AESKULISA tTg-A New Generation

Ref 3503













Product Ref.	3503
Product Desc.	tTg-A New Generation
Manual Rev. No.	004 : 2013-10-10

Instruction Manual

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1 Intended Use

The **AESKULISA tTg-A New generation** is a solid phase enzyme immunoassay for the quantitative and qualitative detection of IgA antibodies against neo-epitopes of tissue transglutaminase (tTG) in human serum. The assay employing human recombinant transglutaminase crosslinked with gliadin-specific peptides displays neo-epitopes of tTg which ensures a significantly increased sensitivity and specificity of the test.

The assay is a tool for the diagnosis and monitoring of celiac disease (gluten-sensitive enteropathy).

2 Clinical Application and Principle of the Assay

Gluten-sensitive enteropathy or celiac disease is characterized by atrophy of the small intestinal villi leading to a so-called flat mucosa. It is caused by a pathological intolerance to gliadin, the alcohol-soluble fraction of gluten in wheat, rye and barley. As celiac disease is caused by the uptake of gluten, consequently a gluten-free diet cures the disease completely and thus has to be maintained for life-time. Renewed consumption of gliadin leads to a return of the symptoms. The disease is HLA-associated (>95% of patients have DQ2 enREFd by DQA1*0501 and DQB1*0201) and manifests at any age with a peak onset in early childhood, even in neonatals. The incidence rates range from 1 in 4000 to 1 in 300 in european countries.

Diagnosis of celiac disease is made by small intestinal biopsy (demonstrating flat mucosa) supported by serological markers. Antibodies against gliadin and anti-endomysium antibodies (EMA) are of major significance. They are detected so far by indirect immunofluorescence, which is restricted to subclass IgA only. The identification of tissue transglutaminase (tTG) as the major target antigen of EMA provided the opportunity of a more easy and reliable diagnosis of celiac disease. tTG is an enzyme that upon wounding is released from cells where it is thought to aid in tissue repair.

Anti-tTG antibodies show higher sensitivity and specificity than anti-Gliadin antibodies. Furthermore they correlate tightly with the activity of the disease and thus are especially useful for diet monitoring. The cross-link of tTg with gliadin-specific peptides results in neoepitopes of tTg. As these neo-epitopes are structurally closer to the physiological antigens, the new generation AESKULISA tTg tests show a markedly increased sensitivity and specificity. These epitopes show no cross-reactivities with gliadin.

The determination of IgG antibodies to tTG is the only available specific serology for those 2% to 5% of patients with IgA deficiency. A high number of subclinical cases have been detected by screening for anti-tTg, fostering the theory that the majority of celiac disease cases is undetected and untreated (Iceberg model).

Principle of the test

Serum samples diluted 1:101 are incubated in the microplates coated with the specific antigen. Patient's antibodies, if present in the specimen, bind to the antigen. The unbound fraction is washed off in the following step. Afterwards anti-human immunoglobulins conjugated to horseradish peroxidase (conjugate) are incubated and react with the antigen-antibody complex of the samples in the microplates. Unbound conjugate is washed off in the following step. Addition of TMB-substrate generates an enzymatic colorimetric (blue) reaction, which is stopped by diluted acid (color changes to yellow). The intensity of color formation from the chromogen is a function of the amount of conjugate bound to the antigen-antibody complex and this is proportional to the initial concentration of the respective antibodies in the patient sample.



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3 Kit Contents

		TO BE	RECONSTITU	JTED
Item	Quantity	Cap color	Solution color	Description / Contents
Sample Buffer (5x)	1 x 20ml	White	Yellow	5 x concentrated Tris, sodium chloride (NaCl), bovine serum albumin (BSA), sodium azide < 0.1% (preservative)
Wash Buffer (50x)	1 x 20ml	White	Green	50 x concentrated Tris, NaCl, Tween 20, sodium azide < 0.1% (preservative)
		RE	ADY TO USE	
Item	Quantity	Cap color	Solution color	Description / Contents
Negative Control	1 x 1.5ml	Green	Colorless	Human serum (diluted), bovine serum albumin (BSA), sodium azide < 0.1% (preservative)
Positive Control	1 x 1.5ml	Red	Yellow	Human serum (diluted), bovine serum albumin (BSA), sodium azide < 0.1% (preservative)
Cut-off Calibrator	1 x 1.5ml	Blue	Yellow	Human serum (diluted), bovine serum albumin (BSA), sodium azide < 0.1% (preservative)
Calibrators	6 x 1.5ml	White	Yellow *	Concentration of each calibrator: 0, 3, 10, 30, 100, 300 U/ml. Human serum (diluted), bovine serum albumin (BSA), sodium azide < 0.1% (preservative)
Conjugate, IgA	1 x 15ml	Red	Red	Containing: Anti-human immunoglobulins conjugated to horseradish peroxidase, bovine serum albumin (BSA)
TMB Substrate	1 x 15ml	Black	Colorless	Stabilized tetramethylbenzidine and hydrogen peroxide (TMB/H ₂ O ₂)
Stop Solution	1 x 15ml	White	Colorless	1M Hydrochloric Acid
Microtiter plate	12 x 8 well strips	N/A	N/A	With breakaway microwells. Refer to paragraph 1 for coating.

MATERIALS REQUIRED, BUT NOT PROVIDED

Microtiter plate reader 450 nm reading filter and recommended 620 nm reference filter (600-690 nm). Glass ware (cylinder 100-1000ml), test tubes for dilutions. Vortex mixer, precision pipettes (10, 100, 200, 500, 1000 µl) or adjustable multipipette (100-1000µl). Microplate washing device (300 µl repeating or multichannel pipette or automated system), adsorbent paper. Our tests are designed to be used with purified water according to the definition of the United States Pharmacopeia (USP 26 - NF 21) and the European Pharmacopeia (Eur.Ph. 4th ed.).

4 Storage and Shelf Life

Store all reagents and the microplate at 2-8°C/35-46°F, in their original containers. Once prepared, reconstituted solutions are stable at 2-8°C/35-46°F for at least 1 month. Reagents and the microplate shall be used within the expiry date indicated on each component, only. Avoid intense exposure of TMB solution to light. Store microplates in designated foil, including the desiccant, and seal tightly.



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5 Precautions of Use

5.1 Health hazard data

THIS PRODUCT IS FOR IN VITRO DIAGNOSTIC USE ONLY. Thus, only staff trained and specially advised in methods of in vitro diagnostics may perform the kit. Although this product is not considered particularly toxic or dangerous in conditions of the intended use, refer to the following for maximum safety:

Recommendations and precautions

This kit contains potentially hazardous components. Though kit reagents are not classified being irritant to eyes and skin we recommend to avoid contact with eyes and skin and wear disposable gloves.

WARNING! Calibrators, Controls and Buffers contain sodium azide (NaN₃) as a preservative. NaN₃ may be toxic if ingested or adsorbed by skin or eyes. NaN₃ may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build-up. Please refer to decontamination procedures as outlined by CDC or other local/national guidelines.

Do not smoke, eat or drink when manipulating the kit. Do not pipette by mouth.

All human source material used for some reagents of this kit (controls, standards e.g.) has been tested by approved methods and found negative for HbsAg, Hepatitis C and HIV 1. However, no test can guarantee the absence of viral agents in such material completely. Thus handle kit controls, standards and patient samples as if capable of transmitting infectious diseases and according to national requirements.

The kit contains material of animal origin as stated in the table of contents, handle according to national requirements.

5.2 General directions for use

In case that the product information, including the labeling, is defective or incorrect please contact the manufacturer or the supplier of the test kit.

Do not mix or substitute Controls, Calibrators, Conjugates or microplates from different lot numbers. This may lead to variations in the results.

Allow all components to reach room temperature (20-32°C/68-89.6°F) before use, mix well and follow the recommended incubation scheme for an optimum performance of the test.

Incubation: We recommend test performance at 30°C/86°F for automated systems.

Never expose components to higher temperature than 37°C/98.6°F.

Always pipette substrate solution with brand new tips only. Protect this reagent from light. Never pipette conjugate with tips used with other reagents prior.

A definite clinical diagnosis should not be based on the results of the performed test only, but should be made by the physician after all clinical and laboratory findings have been evaluated. The diagnosis is to be verified using different diagnostic methods.



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6 Sample Collection, Handling and Storage

Use preferentially freshly collected serum samples. Blood withdrawal must follow national requirements. Do not use icteric, lipemic, hemolysed or bacterially contaminated samples. Sera with particles should be cleared by low speed centrifugation (<1000 x g). Blood samples should be collected in clean, dry and empty tubes.

After separation, the serum samples should be used during the first 8h, respectively stored tightly closed at 2-8°C/35-46°F up to 48h, or frozen at -20°C/-4°F for longer periods

7 Assay Procedure

7.1 Preparations prior to starting

Dilute concentrated reagents:

Dilute the concentrated sample buffer 1:5 with distilled water (e.g. 20 ml plus 80 ml).

Dilute the concentrated wash buffer 1:50 with distilled water (e.g. 20 ml plus 980 ml).

To avoid mistakes we suggest to mark the cap of the different calibrators.

Samples:

Dilute serum samples 1:101 with sample buffer (1x)

e.g. 1000 µl sample buffer (1x) + 10 µl serum. Mix well!

Washing:

Prepare 20 ml of diluted wash buffer (1x) per 8 wells or 200 ml for 96 wells

e.g. 4 ml concentrate plus 196 ml distilled water.

Automated washing:

Consider excess volumes required for setting up the instrument and dead volume of robot pipette.

Manual washing:

Discard liquid from wells by inverting the plate. Knock the microwell frame with wells downside vigorously on clean adsorbent paper. Pipette 300 µl of diluted wash buffer into each well, wait for 20 seconds. Repeat the whole procedure twice again.

Microplates:

Calculate the number of wells required for the test. Remove unused wells from the frame, replace and store in the provided plastic bag, together with desiccant, seal tightly (2-8°C/35-46°F).



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7.2 Pipetting Scheme

We suggest pipetting calibrators, controls and samples as follows:

For *QUANTITATIVE* interpretation

	1	2	3	4
Α	Cal A	Cal E	P1	
В	Cal A	Cal E	P1	
С	Cal B	Cal F	P2	
D	Cal B	Cal F	P2	
E	Cal C	PC	P3	
F	Cal C	PC	P3	
G	Cal D	NC		
Н	Cal D	NC		

For QUALITATIVE interpretation

	1	2	3	4
Α	NC	P2		
В	NC	P2		
С	СС	P3		
D	СС	P3		
E	PC			
F	PC			
G	P1			
Н	P1			

CalA: calibrator A CalD: calibrator D

CalB: calibrator B CalE: calibrator E

CalC: calibrator C CalF: calibrator F

PC: positive control P1: patient 1

NC: negative control P2: patient 2

CC: cut-off calibrator P3: patient 3

7.3 Test Steps

Step	Description
1.	Ensure preparations from step 7.1 above have been carried out prior to pipetting.
2.	Use the following steps in accordance with quantitative/ qualitative interpretation results desired:
	CONTROLS & SAMPLES
3.	Pipette into the designated wells as described in chapter 7.2 above, 100 μl of either:
	a. Calibrators (CAL.A to CAL.F) for <i>QUANTITATIVE</i> or b. Cut-off Calibrator (CC) for <i>QUALITATIVE</i> interp.
	and 100 µl of each of the following:
	 Negative control (NC) and Positive control (PC), and Patients diluted serum (P1, P2)
4.	Incubate for 30 minutes at 20-32°C/68-89.6°F.
5.	WashB → Wash 3x with 300 µl washing buffer (diluted 1:50).



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CONJUGATE					
6.	+100 µl	Pipette 100 μl conjugate into each well.			
7.	30'	Incubate for 30 minutes at 20-32°C/68-89.6°F.			
8.	WASHB →	Wash 3x with 300 μl washing buffer (diluted 1:50).			
		SUBSTRATE			
9.	**************************************	Pipette 100 μl TMB substrate into each well.			
10.	30'	Incubate for 30 minutes at 20-32°C/68-89.6°F, protected from intense light.			
		STOP			
11.	+100 µl	Pipette 100 μl stop solution into each well, using the same order as pipetting the substrate.			
12.	5'	Incubate 5 minutes minimum.			
13.		Agitate plate carefully for 5 sec.			
14.	OD ₄₅₀ OD ₆₂₀	Read absorbance at 450 nm (recommended 450/620 nm) within 30 minutes.			



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8 Quantitative and Qualitative Interpretation

For quantitative interpretation establish the standard curve by plotting the optical density (OD) of each calibrator (y-axis) with respect to the corresponding concentration values in U/ml (x-axis). For best results we recommend log/lin coordinates and 4-Parameter Fit. From the OD of each sample, read the corresponding antibody concentrations expressed in U/ml.

Normal Range	Equivocal Range	Positive Results
< 12 U/ml	12 - 18 U/ml	>18 U/ml

Example of a standard curve

Do NOT use this example for interpreting patient's result

Calibrators IgA	OD 450/620 nm	CV % (Variation)
0 U/ml	0.073	3.1
3 U/ml	0.179	2.3
10 U/ml	0.342	1.2
30 U/ml	0.662	0.1
100 U/ml	1.310	0.9
300 U/ml	2.263	0.3

Example of calculation

Patient	Replicate (OD)	Mean (OD)	Result (U/ml)
P 01	0.808/0.831	0.820	39.6
P 02	1.081/1.071	1.076	66.1

Samples above the highest calibrator range should be reported as >Max. They should be diluted as appropriate and re-assayed. Samples below calibrator range should be reported as < Min

For lot specific data, see enclosed quality control leaflet. Medical laboratories might perform an in-house quality control by using own controls and/or internal pooled sera, as foreseen by national regulations.

Each laboratory should establish its own normal range based upon its own techniques, controls, equipment and patient population according to their own established procedures.

In case that the values of the controls do not meet the criteria the test is invalid and has to be repeated.

The following technical issues should be verified: Expiration dates of (prepared) reagents, storage conditions, pipettes, devices, photometer, incubation conditions and washing methods.

If the items tested show aberrant values or any kind of deviation or that the validation criteria are not met without explicable cause please contact the manufacturer or the supplier of the test kit.

For **qualitative interpretation** read the optical density of the cut-off calibrator and the patient samples. Compare patient's OD with the OD of the cut-off calibrator. For qualitative interpretation we recommend to consider sera within a range of 20% around the cut-off value as equivocal. All samples with higher ODs are considered positive, samples with lower ODs are considered negative.

Negative: OD patient < 0.8 x OD cut-off

Equivocal: $0.8 \times OD \text{ cut-off} \leq OD \text{ patient } \leq 1.2 \times OD \text{ cut-off}$

Positive: OD patient > 1.2 x OD cut-off



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9 Technical Data

Sample material: serum

Sample volume: 10 µl of sample diluted 1:101 with 1x sample buffer

Total incubation time: 90 minutes at 20-32°C/68-89.6°F

Calibration range: 0-300 U/ml

Analytical sensitivity: 1.0 U/ml

Storage: at 2-8°C/35-46°F use original vials only.

Number of determinations: 96 tests

10 Performance Data

10.1 Analytical sensitivity

Testing sample buffer 30 times on AESKULISA tTg-A New Generation gave an analytical sensivity of 1.0 U/ml.

10.2 Specificity and sensitivity

The microplates are coated with recombinant human tissue-transglutaminase and gliadinspecific peptides. No crossreactivities to other autoantigens have been found. To test crossreactivity with gliadin, 7 sera positive for gliadin were tested and did not react with this assay, though this can be different for other gliadin positive sera.

For determination of sensitivity and specificity sera of 165 patients suffering from Celiac disease (n=102) and related diseases (see bottom table) were assessed on the AESKULISA and a predicate device. The results as a comparison to the predicate device and disease information are shown in the table below.

			diagnos	sis
		Pos	Neg	Total
AESKULISA	Pos	62	5	67
tTg-A	Neg	2	96	98
	Total	64	101	165

Agreement: 95.8 %
Sensitivity: 96.9 %
Specificity: 95.0 %

		Pre	dicate d	device
		Pos	Neg	Total
AESKULISA	Pos	25	42	67
tTg-A	Neg	2	96	98
	Total	27	138	165

rel. agreement: 73.3 %
rel. sensitivity: 92.6 %
rel. specificity: 69.6 %



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Disease	# Tested	# positive AESKU	# positive pred dev
Celiac Disease	64 / 64	62 (96.9)	21 (32.8)
Celiac Disease (gluten free diet)	38 / 38	0 (0.0)	1 (2.6)
Disease control (total)	70 / 210	5 (2.4)	5 (7.1)
Crohns Disease	51 / 51	1 (2.0)	0 (0.0)
Crohns Disease	0 / 58	0 (0.0)	n/d
Ulcerative Colitis	4 / 4	0 (0.0)	1 (25.0)
Ulcerative Colitis	0/2	0 (0.0)	n/d
Helminthiasis	2/2	2 (100.0)	2 (100.0)
Lactose Intolerance	2/2	2 (100.0)	2 (100.0)
Gliadin positive sera	0/7	0 (0.0)	n/d
Healthy donors	4 / 4	0 (0.0)	0 (0.0)
Healthy donors	0 / 80	0 (0.0)	n/d

10.3 Linearity

Chosen sera have been tested with this kit and found to dilute linearly. However, due to the heterogeneous nature of human autoantibodies there might be samples that do not follow this rule.

Sample No.	Dilution Factor	Measured (U/ml)	Expected (U/ml)	Recovery (%)
1	1 / 100	76.5	71.0	107.7
	1 / 200	36.6	35.5	103.1
	1 / 400	17.2	17.8	96.8
	1 / 800	8.5	8.9	95.8
2	1 / 100	62.2	59.0	105.4
	1 / 200	29.7	29.5	100.7
	1 / 400	13.3	14.8	90.2
	1 / 800	7.0	7.4	94.9



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10.4 Precision

To determine the precision of the assay, the variability (intra and inter-assay) was assessed by examining its reproducibility on three serum samples selected to represent a range over the standard curve (n=18). The accepted range for CV is 10%. (n=24 / 18)

Intra-assay			
Sample No.	Mean (U/ml)	CV (%)	
1	13.8	7.0	
2	56.7	5.2	
3	166.5	5.5	

Inter-assay		
Sample No.	Mean (U/ml)	CV (%)
1	10.1	2.3
2	38.9	0.8
3	169.4	4.2

10.5 Calibration

Due the lack of international reference calibration this assay is calibrated in arbitrary units (U/mI).



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11 Literature

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