Directions for use

Tissue Transglutaminase Pico-Assay Kit

Assay for the determination of
Tissue Transglutaminase (TG2) activity
in the picogram scale.

Art.-No. M003

For Research & Development Only

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1. Introduction and background

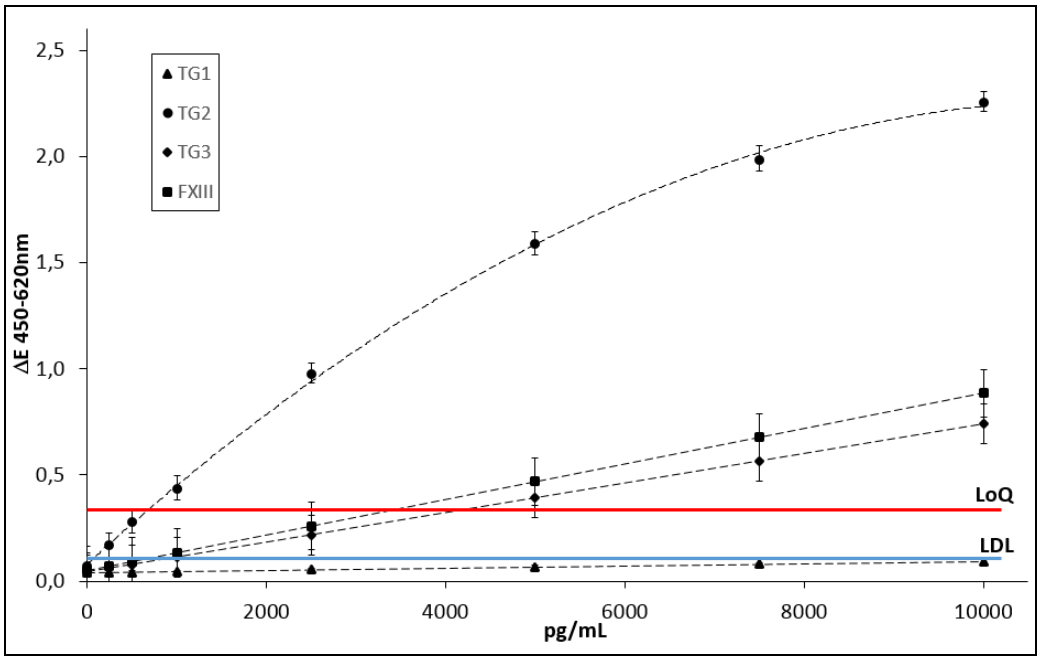
Transglutaminases are a widely distributed and unique family of enzymes that catalyse the posttranslational modification of proteins by inserting isopeptide bonds within or between polypeptide chains (Folk, J. E. and Cole, P. W., 1966).

In mammals, eight active transglutaminase isoenzymes have been described so far. They are calcium dependent and widely distributed in various organs, tissues and body fluids. Among them, **tissue transglutaminase (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. 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.

The **Tissue Transglutaminase Pico-Assay Kit** is based on a high activity high molecular weight substrate (HA-HMS) coated on a solid phase as glutamine-donor and a biotinylated substrate of transglutaminase (Biotinyl-cadaverine) as primary amine. Samples suspected of containing TG2 are incubated with calcium, dithiothreitol (DTT) and biotinyl-cadavarine in the wells of microtiter plates.

The present kit is intended for the quantitative determination of tissue transglutaminase activity in a broad application diversity. The kit can also be used for screening of transglutaminase inhibitors.

Note: The kit is selective, but not specific for tissue transglutaminase i.e. it also measures FXIII and TG3 activity in samples (see figure page 4). Please contact us if calibration for TG3 or FXIII are required. TG1 cannot be measured with this kit.



Lower TG2-Detection Limit (LDL): **0.14 µU/mL*; 100 pg/mL; 5 pg/well**

Limit of TG2-Quantitation (LoQ): **0.41 µU/mL*; 300 pg/mL (3x LDL)**
300 ppt (1 mL ≙ 1 g)

* specific activity ≙ 1.41 U/mg determined with
Zedi*Xclusive* Microbial Transglutaminase Assay Kit (Product No.: Z009)

The detection limit (LDL) is defined as the concentration of analyte that corresponds to the mean net absorbance of sample buffer pus 3-fold standard deviation. The Limit of Quantitation equals three times the detection limit.

Limits of Detection	LDL		LoQ
	[pg/well]	[pg/mL]	[pg/mL]
human tissue transglutaminase (TG2)	5	100	300
human Factor XIIIa, Thrombin activated (FXIIIa)	34	680	2,040
human epidermal transglutaminase, Dispase activated (TG3)	48	960	2,880

2. Warnings and precautions

The test kit is intended for *in vitro* use only. It is applicable only for research and development, not for diagnostic or therapeutic purposes.
The sample buffer contains Na-azide as preservative. Na-azide may react with lead and copper plumbing to form explosive metal azides. On disposal, flush with a large amount of water to prevent azide build-up.
The wash buffer contains bromonitrodioxane (bronidox) and the conjugates methylisothiazolone / bromonitrodioxane as preservatives. The substrate contains 3, 3', 5, 5'-tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2).
The stop solution, 0.5 M sulfuric acid (H_2SO_4), is acidic and corrosive.
All the above mentioned reagents may be toxic if ingested.

Follow routine precautions for handling hazardous chemicals. Avoid all body contact, wear gloves and eye protection. If one of the reagents comes into contact with skin, wash thoroughly with water.

Do not use reagents after expiration date.

Do not mix or use components from kits with different lot numbers.

Replace caps on reagents immediately. Do not swap caps.

Do not pipette reagents by mouth.

3. Principle of the test

The wells of the solid phase are coated with a specially developed high activity high molecular weight transglutaminase substrate (HA-HMS).

1st reaction: Tissue transglutaminase present in the sample covalently links biotinyl-cadaverine to the high molecular weight substrate (HA-HMS) coated on the microtiter plate surface.

2nd reaction: Streptavidin conjugated to horse-radish peroxidase (HRP) binds to the biotinyl-group incorporated into the HA-HMS solid phase.

3rd reaction: HRP converts a substrate (TMB) into a blue product which upon addition of the stop solution turns yellow. Samples containing transglutaminase activity develop the blue colour (which upon addition of the stop solution turns yellow), whereas samples without activity remain colourless.

4. Contents of the kit

a. Microtiter plate (1x), coated with high activity high molecular weight substrate (HA-HMS) and packed in a foil laminate pouch with a dessicant bag. The plate consists of 12 strips, each of which can be broken into 8 individual wells, thus providing maximum flexibility and economy in use of the assay.

b. Sample Buffer, 50 mL. Contains Tris-buffer and Na-azide.

c. Wash Buffer, 50 mL, 10x-concentrate, blue colored. Contains Tris buffered saline (TBS), Tween and bromonitrodioxane (Bronidox).

d. TG2-Standard for preparation of calibrators.

e. DTT, lyophilized. Needed for preparation of Sample Buffer with DTT.

f. Reaction Starter Mix (Biotinyl-cadaverine / Calcium), 210 µL, colourless. Contains Tris-buffer and Na-azide.

g. Reaction Stop, 18 mL, ready-to-use, colourless. Contains Tris-buffer, EDTA and Na-azide.

h. Streptavidin-HRP conjugate, 12 mL, ready-to-use, green colored. Buffered solution containing stabilising protein, methylisothiazolone and bromonitrodioxane (Bronidox).

i. Substrate solution, 14 mL, ready-to-use.
Botteled in a vial impermeable to light.

j. Stop solution (0.5 M H_2SO_4), 14 mL, ready-to-use.
Caution: sulfuric acid is corrosive.

k. Directions for use.

5. Materials required but not supplied

a. Microcentrifuge

b. Deionised or distilled water

c. Graduated cylinder, 500 mL

d. Low protein binding tubes (recommended)

e. Microplate washer (optional)

f. Automatic dispenser for 50 µL – 100 µL (recommended)

g. Microplate photometer fitted with a 450 nm and (optionally, as reference wavelength) a 620 nm filter.

h. Evaluation software (recommended)

6. Storage of the kit

TG2-Calibrator should be stored at -20°C. Shipment is possible at 4 - 8°C.
Store the remaining kit at 4 - 8°C. It is stable up to the expiry date stated on the label of the box. Do not use kit beyond its expiry date. After opening the pouch keep unused microtiter wells resealed to minimize exposure to moisture.

7. Reagent and Calibrator preparation

a. Before opening the pouch of the solid phase it must have reached room temperature. Remove the supernumerary microwells from the frame and immediately put them back into the pouch, together with the dessicant bag. Reseal the pouch hermetically and keep it refrigerated for future use.

b. Dilute the Wash Buffer 10x-concentrate (50 mL, blue) with 450 mL deionised water. Mix thoroughly. The diluted washing buffer is stable for at least four weeks if stored refrigerated.

c. Dissolve the lyophilized **DTT** with 120 µL of water and **transfer 100 µL into the 50 mL of Sample Buffer** to obtain **Sample Buffer with DTT.**
Note: Tick the "Yes" box on top of the yellow lid.
Sample Buffer with DTT should be used within one week.

d. Transfer 5 ml of Sample Buffer with DTT into the Vial of Reaction Starter Mix. Mix thoroughly

e. Preparation of TG2-calibrators: Add **3 mL** of Sample Buffer with DTT to the vial with **TG2-Standard**, mix gently afterwards.
Prepare calibrators using normal laboratory techniques and dilute them according the scheme below. The usage of protein low bind tubes is highly recommended. Calibrators have to be assayed at least in duplicate.

Scheme for calibrator preparation:

Calibrator No.	Activity* [µU/mL]	TG2-Conc. [ng/mL]	TG2/well [pg]	Volume **(taken from calibrator No.)**	Sample Buffer with DTT
1	14	10	500	**30** µL **TG2-Standard**	**870** µL
2	10.5	7.5	375	**300** µL (**1**)	**100** µL
3	7	5.0	250	**150** µL (**1**)	**150** µL
4	3.5	2.5	125	**125** µL (**1**)	**375** µL
5	1.4	1.0	50	**50** µL (**1**)	**450** µL
6	0.7	0.5	25	**25** µL (**1**)	**475** µL
7	0.35	0.25	12.5	**20** µL (**1**)	**780** µL
Negative control	0	0	0	-	**250** µL

* specific activity ≙ 1.41 U/mg determined with
Zedi*Xclusive* Microbial Transglutaminase Assay Kit (Product No.: Z009)

8. Assay procedure

Before starting the assay, all components of the kit (including the desired number of unsealed microstrips) must have reached room temperature (23 ± 3°C).

Put the desired number of microstrips into the frame. All reagents should be mixed by gentle inversion or vortexing prior to use. Do not allow foam formation. To achieve best results, careful washing is essential. It is crucially important to remove the wash solution completely. For that purpose, tap the plate firmly on several layers of absorbent tissue. Automated washers must be verified according to results obtained by manual washing.

a. Immediately prior to use, soak the solid phase once: fill wells with **200 µL Sample Buffer with DTT** each, **soak for about 10 minutes** in the wells and remove the liquid.

b. If suggested transglutaminase concentration in the sample exceeds the highest calibrator, dilute this sample accordingly, using **Sample Buffer with DTT**.

c. Dispense the **calibrators No. 1 – 7**, the **negative control** and the **diluted samples** rapidly into the microwells, **50 µL** per well. Duplicate measurements are recommended.

d. Start reaction by adding **50 µL** of **Reaction Starter Mix** (Biotinyl-cadaverine / Calcium / Sample Buffer with DTT) to each well.
It is highly recommended to use an automatic dispenser on this step.

Incubate the plate for 30 minutes at room temperature (23 ± 3°C).

e. Stop the reaction by adding **150 µL of Reaction Stop** (ready-to-use) to each well and allow to stand for 5 min at room temperature (23 ± 3°C).

Note: Stop the reaction in the same pattern used for starting.

f. Wash the wells 5 times with **300 µL Wash Buffer**.

g. Dispense **100 µL of Streptavidin-HRP conjugate** (ready-to-use) per well.

Incubate the plate for 15 minutes at room temperature (23 ± 3°C).

h. Wash the wells **5 times with 300 µL Wash Buffer**.

i. Rapidly (preferably using an automatic dispenser) dispense **100 µL** of **Substrate Solution** (ready-to-use) per well. Incubate the plate for **15 min at**

room temperature (23 ± 3°C). As the substrate is photosensitive, avoid intense light exposure (e.g. direct sunlight).

j. Rapidly (preferably using an automatic dispenser) dispense **100 µL Stop Solution** (ready-to-use) per well. Use the same sequence as for the substrate. Agitate the plate, preferably on an orbital shaker, for about 10 seconds. Avoid air bubbles.

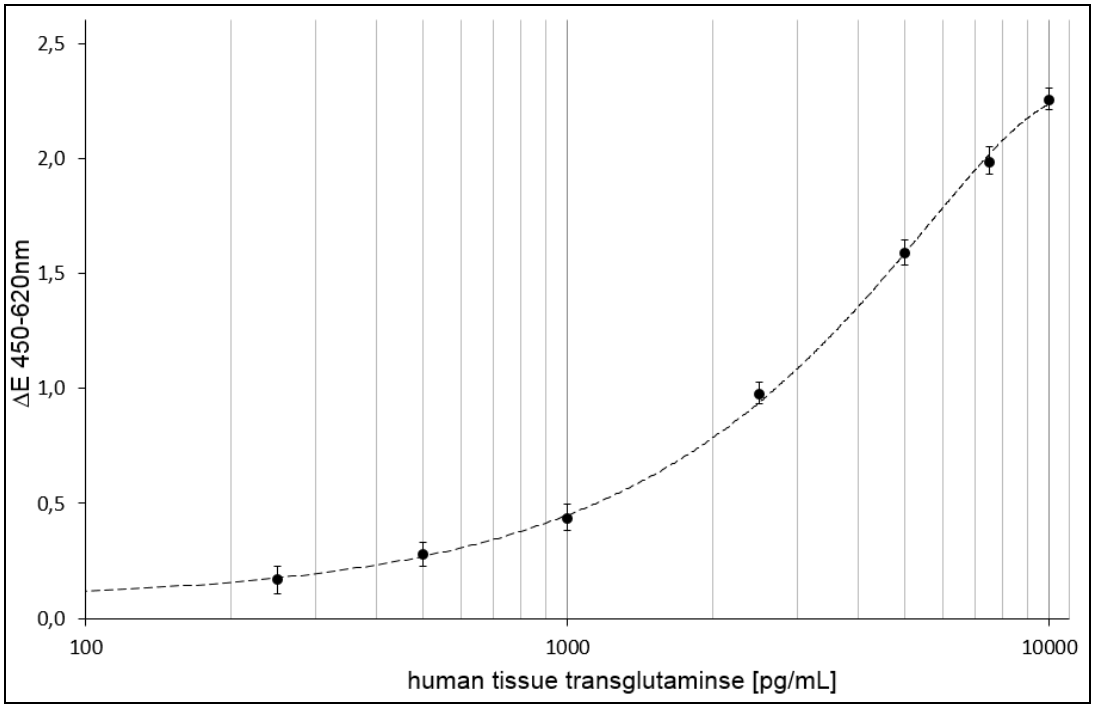
k. Immediately read the absorbance at 450 nm or preferably at **450 nm** and **620 nm**.

l. Store the remainder of the reagents refrigerated if they are to be used again.

9. Evaluation

If adsorbance at 450 nm and 620 nm was measured, subtract the 620 nm value from the 450 nm-value for compensation of the microtiter plate background. Otherwise use the 450 nm-value for further evaluation.
The data obtained are quantitatively evaluated with the standard curve, as shown below. However, the depicted curve can only serve as a model. It can not substitute the measurement of the calibrators, together with the control and actual samples. The curve has been constructed with a conventional evaluation program, using a 4-parameter function. The Spline approximation is also appropriate.

Do not use for calibration



If no computer supported evaluation is possible, the standard curve may be drawn by hand. It allows transformation of the absorbance value of a sample into its concentration, i.e. into [µU TG2 / mL] or [ng TG2 / mL] sample.

10. Warranty

Zedira GmbH guarantees that the product delivered has been thoroughly tested to ensure that its properties specified herein are fulfilled. No further warranties are given.
The performance data presented here were obtained using the procedure indicated. Any modification in the procedure may affect the results in which case Zedira GmbH disclaims all warranties whether expressed, implied or statutory. Moreover, Zedria GmbH accepts no liability for any damage, whether direct, indirect or consequencial, which results from inappropriate use or storage of the product.

11. Summary flow chart

a. Dissolve lyophilized DTT by adding 120 µL of water and mix.
   Transfer 100 µL DTT into the 50 mL Sample Buffer.

b. Dilute the sample in Sample Buffer with DTT (50 mL, ready-to-use) and mix.

c. Dilute the Wash Buffer 10x-concentrate (50 mL, blue) with 450 mL water and mix.

d. Dilute the Reaction Starter Mix with 5 mL Sample Buffer with DTT.

e. Soak the wells once with 200 µL Sample Buffer with DTT incubate for 10 min at room temperature (23 ± 3°C), remove liquid.

f. Dispense 50 µL of the calibrators, negative control and of diluted samples into the wells of the solid phase.
   Duplicate measurements are recommended.

g. Start reaction by adding 50 µL of Reaction Starter Mix (Biotinyl-cadaverine / Calcium / Sample Buffer with DTT) to each well. Incubate 30 min at room temperature (23 ± 3°C).

h. Stop reaction by adding 150 µL of Reaction Stop (ready-to-use) to each well. Use the same sequence as for the Reaction Starter Mix.
   Allow the reaction to stand for 5 min.

i. Wash the wells 5 times with 300 µL Wash Buffer each.

j. Dispense 100 µL of Streptavidin-HRP conjugate (ready-to-use) per well. Incubate for 15 min at room temperature (23 ± 3°C).

k. Wash the wells 5 times with 300 µL Wash Buffer each.

l. Dispense 100 µL of Substrate Solution (14 mL, ready-to-use, black vial) per well. Incubate the plate for 15 min at room temperature (23 ± 3°C).
   Then, add 100 µL Stop Solution (14 mL, ready-to-use, colourless) per well. Use the same sequence as for the substrate solution. Agitate the plate briefly and avoid air bubbles.

m. Immediately measure the absorbance at 450 nm (or preferably at 450 nm and 620 nm).

n. Quantitative evaluation: Determine the standard curve from the absorbance of the calibrators. Using this curve, transform the absorbance of the samples into their respective activity or concentration (µU/mL or ng/mL).