PRODUCT DATA SHEET

T055 ("TG2-CovTest")

TISSUE TRANSGLUTAMINASE MICROASSAY KIT, COLORIMETRIC

Kit produced and Developed by CovalAb, France

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NOTE: INTENDED FOR RESEARCH USE ONLY, NOT FOR USE IN HUMAN, THERAPEUTIC OR DIAGNOSTIC APPLICATIONS.

Introduction

Transglutaminases (EC. 2.3.213, R-glutamyl-peptide: amine γ-glutamyltransferase) are a family of calcium dependent enzymes which catalyse an acyl transfer reaction between the γ-carboxamide group of peptide bound glutamine and various primary amines.

In mammals, at least eight active transglutaminase (TGs) isoenzymes have been described so far. They are widely distributed in various organs, tissues and body fluids. Among them, tissue transglutaminase (tTG, TG2) is distinguished from others TGs by its functional versatility and ubiquitous expression pattern in mammalian tissues.

This isoenzyme is involved in a variety of roles including stabilization of intra and extracellular matrices and cross linking of cell envelopes in apoptosis. It has also been associated with a large number of pathological conditions such as fibrosis, celiac disease, neurodegenerative disorders, inflammatory processes in sepsis, and in carcinogenesis of hepatocellular and ovarian carcinoma.

Solid phase assays developed so far in similar principles have been compromise by a high background signal, low sensitivity compare to radiolabeling methods and the lack of the specificity. **TG2-CovTest** overcomes these problems and constitutes a highly sensitive and specific TG2 solid-phase microassay.

The kit is designed to have the following advantages:

- Measurement of TG2 with low or no interference with other TGs isoforms.
- High sensitivity (equivalent to commonly used radiometric filter paper method).
- Simultaneous screening of TG2 activity using 96 wells (12 x 8-well strips).

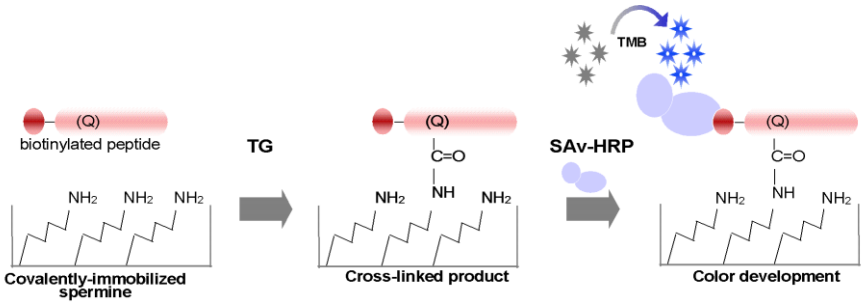
Principle of the assay

The **TG2-CovTest** uses a biotinylated preferred glutamine substrate of TG2 (biotin-pepT26) as amine-acceptor, and spermine as second substrate (amine-donor) of the enzyme. Samples suspected of containing TG2 are incubated with calcium, dithiothreitol (DTT) and biotin-pepT26 in the wells of microtiter plates to which spermine has been covalently coupled.

In the presence of TG2, spermine is incorporated into the γ-carboxamide of the glutaminyl residue of biotin-pepT26 to form a biotin-pepT26- γ-glutamyl spermine.

Enzymatic reaction is determined by its interaction with Streptavidin labelled peroxidase (SAv-HRP). Following a wash step to remove any unbound enzyme reagent, a substrate solution for SAv-HRP containing H_2O_2 and tetramethyl benzidine as electron acceptor (chromogen) is added and colour developed. The colour intensity is directly proportional to the TG2 activity in the sample.

The reaction scheme is shown below:



Contents of the Kit

Strips/Plate and Reagents	Quantity
Microtiter strips with covalently bound spermine	12x 8-wells strips
R1: DTT	0.2 mL
R2: EDTA (Negative Control)	1 mL
R3: Reaction Buffer (biotin-pepT26/ $CaCl_2$)	2 vials (Lyophilized powder)
R4: Enzyme Tracer (SAv-HRP)	50 µL
R5: Wash Buffer 10X	30 mL
R6: Diluent Buffer 10X	10 mL
R7: HRP Substrate Solution	12 mL
R8: Blocking Reagent	12 mL

The Kit contains also 2x 5 µg recombinant tissue transglutaminase (Zedira, T058, opr0036) for calibration purposes.

Storage and stability

The kit is shipped on ice. Upon arrival, the recombinant tissue transglutaminase (opr0036) and Enzyme Tracer (R4) should be stored at –20°C. All the other components of the kit should be kept at +4°C. When stored properly, these stock solutions are stable for at least 24 months.

Other supplies required

Microplate reader capable of measuring absorbance at 450 nm.
Pipettes, multi-channel pipette, and pipette tips.
Ultra-pure or deionized water
Squirt bottle, manifold dispenser, or automated microplate washer.

Assay procedure

Preliminary Operations

1. Identify a sufficient number of wells/strips to run in duplicate according to the **Reaction Scheme** (see below):

a) Blank
b) samples and transglutaminase control
c) negative control.

(Controls and samples should be subjected to the same assay procedure.)

2. Wash Buffer 10X (R5) and Diluent Buffer 10X (R6) should be diluted 1:10 with ultrapure water to obtain 1x solution, respectively. For 1x 8-well strip prepared at least 15 mL of solution Wash Buffer 1X. Place these solutions on ice until use.

3. Reconstitute the Reaction Buffer (R3) (lyophilised powder) in 3 mL of ultra-pure water. Place on ice until use, or store at -20° C.

4. Prepare a sufficient volume of **Assay Mixture**

   For 1x 8-well strip: Add 10 µL of DTT per 0.5 ml of reconstituted Reaction Buffer (R3) and place on ice until use. Of note, unused Assay Mixture should be discarded.

5. Sample (cell lysates, tissue extracts, human fluids, cellular culture medium, etc...) should be centrifuged at +4°C before use to remove particulates. Dilute your samples at a suitable concentration using the Diluent Buffer 1X. The supernatant must be immediately used or aliquoted and stored at ≤-20°C. Avoid repeated freeze-thaw cycles.

Reaction Scheme

	Assay Mixture	EDTA	Dilution Buffer	Sample / Enzyme
Blank	50 µL	-	50 µL	-
Test	50 µL	-	50 – x*µL	x*µL
Negative Control	50 µL	10 µL	-	50 µL

x*: *Volume of test sample added to the reaction*

GENERAL PROCEDURE

STEP I - HYDRATATION OF THE STRIPS/PLATES (15 min)

1. Dispense 150 µL per well of Wash buffer 1X. Incubate 15 minutes at 37°C.
2. Remove the Wash Buffer 1X from the strips.

STEP II. - TG2 REACTION (15-30 min)

3. According to the **Reaction Scheme**, dispense 50 µL per well of ice cold Assay Mixture.
4. Add 10 µL of EDTA (R2) for the Negative Control and mix well by pipetting up and down.
5. Add into the corresponding wells 50 µL of ice cold prepared Sample/Enzyme.
6. Incubate for 15-30 minutes at 37°C and shake gently.

STEP III. - SAv-HRP INTERACTION (15 min)

7. During incubation, dilute the Enzyme tracer (R4) solution at 1:2000 in **"Wash Buffer 1X"** and mix gently by inversion. Place on ice until use.
8. Wash the wells three times with 200 µL/well of Wash Buffer 1X
9. Dispense 100 µL of the freshly prepared Enzyme Tracer (R4) solution per well.
10. Incubate for 15 minutes at 37°C and shake gently.
11. Wash the wells three times with 200 µL/well of Wash Buffer 1X

STEP IV. - DEVELOPMENT OF COLOR (1-5 min)

12. Dispense 100 µL per well of HRP Substrate Solution (R7). Allow the reagent to warm to room temperature before use.
13. Incubate for 1 to 5 minutes at room temperature and shake gently. Protect from light.
14. Dispense 50 µL per well of Blocking Reagent (R8).
15. Measure the optical density of each well within 30 minutes, using a microplate reader set to 450 nm.

Quantitative analysis of data

Planning of a standard curve using TG2 enzyme of known activity is recommended for each set of samples (See **Example of Data** below). For accurate results we recommmend to duplicate the assays.

Average the duplicate readings for each standard, control, and sample and substract the average zero standard optical density. Construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph.

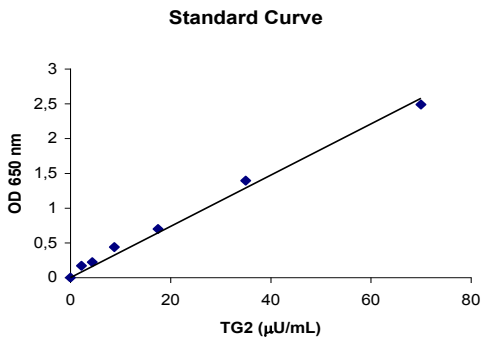
If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Several experiments using human recombinant TG2 (Zedira, T002) [Activity: 0.59 units/mg; one unit catalyse the formation of 1µmole of hydroxamate per minute from Z-Gln-Gly-OH and hydroxylamine at pH 6.0 at 37°C] have shown that 35 µU/mL correspond to an absorbance value (OD) of 1.4 ± 0.06 at 450 nm.

Example of Data

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.

Sample	TG2 (µU/mL)	O.D.	Average	Corrected Data*
		2,580		
1	70	2,596	2,606	2,490
		2,642		
		1,480		
2	35	1,513	1,509	1,393
		1,534		
		0,799		
3	17,5	0,810	0,816	0,700
		0,839		
		0,525		
4	8,75	0,579	0,556	0,440
		0,564		
		0,318		
5	4,37	0,331	0,337	0,221
		0,362		
		0,275		
6	2,18	0,289	0,286	0,170
		0,294		
		0,110		
Blank	0	0,121	0,116	___
		0,117		



(Corrected Data* = Average O.D. Sample – Average O.D. Blank)

Technical Hints

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each sample/enzyme, and between reagent additions. Also use separate reservoirs for each reagent.
- Ensure that HRP Substrate (R7) reached room temperature before use. Work with all the other reagents, solutions and sample/enzyme on ice while performing the assay.
- HRP Substrate (R7) should remain colourless until added to the plate. Keep this reagent protected from light. HRP Substrate should change from colourless to gradations of blue.
- Blocking Reagent (R8) should be added to the plate in the same order as the HRP Substrate (R7). The colour developed in the wells will turn from blue to yellow upon addition of the Blocking Reagent.

TG2 Inhibition Assay

The kit can be used to assay enzyme inhibitors. As a reference we recommend using Zedira inhibitor Boc-DON-Gln-Ile-Val-OMe (B003). To perform inhibition studies, first optimize the concentration of the enzyme to be used, and perform the **TG2-CovTest** as follows**:**

a) Perform the Hydratation Step as described in the **General Procedure** (see above).

b) Load 50µL of Assay Mixture into the wells, and 10µL of EDTA (R2) or known TG2 inhibitor for the negative control.

c) Add 1-25µL of sample/Enzyme and 1-25µL inhibitor of choice in an reaction tube, and adjust the volume to 50µL with Diluent Buffer 1X.

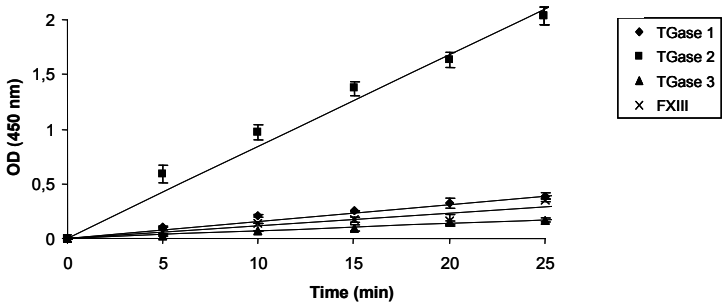
d) Pre-incubate the enzyme with the inhibitors for 1-5 minutes on ice, and load into the appropiate wells.

e) Continue from step 6 of the **General Procedure.**

Examples of results

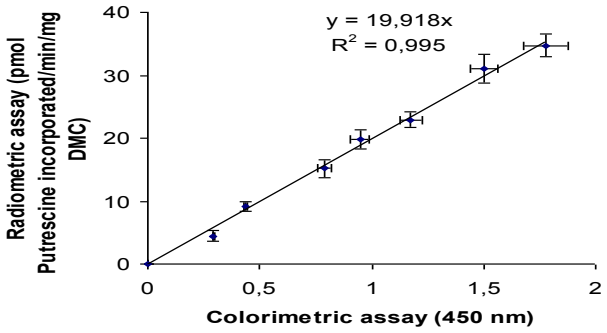
Ex. 1: Assay specificity for TG2.

This experiment was carried out using a similar enzymatic activity of the enzymes (0,33 mU/mL)



Ex. 2: Correlation of the Colorimetric TG2-CovTest with Standard Radiometric TG Assays

The standard radiometric assay is based on the ability of the enzyme to catalyse the incorporation of radiolabelled putrescine into dimethylcasein.



Sensitivity

Detection Limits of the TG2-CovTest (Comparison with the radiometric assay)

	TG2-Cov Test	TG radiometric assay
Rec.human TG 2	< 4 ng	10 ng
Purified guinea pig TG 2	< 5 ng	10 ng
Mouse embryonic fibroblast (MEF cells)	6 ng	18 ng
Guinea pig crude liver extracts	1,25 µg	5 µg

Safety procedure

- The product is not licensed or approved for administration to humans or to animals.
- Standard Laboratory Practices should be followed when handling this material.
- **Handle with care HRP substrate and blocking reagent.** These reagents are irritating to eyes and skin. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.

References

1. Perez Alea M. *et al*. Development of an isoenzyme-specific colorimetric assay for tissue transglutaminase 2 cross-linking activity. *Anal. Biochem.*, 2009; 389, 150-156.
2. Sugimura Y. *et al*. Screening for the Preferred Substrate Sequence of Transglutaminase Using a Phage-displayed Peptide Library: identification of peptide substrates for TGase 2 and factor XIIIA. *J Biol. Chem*; 2006; 281, 17699–706
3. Thomas V. and al. Development and evaluation of a modified colorimetric solid-phase microassay for measuring the activity of cellular and plasma (Factor XIII) transglutaminases *Biotechnol. Appl. Biochem. (2006) 43, 171–179.*
4. Lorand L *et al*. A filter paper assay for transamidating enzymes using radioactive amine substrate. *Anal . Biochem*., 1972; 50, 623-631.