Directions for use

TRANSGLUTAMINASE ASSAY KIT, FLUORESCENT

Transamidase-activity based TG assay
Transamidation Assay, “DCC”
(Fluorogenic: λ_{ex} = 332 nm; λ_{em} = 500 nm)

Art.-No. T036

For Research & Development Only

Revision Number: 2.0 **Release Date: 15 June 2023**

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1. Assay principle

Transglutaminases (TG, TGM) catalyze acyl transfer reactions from glutamine residues in proteins or peptides to primary amines.

The TRANSGLUTAMINASE-ASSAY KIT (T036) records the course of enzymic transamidation reactions by determination of the continuous fluorescence increase (excitation wavelength 332 nm; emission wavelength 500 nm) upon transglutaminase-catalyzed incorporation of monodansylcadaverine into glutamine-donor substrate N,N-dimethyl casein ("DCC assay") as shown in figures 1 and 2 [1-3].

The covalent coupling is accompanied by marked changes in the fluorescence emission shift and an increase in intensity of fluorescence of the dansyl group.

The fluorescence-based assay is non-specific and therefore suitable for the measurement of the majority of transglutaminase (hTG1, hTG2, hTG3a, hTG6, hTG7, hFXIIIa) family members, e.g. for the determination of selectivity.

2. Intended use

Determination of transglutaminase (hTG1, hTG2, hTG3a, hTG6, hTG7, hFXIIIa) activity.

3. Test sample

Samples containing transglutaminases (hTG1, hTG2, hTG3a, hTG6, hTG7, hFXIIIa) from human or mammals (mouse, rat, dog, cynomolgus, pig, and cat tissue transglutaminases or guinea pig liver transglutaminase).

Application: Inhibitor screening; determination of inhibition data.

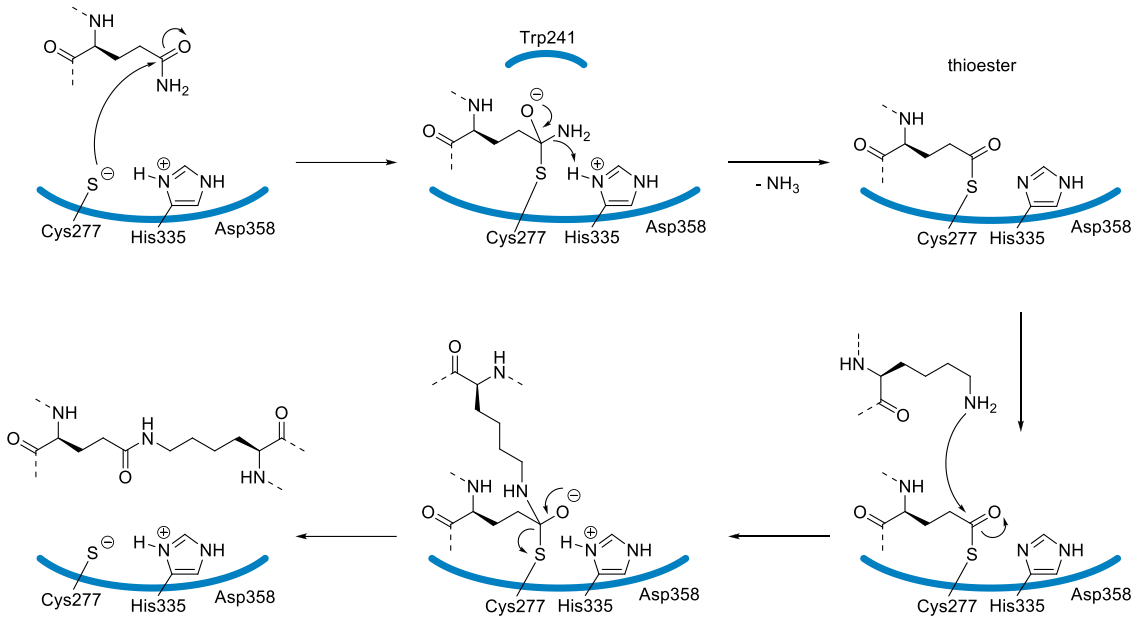


Figure 1: Transglutaminase reaction mechanism (transamidation): the catalytic triad of human tissue transglutaminase is formed by the amino acids Cys277-His335-Asp358. The proposed thiolate-imidazolium ion pair is exceptionally nucleophilic enabling the attack of the otherwise inert carboxamide side-chain of protein bound glutamine to yield the thioester intermediate that itself is prone to react with the ε-amino group of lysine. The proposed tetrahedral oxyanion is stabilized by Trp241 and by the backbone nitrogen of Cys277; the driving force of the reaction is the release of ammonia. Hydrolysis of the reactive thioester is suppressed by the narrow hydrophobic tunnel excluding water from the catalytic site [3, 4].

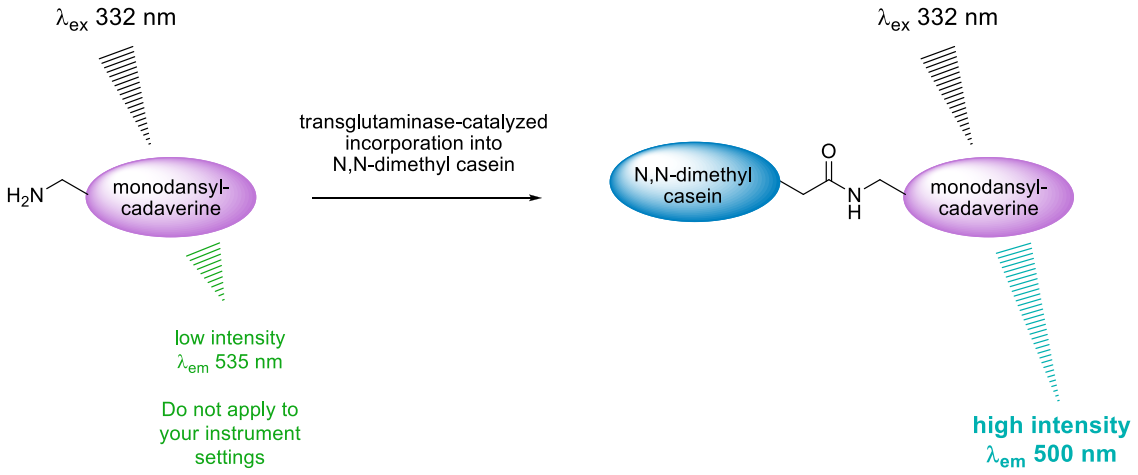


Figure 2: Transglutaminase-catalyzed incorporation of monodansylcadaverine into glutamine-donor substrate N,N-dimethyl casein ("DCC assay") yielding in a fluorescence emission shift and an increase in intensity of fluorescence of the dansyl group [1-3].

4. Reagents in the kit

(1) T036/SR — SUBSTRATE REAGENT (**SR**):
2 x 0.185 mg monodansylcadaverine.

(2) T036/BR — BUFFER REAGENT (**BR**):
2 x 20 mL TRIS buffer pH 8.0 containing calcium chloride, sodium chloride, N, N-dimethylcasein, glutathione (lyophilizate).

Optionally available at Zedira (not included in the kit):

- T009 — hTG1 REFERENCE, Human keratinocyte transglutaminase, rec. in *E. coli*
- T002 — hTG2 REFERENCE, Human tissue transglutaminase, rec. in *E. coli*
- T022 — hTG2 REFERENCE, Human tissue transglutaminase, rec. in insect cells
- T040 — mTG2 REFERENCE, Mouse tissue transglutaminase
- T038 — rTG2 REFERENCE, Rat tissue transglutaminase, rec. in *E. coli*
- T013 — TG3a REFERENCE, Human epidermal transglutaminase, Dispase activated rec. in insect cells
- T021 — hTG6 REFERENCE, Human neuronal transglutaminase, rec. in insect cells
- T011 — hTG7 REFERENCE, Human transglutaminase 7, rec.in *E. coli*
- T070 — hFXIIIa REFERENCE, Human Factor XIIIa, Thrombin activated, A subunit, rec. in insect cells
- Z006 — ("Z-DON", TG2-INHIBITOR)

Please refer to our catalogue (https://zedira.com) for selected transglutaminase family members (hTG1, hTG2, hTG3a, hTG6, hTG7, hFXIIIa) from human or mammals (mouse, rat, dog, cynomolgus, pig, and cat tissue transglutaminases or guinea pig liver transglutaminase). Please feel free to contact us for assistance (contact@zedira.com).

5. Reagent preparation, storage, and stability

In their original packing box, when stored at 2-8 °C, the unopened reagents are stable until the expiration date printed on the box.

Shipment at ambient temperature is possible.

Table 1: Reconstitution of kit components

Component	Preparation	Storage
BUFFER REAGENT (**BR**) 2 x 20 mL TRIS buffer lyophilizate (TRIS, $CaCl_2$, NaCl, N,N-dimethylcasein, glutathione, pH 8.0)	Add 19.5 mL of deionized water per vial and mix carefully	Consume within one day or store frozen at -20°C for at least 4 weeks
SUBSTRATE REAGENT (**SR**) 2 x 0.185 mg monodansylcadaverine	Dissolve one vial in 550 µL DMSO	Store at 2-8°C Stable for at least 6 months

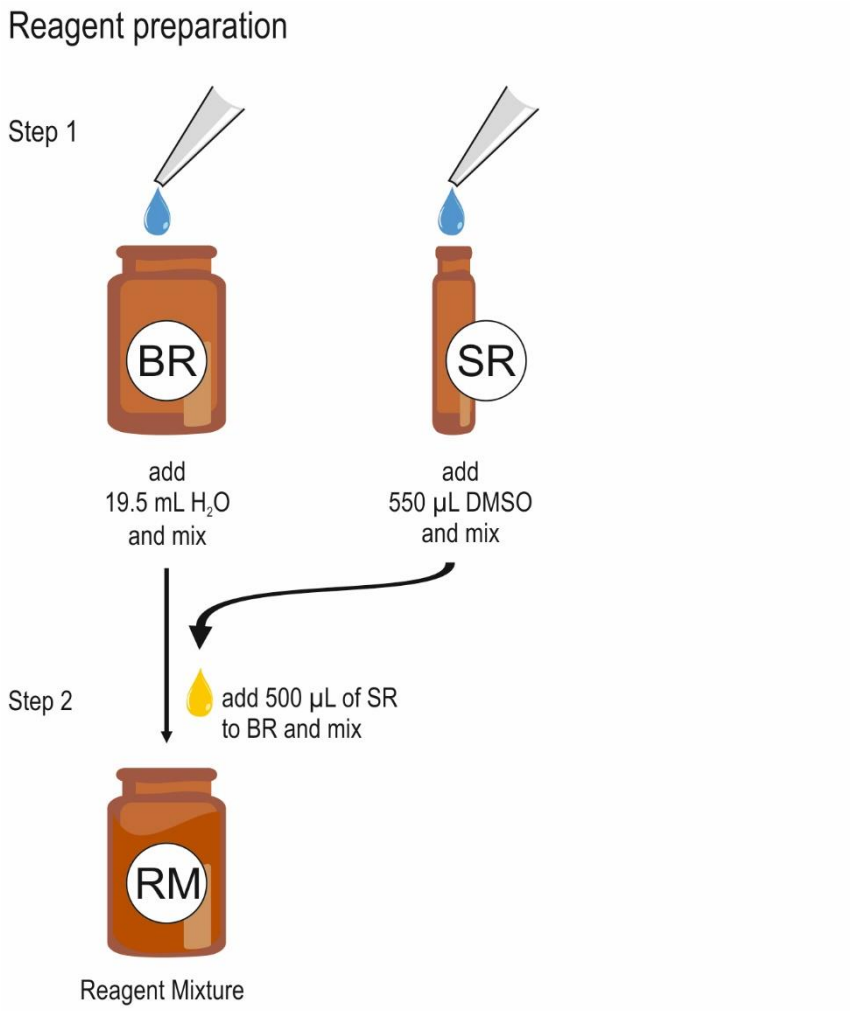
Table 2: Preparation of REAGENT MIXTURE (**RM**)

Component	Preparation	Storage
SUBSTRATE REAGENT (**SR**) BUFFER REAGENT (**BR**)	Add 500 µL **SR** to 19.5 mL prepared **BR** and mix	Stability of the REAGENT MIXTURE after reconstitution: 20°C – 8 hours -20°C – 1 month

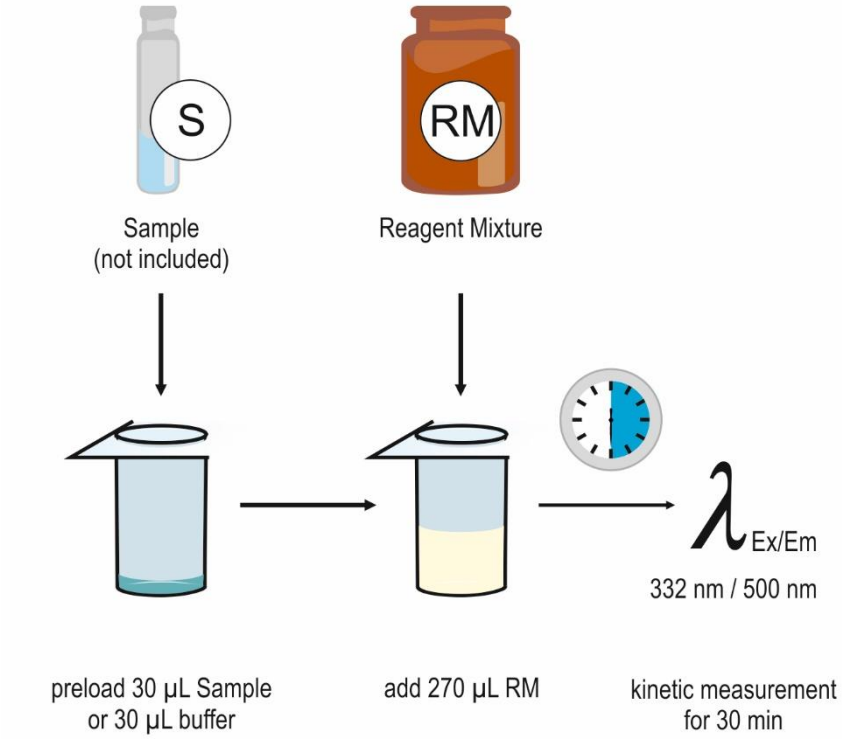
Table 3: Components optionally available at Zedira (not included in the kit)

Component	Preparation	Storage
TG REFERENCES	Dissolve as indicated in the respective CoA Serial dilution 500-8.0 nM (38.7-0.6 µg/mL) in buffer Final assay concentration will be one-tenth	Prepare enzyme diluent directly before use Do not vortex! Store diluent on ice
Z006 TG2-INHIBITOR "Z-DON"	Prepare a DMSO stock solution or serial dilution according to your desired assay concentration as indicated in the respective PDS	DMSO stock solutions can be stored at -20°C for at least 6 months

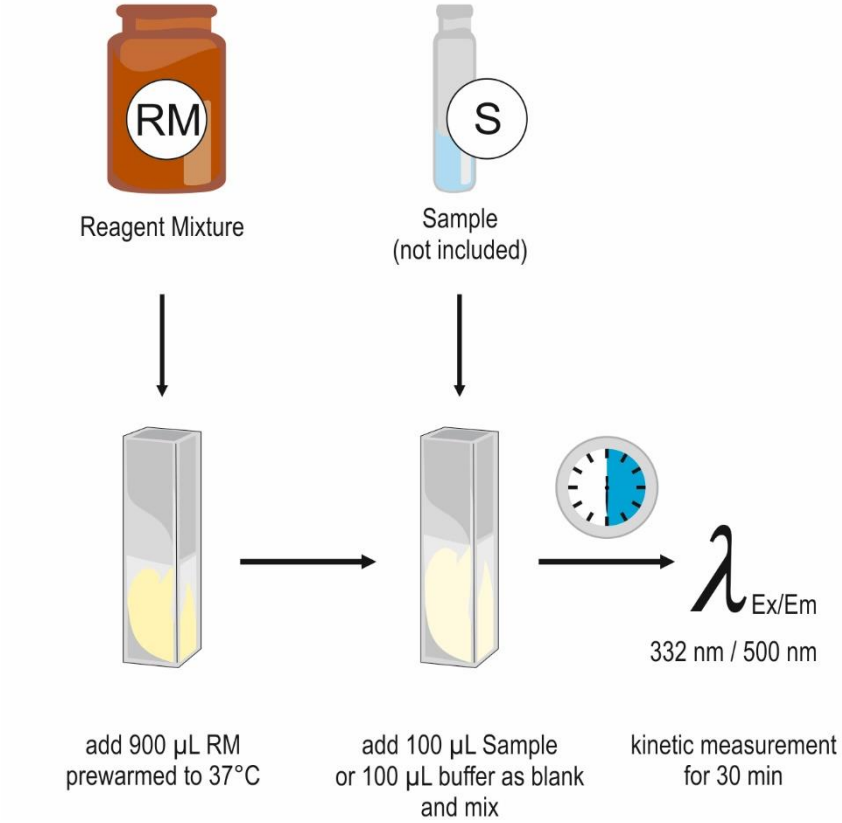
6. Schematic assay overview



Assay procedure (96 well microtiter plate)



Assay procedure (cuvette)



7. Procedure and Equipment

Set the fluorescence spectrophotometer temperature to 37°C, if applicable. Data shown in the manual are obtained at this temperature.

The TRANSGLUTAMINASE-ASSAY KIT (T036) can be used in fluorescence plate readers using microplates as well as in standard fluorescence spectrophotometers with cuvettes. Refer to the instructions of the manufacturer.

Add SAMPLE (**S**) and REAGENT MIXTURE (**RM**) depending on your assay format:

Microtiter plate (96 well, 300 µL): Select a microplate that is rated for fluorescence-based assays and exhibits little or no autofluorescence in the emission range of the reagent you wish to use. Black plates are typically recommended.

Preload the wells with 30 µL of your SAMPLE (**S**). Start the reaction by adding 270 µL of REAGENT MIXTURE (**RM**) to SAMPLE (**S**) and mix thoroughly.

Fluorescence cuvette (1 mL): Start the reaction by adding 100 µL of SAMPLE (**S**) to 900 µL of REAGENT MIXTURE (**RM** - prewarmed to 37°C), mix thoroughly.

Use a TRIS BUFFER instead of SAMPLE (**S**) to generate a blank (e.g. 20 mM TRIS, 300 mM NaCl, pH 7.2). Measurement of samples in duplicate or triplicate is recommended.

Start the kinetic measurement after starting the reaction using the instrument settings shown in table 4.

Table 4: Instrument settings for fluorescence spectrophotometer.

Excitation wavelength	332 nm
Emission wavelength	500 nm
Assay time (min)	30

Determine the slope of fluorescence increase over a reaction time of 30 min. Use the linear part of slope for assessment.

Unlike absorbance, fluorescence is not an absolute measurement. The RFU scale cannot be standardized. Accordingly, absolute counts cannot be compared between readers of different manufacturers. The intensity of a fluorescent signal is usually relative to other measurements, to a reference measurement, or to the gain settings [5].

Adjust fluorescence gain of the sample with the expected highest signal output (e.g. your positive control) to avoid saturation but still cover a good assay dynamic range.

NOTE: Transglutaminase references are optionally available (see section 4) for quantification purposes.

8. Number of sample measurements

The assay reagents per kit are sufficient for 140 measurements in microtiter plates and 40 measurements in cuvettes.

9. Results

Fluorescence increase is proportional (2nd polynomial fit) to the transglutaminase activity. The correlating transglutaminase amount can be calculated with a reference curve. Figure 3 shows a typical plot.

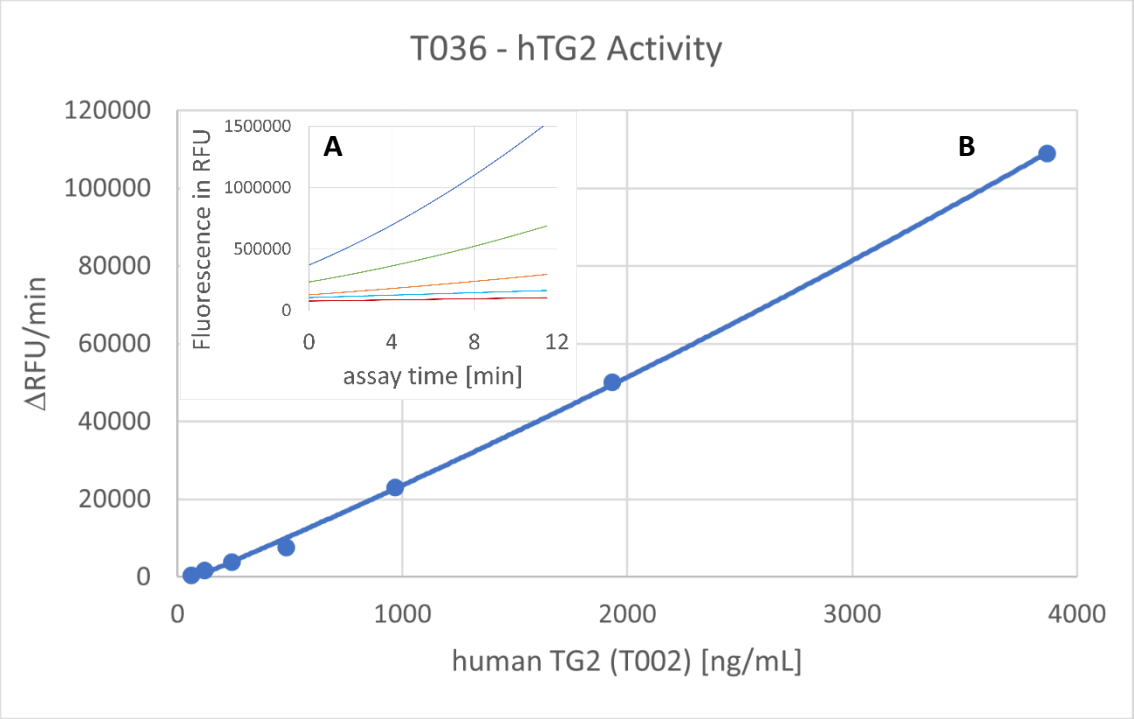


Figure 3A: **Recombinant human tissue transglutaminase** (T002, hTG2 REFERENCE) dependent increase in fluorescence emission (RFU: relative fluorescence units) for a serial dilution from 3.9 to 0.06 ng/mL (50 to 0.8 nM) over 30 min. The increase in fluorescence was found to be linear between 2 and 10 min. Each concentration was determined in triplicate. The control without hTG2 did not yield any increase in signal intensity (not shown). The graph represents the average curves of triplicate measurements (error bars are not shown due to readability).

Figure 3B: Plotting ΔRFU/min (taken from figure 3A, assessed between 2 and 10 min) against the indicated hTG2 concentration, a non-linear regression fit (2nd polynomial fit, $R^2 > 0.99$) was obtained. The lower limit of quantification of the assay (LLOQ) was found to be 0.77 µg/mL (10 nM) hTG2.

10. Reference Range

The TRANSGLUTAMINASE-ASSAY KIT (T036) is suitable for measurements of 10 nM to 50 nM (0.77 µg/mL to 3.9 µg/mL) of hTG2 (T002).

The lower limit of quantification (LLOQ) depends on the analytical system used (CLARIOstar, Serial Number: 430-0347; BMG LabTech, MARS, Version 4.00 R2) and was found to be 0.77 µg/mL (10 nM) hTG2.

11. Specifity

The transglutaminase catalyzed incorporation of dansylcadaverine into N,N-dimethyl-casein is a non-specific reaction and therefore suitable for the measurement of the majority of transglutaminase family members, e.g. for selectivity measurements. Relative activities (ΔRFU/min) of selected transglutaminase family members are shown in figure 4.

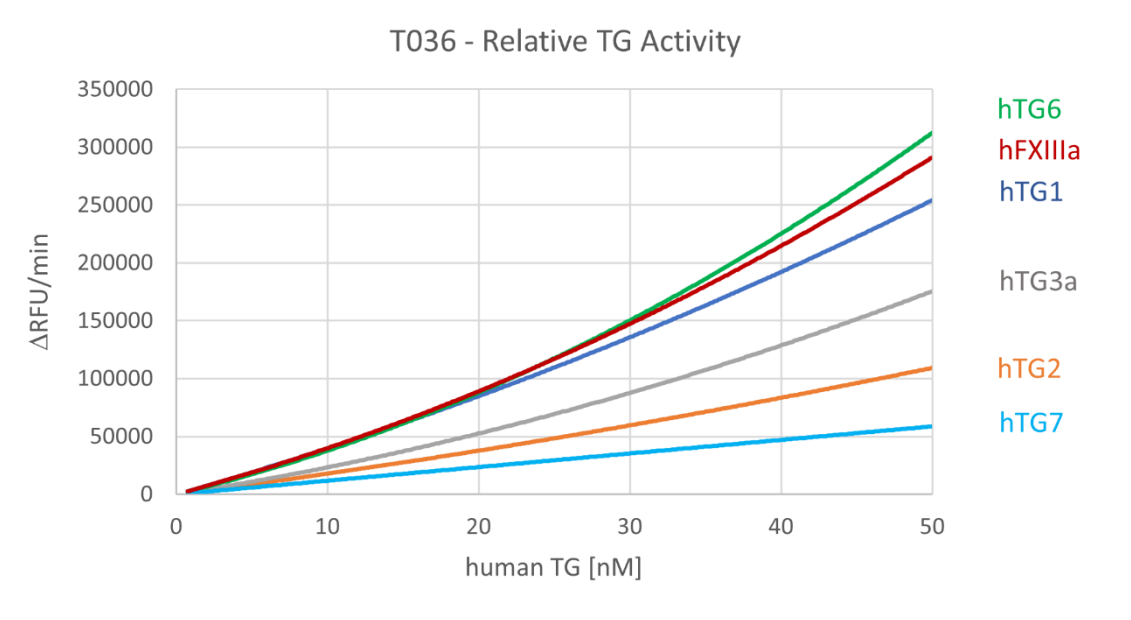


Figure 4: Relative activities (ΔRFU/min) of selected transglutaminase family members (hTG1, hTG2, hTG3a, hTG6, hTG7, hFXIIIa) from human.

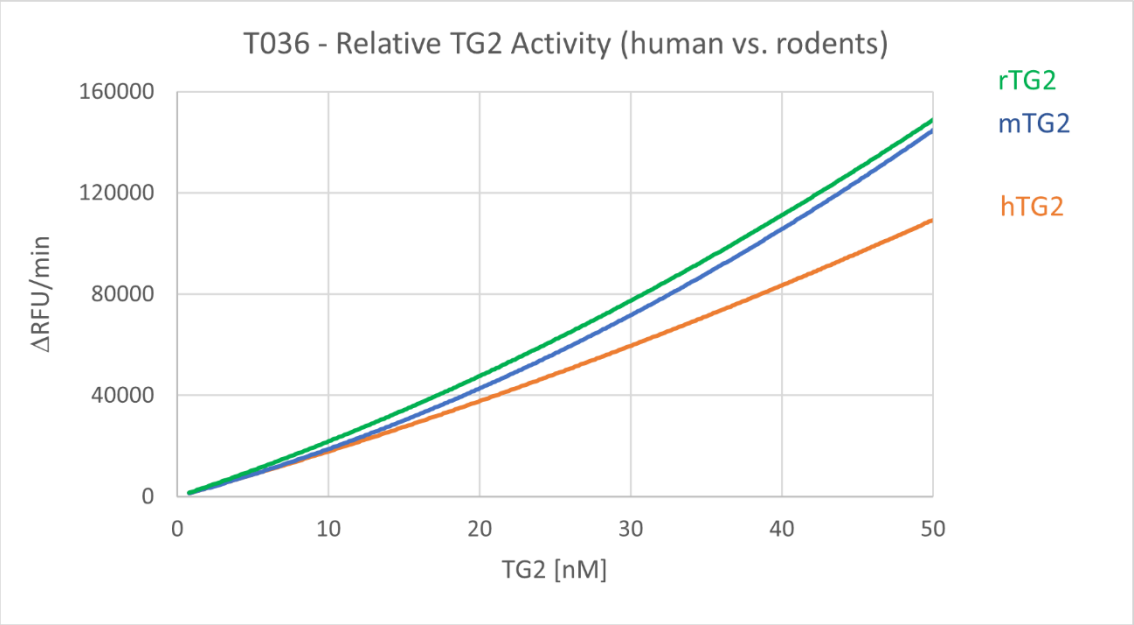


Figure 5: Relative activities (ΔRFU/min) of tissue transglutaminases from human compared to rodents (mouse, rat).

12. Limitations

The TRANSGLUTAMINASE-ASSAY KIT (T036) is meant for research and development only. The kit has been optimized for the measurement of purified hTG2 (T002) in buffer.

13. Precision

All performance studies were conducted on CLARIOstar^{Plus}, Serial Number: 430-4064; BMG LabTech using the MARS Software package, Version 4.00 R2 and black 96 well plates (PS black, Greiner Bio One, Art-Nr.: 655076). Performance was assessed using rec. hTG2 (T002, lot 1918aT002, Zedira GmbH) from 50-0.0.8 nM (3.9-0.06 µg/mL).

The intra-assay-variance (coefficient of variation, CV%) determined during kit development was found to be 2.73 % for 50 nM (3.9 µg/mL) recombinant human tissue transglutaminase (T002, hTG2). The day-to-day variance was found to be 4.61 % for 50 nM (3.9 µg/mL) over a 3-day period, 2 series per day and 4 repetitions within each series.

14. Inhibitor screening

The TRANSGLUTAMINASE-ASSAY KIT (T036) is suitable for compound screening and determination of inhibition data towards transglutaminase.

The "DCC-assay" setting is one of the most challenging one due to the exceptionally competitive N,N-dimethyl casein substrate. Accordingly, the resulting inhibition data will be quite robust.

For the determination of inhibition data (IC_{50} values; inhibitor concentration blocking 50 % of enzymatic activity) microtiter plates as mentioned in chapter 7 are used.

Dilute the inhibitor (DMSO, if applicable) to the desired start concentration and subsequently dilute it stepwise to get 11 serial diluted concentrations.

Inhibitors are usually present in DMSO stock solutions of e.g. 10 mM. Prepare a serial dilution in DMSO. Dilute each of these aliquots in a second step with a suitable buffer available (e.g. phosphate-buffered saline [PBS, pH 7.4] or TRIS buffer [pH 8.0]). Take care not to exceed a DMSO concentration of 20 % (v/v) in this second step. That means your dilution factor should be at least 5. This corresponds to a final assay concentration of 1 % (v/v).

Attention: please **do not use** BUFFER REAGENT (**BR**).

Preload the wells with 15 µL of the inhibitor concentrations, serial diluted. One row per dilution series. A measurement in triplicates is recommended resulting in three rows. As blank, please use the buffer (including max. 20 % DMSO as mentioned above) according to the "second step" dilutions as control (position without inhibitor) and for gain adjustment.

Prepare the REAGENT MIXTURE (BUFFER REAGENT and SUBSTRATE REAGENT) as mentioned in Table 2.

Start the reaction by adding the transglutaminase to the REAGENT MIXTURE (dilution factor 20, e.g. 0.6 mL TG to 11.4 mL REAGENT MIXTURE for the measurement of one inhibitor in triplicates) and mix carefully.

Add 285 µL/well TG- REAGENT MIXTURE using a multichannel pipette to the inhibitor and start the kinetic measurement 3 minutes after start of the reaction.

Determine the slope of fluorescence increase over a reaction time of 30 min. Use the linear part of slope for assessment.

Set the slope of fluorescence with no inhibition (control) to 100 % and calculate the residual activity slopes to percentage values.

Plot the residual TG activity [%] over the inhibitor concentration and calculate the IC_{50} value by using the 4-Parameter fit as shown in figure 6.

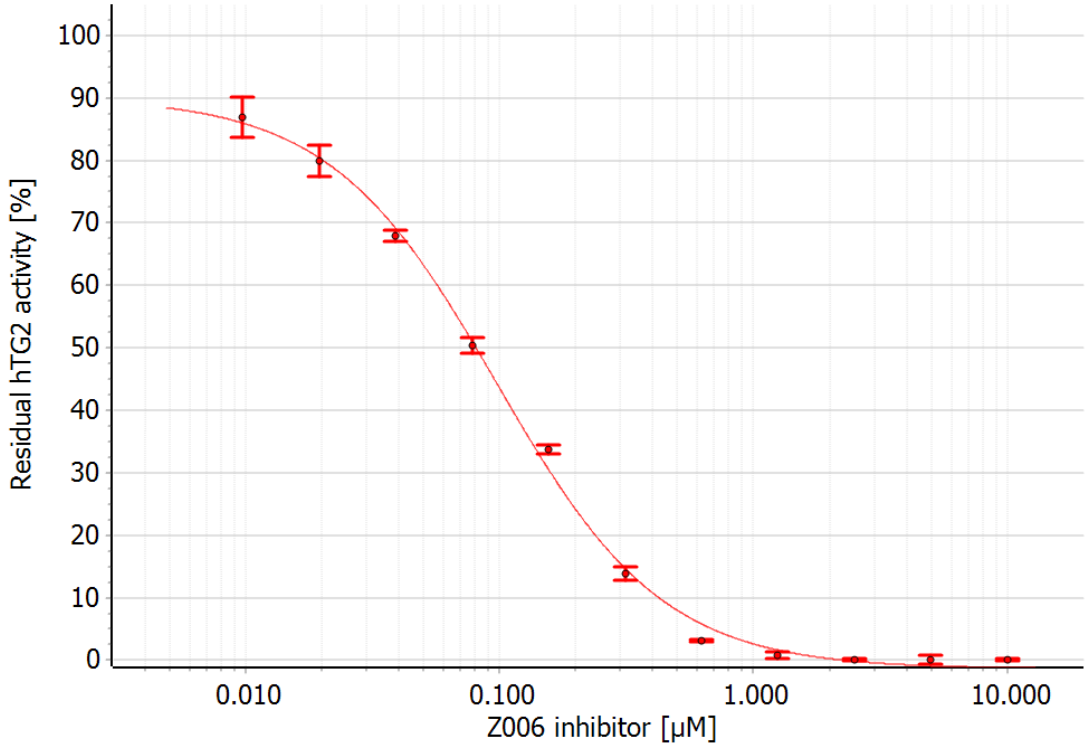


Figure 6: Potency of TG2-INHIBITOR "Z-DON" (Z006) with Human TG2 (T022), final assay concentration 10 nM measured on a CLARIOstar® fluorescence reader (BMG Labtech). The IC_{50} value was calculated to 81 nM using the MARS software package (BMG Labtech).

15. References

1. Lorand, L., et al., *Transamidating enzymes: II. A continuous fluorescent method suited for automating measurements of factor XIII in plasma.* Analytical Biochemistry, 1971. **44**(1): p. 221-231.
2. Pasternack, R., et al., *Novel inhibitor ZED3197 as potential drug candidate in anticoagulation targeting coagulation FXIIIa (F13a).* J Thromb Haemost, 2019: p. e14646.
3. Büchold, C., et al., *Features of ZED1227: The first-in-class tissue transglutaminase inhibitor undergoing clinical evaluation for the treatment of celiac disease.* Cells, 2022. **11**(10): p. 1667.
4. Pinkas, D.M., et al., *Transglutaminase 2 undergoes a large conformational change upon activation.* PLoS Biol, 2007. **5**(12): p. e327.
5. BMG Labtech "How to optimise fluorescence gain"