INSTRUCTION MANUAL

REF 8064

December 22, 2015

CytoBead® CeliAK

- 48 determinations -

IVD *In vitro* diagnostic device

Indirect immunofluorescence test for the determination of IgA or IgG antibodies against endomysium, tissue transglutaminase and deamidated gliadin as well as for positive control of IgA antibodies in human serum

Substrate: monkey esophagus, completed with tTG, DG and anti-IgA coated beads

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

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Manufactured under license to patents EP2362222; AU2011217190.

INTENDED USE

CytoBead® CeliAK is a reagent set for the qualitative and semi-quantitative determination of endomysial antibodies (EmA) of the IgA and IgG classes, using sections of monkey esophagus, as well as the determination of IgA/IgG antibodies against tissue transglutaminase (tTG; Transglutaminase 2), deamidated gliadin (DG) and a control of IgA antibodies in human serum.

Celiac disease (gluten induced enteropathy) is an intolerance to gluten, an adhesive protein found in various types of wheat. This intolerance leads to extended lesions in the mucus membranes, which manifests as "flat" mucosa. Due to these extensive lesions (villous atrophy and extension of the crypts) malabsorption frequently occurs, with the associated deficiency symptoms. Gliadin, the alcohol soluble fraction of gluten, is responsible for the emergence of celiac disease. Gliadin induces inflammatory processes in the small intestinal mucosa with the involvement of humoral and cellular immune processes.
Celiac disease is one of the most common chronic enteropathies in small children. Patients with advanced celiac disease have an increased risk of developing T-cell lymphoma.
Antibody determination (clinical guidelines from ESPGHAN*):
Diagnosis of celiac disease is characterized through highly specific autoantibodies (AAb) against tissue transglutaminase, the autoantigen of celiac disease, and deamidated gliadin. EmA are directed against extracellular tTG. AAb in celiac disease are typically IgA class antibodies (except Ab against DG). In patients with an IgA antibody deficiency, AAb of IgG type can be used as diagnostic tool.

TEST PRINCIPLE

CytoBead® CeliAK is an indirect immunofluorescence test for the parallel qualitative and semi-quantitative determination of IgA-EmA, IgA-tTG and IgA-DG-AAb, as well as total IgA. With clinically suspected or confirmed IgA deficiency and/or no detection of IgA, celiac specific AAb of IgG type can be detected using an additional conjugate.
The AAb in diluted patient samples and controls react in the first step in parallel with the antigens of the monkey esophagus and the anti-IgA, tTG and DG coated beads. After 30 minutes incubation at room temperature unbound components are removed in a wash step.
The bound AAb are specifically detected in a second reaction step with anti-human IgA, or alternatively anti-human IgG, conjugates with fluorescein-isothiocyanate (FITC). Excess conjugate molecules are removed from the immune complexes bound to the solid phase with a further wash step, following 30 minutes incubation at room temperature.
Slides are covered, and read under a fluorescence microscope (excitation wavelength 490nm, emission wavelength 520nm). According to the histological orientation of the endomysial antigens, a positive patient sample (diluted 1:10) gives a specific fluorescence pattern: a net or honeycomb formed staining of the connective tissue cases in the muscle fiber bundles of the muscularis mucosa of monkey esophagus.

PATIENT SAMPLES

Separation and Storage

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

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

Screening
Dilute samples to be tested **1:10** (v/v), **e.g. 20 µl sample + 180 µl PBS (C)**. 70 µl of diluted patient sample is required per well.

TEST COMPONENTS 48 DETERMINATIONS

A (5418) Ag 8	**Substrate slides** 8 wells coated with cryostat sections of monkey esophagus, and tTG, DG and anti-IgA beads	6 sealed in a foil pouch
C (9018) BUF PBS	**PBS Buffer** for 3 x 1000 ml PBS solution, pH 7,4,	3 x 10 g dry substance
D (5510) CONJ IgA	**Conjugate IgA** anti-human IgA (sheep), FITC labeled	10 ml ready for use capped blue
D1 (5502) CONJ IgG	**Conjugate IgG** anti-human IgG (sheep), FITC labeled	2.0 ml ready for use dropper bottle capped blue
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 capped white
F (8075) TEMPL	**Blotting templates**	6
G (9318) COVER	**Coverslips** 70 x 22 mm	12
P (5102) CONTROL +	**Positive control** diluted human serum	2.0 ml ready for use dropper bottle capped red
N (5302) CONTROL –	**Negative control** diluted human serum	2.0 ml ready for use dropper bottle capped green

Additional materials and equipment required

- Adjustable micropipettes (10, 100, 1000 µl) with tips
- Sample dilution tubes
- Destilled (or deionized) water
- Measuring cylinder or beaker 1 l
- Moist chamber
- Vacuum pump
- Staining dishes or Coplin jar
- Fluorescence microscope with excitation wavelength 490 nm and emission wavelength 520 nm, magnification 100x (recommended)

Size and storage

Each CytoBead® CeliAK 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® CeliAK 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 appropriately before use with PBS (B)**
  20 µl serum + 180 µl PBS (C) → 70 µl / Well
- **Do not allow slides/wells to dry out while processing**

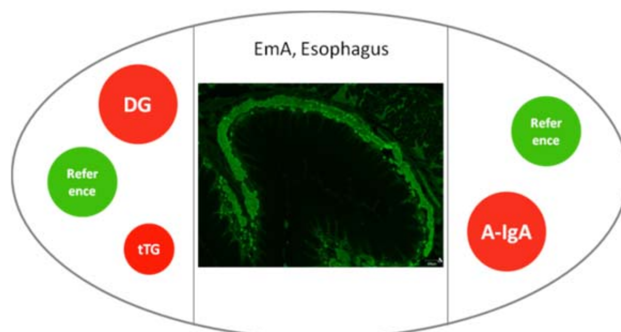
1. Bring test reagents to room temperature (RT, 20-25°C), slides should be labelled and only removed from packaging immediately before use.
2. Pipette:
   - **1-2 drops (50-70) µl** of each control (P, N)
   - **70 µl** diluted patient sera

   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 consecutively with each slide (take one slide after another).
5. Wash for **5 x 2 min** with fresh PBS solution in a coplin jar.
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 (50 µl)** conjugate (D) or (D1) onto each well, covering them completely.
8. Incubate slides for **30 minutes** at RT in a moist chamber. Protect slides from direct light.
9. Aspirate the liquid from each well using a vacuum pump (take one slide after another).
10. Wash for **5 x 2 min** with fresh PBS solution in a coplin jar.
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 cover slip, 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 cover slip, 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, magnification 100x. Avoid longer exposition of one viewing field to minimize bleaching of fluorescence

INTERPRETATION of the RESULTS

Fluorescence Patterns

The middle section of the wells contains tissue of the monkey oesophagus, the right hand section of the wells contains different sized antigen coated beads: anti-IgA (Ø ca. 15 µm) and the „Size and Location Reference" (SLR). The left hand section contains tTG coated beads (Ø ca. 10 µm), DG coated beads (Ø ca. 15 µm) and 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 homogenous fluorescence. The red base fluorescence of the tTG, DG and anti-IgA beads can be visualized according to the filter used on the fluorescence microscope.



Representation of a well used for determination of celiac-specific AAb with CytoBead® CeliAK. Each well contains tissue transglutaminase (tTG) and deamidated gliadin (DG) beads in the left hand section, anti-IgA beads in the right hand section and in the centre tissue sections from monkey esophagus.

Fluorescence Intensity of the EmA pattern

The fluorescence intensity can be classified according to the recommendations of the CDC, Atlanta, USA (5):

4+ = maximal fluorescence, brillant 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

The patient sample is classed as IgA-/IgG-EmA negative if the characteristic honeycomb fluorescence pattern is not present. The tTG and DG beads do not show ring fluorescence. Since patient sera normally contain IgA antibodies the A-IgA beads emits a green ring fluorescence. The reference bead population shows a homogenous green fluorescence.

Negative Result with IgA Antibody Deficiency

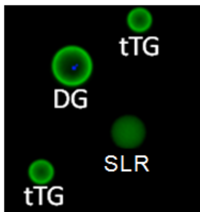
If the anti-IgA bead population in the right part of the well shows no ring fluorescence, there is the possibility of an IgA deficiency. In these cases, the measurement of EmA IgA as well as IgA tTG and IgA DG are not possible. We recommend that the test is repeated using the IgG specific conjugate. Please be aware that the standard method of IgA antibody quantification is nephelometry.

Positive Result

The patient sample is classed as IgA-/IgG-EmA positive if a net or honeycomb formed staining of the connective tissue cases in the muscle fiber bundles of the muscularis mucosa of monkey esophagus with a intensity of +1 is present. The tTG and DG beads in the left hand section show ring fluorescence. Since patient sera normally contain IgA antibodies the A-IgA beads emits a green ring fluorescence. The reference bead population shows a homogenous green fluorescence.

Anti-tTG and/or anti-DG

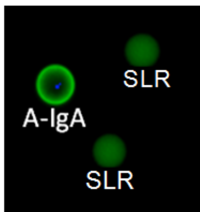
If the patient sample has IgA antibodies against tTG, the smaller bead population on the left emits green ring fluorescence. If the larger bead population on the left part of the well shows a green ring fluorescence, IgA antibodies against DG are detected. The middle-sized reference bead population aids orientation and the differentiation between the smaller (tTG) and the larger (DG) bead populations. The reference bead population emits a homogenous green fluorescence and no ring fluorescence.



Example image for the identification of IgA antibodies against tTG and DG using CytoBead® CeliAK. Smaller tTG and larger DG beads each with green ring fluorescence, and reference beads with homogenous green fluorescence.

Anti-IgA bead population

If the patient sample contains IgA antibodies, the larger bead population on the right part of the wells emits green ring fluorescence. The reference bead population aids orientation and emits a homogenous green fluorescence.



Example image for the identification of IgA antibodies using CytoBead® CeliAK. Anti-IgA beads with green ring fluorescence and reference beads with homogenous green fluorescence.

Tip: Where the fluorescence microscope used has a strong light source, a weak homogenous fluorescence of the tTG and DG beads can result. This fluorescence can clearly be differentiated from the ring fluorescence seen with a positive tTG and DG result and is to be reported negative.

Furthermore, open emission filters of > 520 nm can cause a yellow to reddish homogenous fluorescence of the IgA, tTG and DG beads. This fluorescence has no influence on the green-emitting ring fluorescence. The reference population can, depending on the emission filter, appear green to yellowish.

Background: In cases of strong fluorescent background staining, titration of the patient serum is recommended to rule out false positive or false negative reactions.

REFERENCE VALUES

IgA-/IgG-EmA	Titer
negative	< 10
positive	≥ 10

Due to variations between populations, it is recommended that each laboratory establishes its own pathological and normal IgA-/IgG-EmA reference ranges. The values given above are only a recommendation.

Test Validation

The positive and negative controls of the kit should be included in each run. The controls react as follows:

Negative control

No, or very weak, fluorescence of the tissue is observed. The tTG and DG beads show no ring fluorescence. The negative control contains antibodies of the IgA class, so the A-IgA Beads emit a green ring fluorescence. The reference beads emit a homogenous green fluorescence.

Positive control

Net or honeycomb formation fluorescence of the muscularis mucosa, as well as the musculature of the esophagus is observed. The anti-IgA in the right part, as well as the tTG and DG in the left part show a green ring fluorescence. The reference beads emit a homogenous green fluorescence.

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

In small children, delayed IgA formation can be observed.
Antibodies against the smooth muscle react with the myofibrils of the smooth muscle, and therefore do not show the net/honeycomb structure of the endomysium.
Antibodies against other components of the tissue section can display their respective fluorescence patterns (e.g. nuclei, epidermal basement membranes, intercellular substance). These fluorescence patterns are reported as negative regarding EmA, however they can indicate the presence of another autoimmune disease.
The IgG conjugate can show crossreactivity on the A-IgA Beads (weak green ring fluorescence).
The detection of IgA endomysial antibodies is dependent on the esophagus section used. If the section present on a slide contain esophagus without smooth muscle, please contact your supplier.
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.

ASSAY PERFORMANCE

Intra and inter assay precision
Intra assay precision of CytoBead® CeliAK was determined with three serum dilutions testing eight times, respectively. The analysis of reactivities for EmA, anti tTG as well as anti DG was investigated with AKLIDES®. Intra assay precision was 10 - 20 %. Inter lot precision were obtained assessing two lots of CytoBead® CeliAK using same serum dilutions. Inter lot precision was 1 - 16 %.

Diagnostic sensitivity and specificity
154 biopsy proven celiac disease patient sera and 217 sera of control groups (59 cystic fibrosis, 68 eye diseases, and 90 blood donors) were investigated with CytoBead® CeliAK to determine endomysial antibodies and antibodies to tTG, DG (each of IgA class). Sensitivities and specificities were determined as follows:

Patient cohort	n	EmA	Anti-tTG	Anti-DG
Celiac disease	154	151	153	140
Sensitivity	154	98 %	99 %	91 %
Cystic fibrosis	59	1	3	7
Eye diseases	68	0	1	2
Blood donors	90	0	0	6
Specificity	217	99 %	98 %	93 %

ASSAY - SCHEMA

CytoBead® CeliAK (8064)

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 (P,- N)	**1-2 drops** (50 - 70 µl)	
	Diluted sera		**70 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 fresh 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 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* diagnostic use 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 and external control sera.

LITERATURE

(1) Kumar V, Beutner EH, Chorzelski TP: Distribution of monkey esophagus anitgens reactive with IgA-class antibodies in the sera of dermatitis herpetiformis patients. Arch Dermatol Res, 1984, 276; 293-6

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(5) Lyerla HC, Forrester FT: The Immunofluorescence (IF) test. In: Immunofluorescence methods in virology, USDHHS, Georgia, 1979, 71-81

(6) Husby S, Koletzko S, Korponay-Szabó IR, Mearin ML, Phillips A, Shamir R, Troncone R, Giersiepen K, Branski D, Catassi C, Lelgeman M, Mäki M, Ribes-Koninckx C, Ventura A, Zimmer KP; ESPGHAN Working Group on Coeliac Disease Diagnosis; ESPGHAN Gastroenterology Committee; European Society for Pediatric Gastroenterology, Hepatology, and Nutrition. J Pediatr Gastroenterol Nutr. 2012 Jan;54(1):136-60.