10 Individually sealed
C (9018) BUF PBS	**PBS Buffer** PBS, pH 7,4, for 3 x 1 L buffer	3 x 10 g Solid substance
D (9510) CONJ	**Conjugate** Anti-human-IgG (heavy and light chain specific), FITC marked	10 ml Ready for use Blue cap
E (8008) MOUNT	**Mounting Medium** Glycerin solution with fluorescence stabilizer, phosphate buffered, pH 7.4 ± 0.2	3.0 ml Ready for use Dropper bottle White cap
F (8075) TEMPL	**Blotting Paper Templates**	10 x
G (9318) COVER	**Coverslips** **70 x 22 mm**	12x
P I (9712) CONTROL	**Positive Control** Diluted pooled human serum, Bead control +	2.0 ml Ready for use Dropper bottle Red cap
P II (9102) CONTROL	**Positive Control** Diluted human serum, cell control +	2.0 ml Ready for use Dropper bottle Red cap
N (9302) CONTROL	**Negative Controls** Diluted human serum –	2.0 ml Ready for use Dropper bottle Green cap

Additional Materials and Equipment Required

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

Size and Storage

Each CytoBead® ANA 2 Kit contains sufficient reagents for 80 determinations.

The expiry date of the complete reagent set is given on the outer label of the kit container. The expiry dates of individual reagents can exceed this and are marked on the individual reagents.

Until use, all reagents of the CytoBead® ANA 2 kit can be stored at 2 - 8 °C. The solid substance PBS can be stored at room temperature. All opened reagents are stable for at least 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 substance PBS (10 g) into a measuring beaker or cylinder (1000ml) and fill to the mark with destilled water. Dissolve solids by mixing or stirring. PBS buffer solution prepared in this way can be stored at room temperature in a sealed glass container. Cloudy or contaminated solutions, or those with altered pH values, are to be discarded.

Protect the conjugate from light.

TEST PROCEDURE

- **Dilute patient samples appropriately before use with sample buffer (B)**
- **Do not allow slides/wells to dry out during the test procedure!**

1. Bring test reagents to room temperature (RT, 20-25°C), open slides immediately before use and label.
2. Pipette
   - **2 drops (80 µl)** controls (PI, PII, N)
   - **80 µl** diluted patient serum

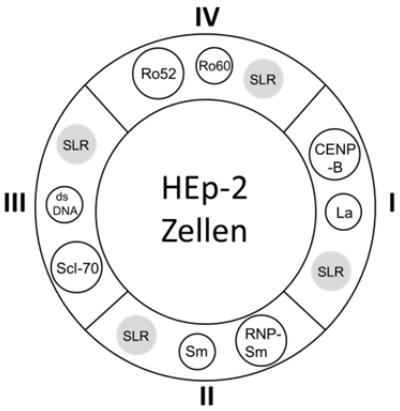
   onto each required well. Ensure wells are completely covered, without touching the surfaces.
3. Incubate slides for **30 minutes** at RT in a moist chamber.
4. Aspirate fluid from wells with a vacuum pump or a 1000 µl pipette.
5. Wash for **5 x 2 min** with fresh PBS solution in a staining chamber, agitating lightly at the start and when changing PBS.
6. Remove slides **individually**, shake off PBS buffer, carefully remove remaining buffer with the enclosed blotting paper templates (F) (*→**TIP:** use the holes in the blotting paper to dry the Teflon between the wells and around their edges).*
7. Apply **2 drops (80 µl)** of conjugate (D) to each well, covering it completely.
8. Incubate slides for **30 minutes** at RT in a moist chamber. Protect from direct light.
9. Aspirate fluid from wells with a vacuum pump.
10. Wash for **5 x 2 min** with fresh PBS solution in a staining chamber, agitating lightly at the start and when changing PBS.
11. Shake off PBS buffer, carefully remove remaining buffer with the enclosed blotting paper templates (F) (see step 6).
12. Apply **1 drop** of mounting medium per well. Carefully place a coverslip onto the slide, so that the medium forms a continuous bubble-free layer. Use tweezers if desired. Wipe excess mounting medium from the edges of the slide. ***Pressing hard on the coverslip or tapping the slide to remove any air bubbles occurring should always be avoided, as this carries the risk of damaging beads.***
13. Read slides under a fluorescence microscope. Do not remain in one visual field for too long, to avoid bleaching out the fluorescence.

INTERPRETATION of the RESULTS

Fluorescence Patterns

HEp-2 cells are found in the middle compartment of the wells. Antigen coated red fluorescent beads are localized in the smaller compartments around the large well, which differ in size and antigen: **Compartment I** contains La/SS-B (Ø ca. 9 µm), Jo-1 (Ø ca. 15 µm) and Size and Location Reference beads (SLR). With a diameter of 13 µm, their size is between that of each antigen bead. **Compartment II** contains Sm (Ø ca. 9 µm), RNP-Sm (Ø ca. 15 µm) and SLR. **Compartment III** contains dsDNA (Ø ca. 9 µm), Scl-70 (Ø ca. 15 µm) and SLR. Compartment IV contains Ro60 (Ø ca. 9 µm), Ro52 (Ø ca. 15 µm) and SLR.

SLR beads always show a green fluorescence, from a polymerized dye. Depending on the filter of the fluorescence microscope used, red background fluorescence of the 9 µm and 15 µm antigen beads may be seen.



Fluorescence Intensity of the ANA Patterns (HEp-2 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)

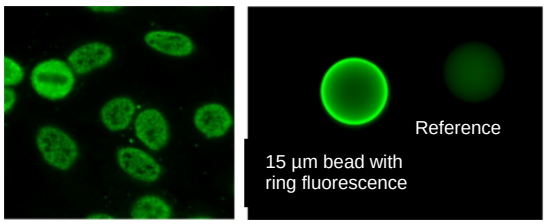
+ = Ring fluorescence visible
- = No ring fluorescence visible

Negative Result

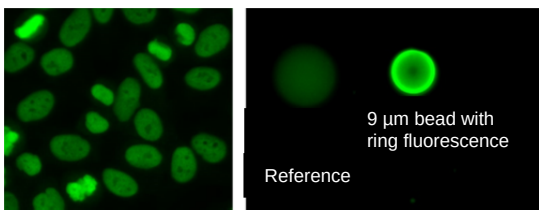
A sample dilution is reported as ANA negative if HEp-2 cells demonstrate a fluorescence of less than 1+ with no apparent pattern.
The absence of ring fluorescence of the antigen coated beads shows a negative result regarding this antibody.

Positive Result

A sample dilution is reported as ANA positive, if HEp-2 cells demonstrate a fluorescence of 1+ or greater, as well as a clearly visible fluorescence pattern.
Ring fluorescence of the antigen coated beads shows the presence of the respective antibody:



Example of an ANA positive result for RNP-Sm: the HEp-2 cells display clear, green fluorescence as well as a clearly recognizable course speckled pattern. The 15 µm bead in compartment II (RNP-Sm) shows a clear, green ring fluorescence.



Example of an ANA positive result for dsDNA: the HEp-2 cells display clear, green fluorescence as well as a clearly recognizable homogenous pattern. The 9 µm bead in compartment III (dsDNA) shows a clear, green ring fluorescence.

The following combinations of fluorescence patterns are possible:

HEp-2 cell pattern	Possible antigens	Possible bead fluorescence	Clinical relevance
Homogenous	DNA, histone	dsDNA	High titers specific for SLE, histone antibodies for medication induced lupus (6)
	Scl-70	Scl-70	Marker for PSS (8)
Speckled	Sm, nRNP	Sm, RNP-Sm	Sm antibodies highly specific for SLE; high anti-nRNP titers specific for MCTD, together with other ANAs in SLE, RA, PSS (8)
	SS-A, SS-B	SSA/Ro60, SSA/Ro52, SS-B/La	Often in primary Sjögren's syndrome, anti-SS-A often in neonatal lupus and congenital heart block (9)
Centromere	Centromeric proteins of the chromosomes		Marker for CREST syndrome, rarely in diffuse scleroderma and Raynaud's phenomenon (11, 12)
Nucleolar	PMScl, RNA polymerase I, fibrillarin		High titers specific for PSS, polymyositis-dermatomyositis overlap, low titers in SLE, Sjögren's syndrome and Raynaud's phenomenon (13)
Cytoplasmic	Ribosomal RNP		In rare cases SLE (15)
	Jo-1 (PL-7, PL-12)	Jo-1	Polymyositis, dermatomyositis (16)
	Mitochrondrial		Marker of primary biliary cirrhosis (PBC)
	Cytoskeleton		Various, anti-actin common in autoimmune hepatitis and infectious disease

REFERENCE VALUES

ANA	Titer
Negative	**< 80**
Positive	**≥ 80**

Therefore titers of 80 and 160 count as low positive, 320 and 640 are seen as medium titers, and titers of 1280 and above are high positive.
Due to variations between populations it is recommended that every laboratory establishes its own pathological and normal reference ranges for ANA. The values given above are only a recommendation.

Test Validation

One positive and one negative control must be included in every test run. The controls included in the test kit must show the following results:
Negative control: fluorescence less than 1+
Positive control Bead (P I): positive staining of the nucleus (coarse speckled, 3+ to 4+) and the cytoplasm and ring fluorescence of SS-B/La, Jo-1, RNP-Sm, (Sm beads borderline), dsDNA, Scl-70 , SS-A/Ro52 and SS-A/Ro60 beads
Positive control cell (P II): positive staining of the nucleus (homogenous, 3+) and ring fluorescence of dsDNA, SS-A/Ro52 and SS-A/Ro60 beads

If the controls do not show the expected results, the test is invalid and must be repeated. Ensure that the test instructions are followed strictly (correct reagent preparation, incubation times and temperatures, sufficient washing). If the validation criteria are not met when the test is repeated, please contact the manufacturer. An information sheet is available for troubleshooting.

Limitations of the Method

If the HEp-2 cells are negative, but the beads show a clear ring fluorescence, test should be repeated with lower dilution. With titrations especially, positive fluorescence can remain for longer on the beads than on the cells.
It is however also possible that the cell results are positive, but the beads show no ring fluorescence. As the beads are not coated with all possible ANA/ENA relevant antigens, this does not mean that the result is ANA negative. Here, further investigative tests are necessary.

The fluorescence intensity does not reflect the antibody concentration, and has no clinical relevance. Differences in optics, filters and light sources between microscopes can lead to differences in fluorescence intensity.
Samples or wash buffer solution contaminated with bacteria can lead to unspecific staining of the cell substrates. Proteolytic enzymes in the samples can lead to damage or loss of the cell substrate, and also attack the antigen-coated bead surfaces.
A clinical diagnosis should not be made from the results of individual diagnostic methods alone. To make a diagnosis clinicians should consult all available clinical and laboratory findings.

TEST CHARACTERISTICS

438 pretested patient sera and 104 healthy controls were analysed with the CytoBead® ANA 2 and compared to the respective ELISA´s. Overall a relative sensitivity of 94% and a relative specificity of 96% for ANA was found. Furthermore the respective antibody specificities were analysed in detail, that means each antigen specific bead was compared with the respective ELISA. The relative sensitivity was in a range of 87-100% whereas the relative specificity was in a range of 94-99%. More detailed data can be provided.

ASSAY PROTOCOL

CytoBead® ANA 2 (8220)

Dilute patient samples appropriately with sample buffer (B)

1	Bring test reagents and slides to room temperature			
			Controls	Patient samples
2	Pipette	Controls (P I, P II, N)	**1-2 drops (80 µl)**	
		Prediluted sera		**80 µl**
3	Incubate		30 minutes, room temperature (20-25°C), moist chamber	
4	Aspirate fluid from the wells			
5	Wash		5 x 2 min in fresh PBS solution (from C)	
6	Pipette conjugate (D)		**2 drops (80 µl)**	
7	Incubate		30 minutes, room temperature (20-25°C), moist chamber, protected from direct light	
8	Aspirate fluid from the wells			
9	Wash		5 x 2 min in fresh PBS solution (from C)	
10	Mounting; 1 drop mounting medium (E) per well, carefully apply coverslip (G), **do not tap or press hard**			
11	Read under a fluorescence microscope			

GENERAL ADVICE and SAFETY PRECAUTIONS

- The test kit is for in vitro diagnostic use only, and must be performed by trained laboratory personnel. The instructions must be followed strictly.
- The test kit or its opened components are only to be used within the stated stability periods.
- Slides in perforated packaging must not be used in the test.
- The mixing of test components from different lots, or the use of reagents from other manufacturers, can lead to altered results.
- Some reagents contain small amounts of sodium azide (< 0.1%) as a conservative. Do not swallow reagents, and avoid contact with mucus membranes.
- 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 material and hazardous chemicals must be used. In particular the following rules:
  - Do not eat, drink or smoke!
  - Never 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 and external control sera

LITERATURE

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