Directions for use

ZEDI*XCITE* 360/460 FLUOROGENIC TG2-ASSAY KIT

Isopeptidase-activity based TG2 assay
(Fluorogenic: λ_{ex} = 360 nm; λ_{em} = 460 nm)

Art.-No. F016

For Research & Development Only

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Watch the video on YouTube, describing
how to run ZEDI*XCITE* FLUOROGENIC TG2-ASSAY KIT **F014**
which is performed comparable to the present Kit **F016**

Note: **F014** is performed at different instrument settings (wavelengths)

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1. Assay principle

TISSUE TRANSGLUTAMINASE (TG2, transglutaminase 2) is a multifunctional enzyme best known for its crosslinking activity, but it can also cleave isopeptide bonds. This feature is used to provide an **easy to handle**, **robust, sensitive,** and **precise fluorogenic assay** to measure TG2 activity. The assay is suitable for research purposes, drug discovery programs, and quality assurance.

TISSUE TRANSGLUTAMINASE cleaves a dark quencher molecule from the side chain of a TG2 specific peptide (SUBSTRATE REAGENT **SR**). The TG2-substrate complex is cleaved by the incorporation of a glycine methyl ester molecule. Subsequently, the fluorescence of an N-terminal coupled fluorophore increases and can be continuously monitored (excitation wavelength 360 nm; emission wavelength 460 nm). The assay principle is shown in Figure 1.

2. Intended use

Specific and sensitive measurement of TISSUE TRANSGLUTAMINASE (TG2) activity in biological samples like tissue homogenates. Suitable for determination of TG2 concentrations of 1.25 to 10 nM (100 to 780 ng/mL or 3.7 to 46 U/L *) using a REFERENCE TG2 preparation.

* For the definition of Unit, please refer to chapter 10

3. Test sample

Sample containing human TISSUE TRANSGLUTAMINASE (hTG2).

Please note: In case of measurement of undiluted biological samples, optionally, consider the addition of dithiothreitol (DTT) to the REAGENT MIXTURE (**RM**). For details, please refer to section 7.

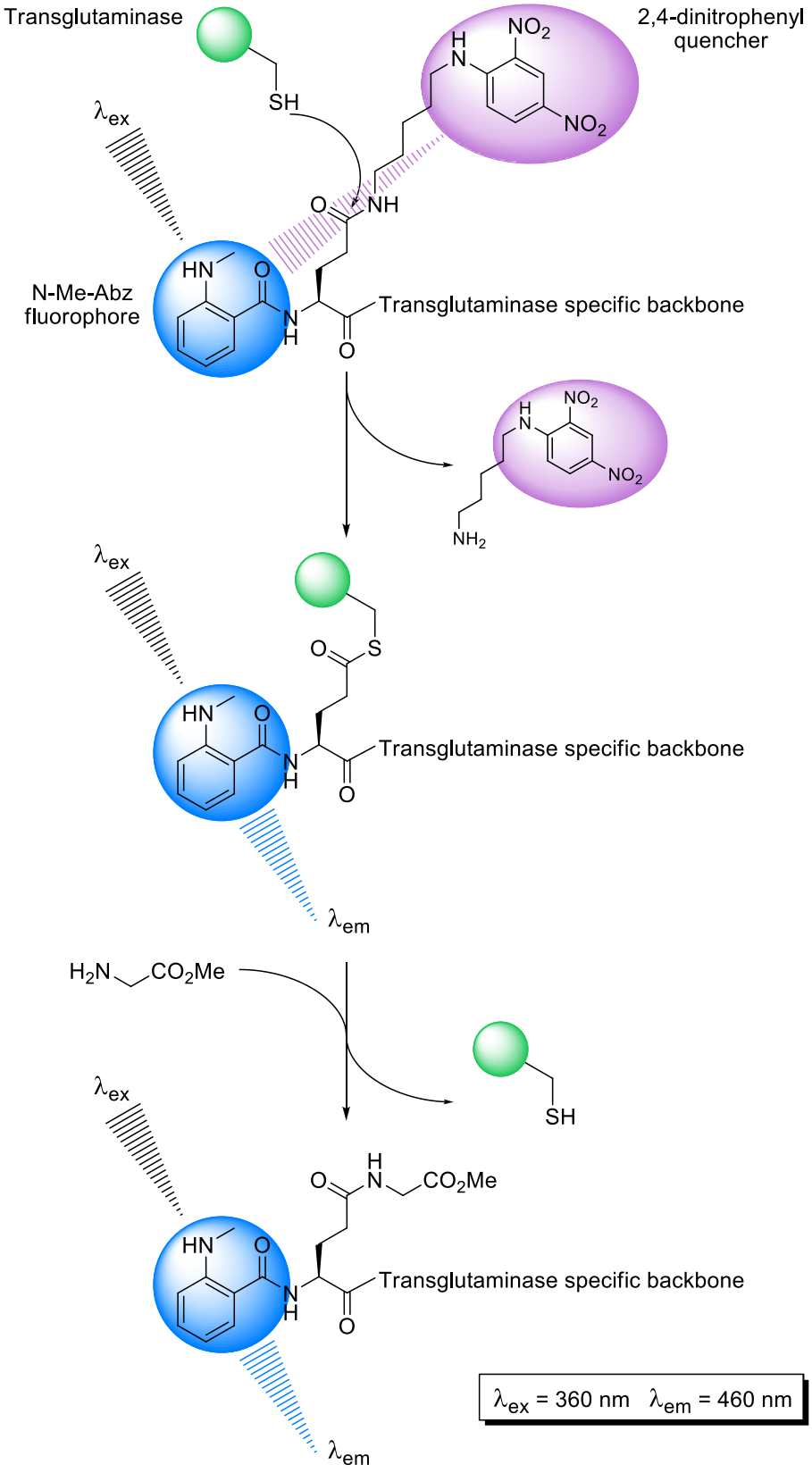


Figure 1: Cleavage of a carboxamide bond in the side chain of a specific peptidic backbone is catalyzed by tissue transglutaminase. The release of the dark quencher is followed by incorporation of glycine methyl ester. After the dark quencher is released, the fluorescence of the fluorophore increases.

The assay continuously monitors TG2 activity.

4. Reagents in the kit

(1) A170/SR — SUBSTRATE REAGENT (**SR**):
2 x 23 µL TG2 specific assay peptide (DMSO solution)

(2) B146/BR — BUFFER REAGENT (**BR**):
2 x 18 mL TRIS buffer pH 7.5 containing 10 mM calcium chloride, 100 mM sodium chloride, 5 mM glycine methyl ester (lyophilizate)

(3) D146/DB — DILUTION BUFFER (**DB**): 1 x 18 mL 10 mM TRIS buffer pH 8.1 containing 150 mM sodium chloride, 1 mM EDTA, 5 mM DTT (lyophilizate)

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

- T022 — hTG2 REFERENCE, Human tissue transglutaminase
- T040 — mTG2 REFERENCE, Mouse tissue transglutaminase
- T038 — rTG2 REFERENCE, Rat tissue transglutaminase
- A171 — N-ME-ABZ-PEPTIDE-CALIBRATOR, reaction product formed for calibration purposes
- Z006 — "Z-DON", TG2-inhibitor

5. Reagent preparation, storage, and stability

ZEDI*XCITE* 360/460 FLUOROGENIC TG2-ASSAY KIT F016 (BUFFER REAGENT **BR** and DILUTION BUFFER **DB** lyophilizates and SUBSTRATE REAGENT **SR**) must be stored at 2-8°C. Shipment at ambient temperature is possible. The unopened reagents are stable according to the retest date printed on the box.

Table 1: Reconstitution of Kit components

Component	Preparation	Storage
BUFFER REAGENT (**BR**) 2 x 18 mL TRIS buffer lyophilizate (50 mM TRIS, 10 mM $CaCl_2$, 100 mM NaCl, 5 mM Gly-OMe, pH 7.5)	Add 18 mL of deionized water per vial and mix carefully	Consume within one day or store frozen at -20°C for at least 4 weeks
DILUTION BUFFER (**DB**) 1 x 18 mL TRIS buffer lyophilizate (10 mM TRIS, 150 mM NaCl, 1 mM EDTA, 5 nM DTT, pH 8.1)	Add 18 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 23 µL TG2 specific assay peptide (DMSO solution)	Ready-to-use DMSO solution Bring to ambient temperature	Store at 2-8°C Stable for at least 6 months

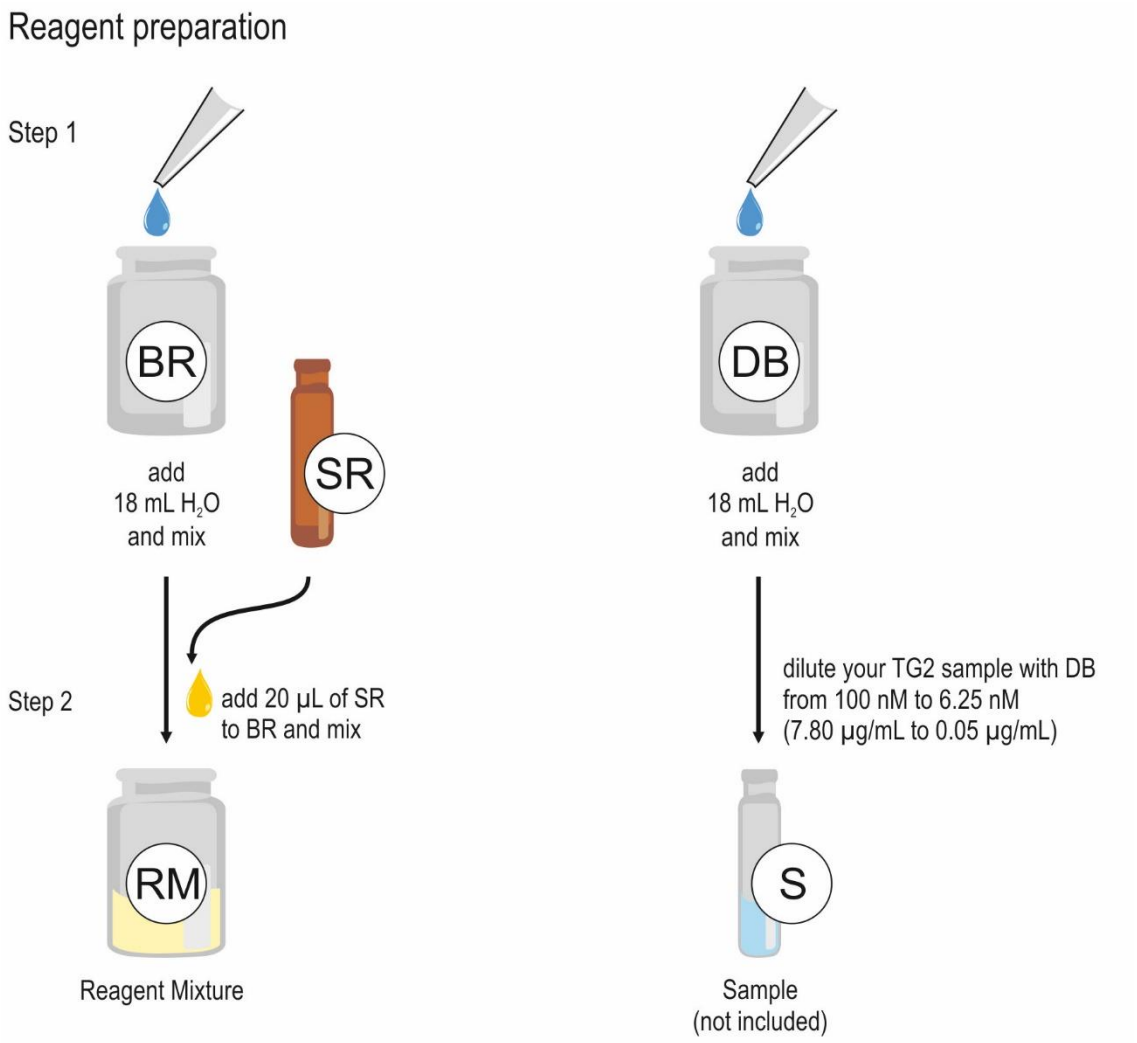
Table 2: Preparation of REAGENT MIXTURE (**RM**)

Component	Preparation	Storage
SUBSTRATE REAGENT (**SR**) BUFFER REAGENT (**BR**)	Add 20 µL **SR** to 18 mL prepared **BR** and mix Optionally, add 90 µL of DTT (1 M in water, details: section 7)	Consume within 2 hours Protect from light Keep **RM** at ambient temperature until usage

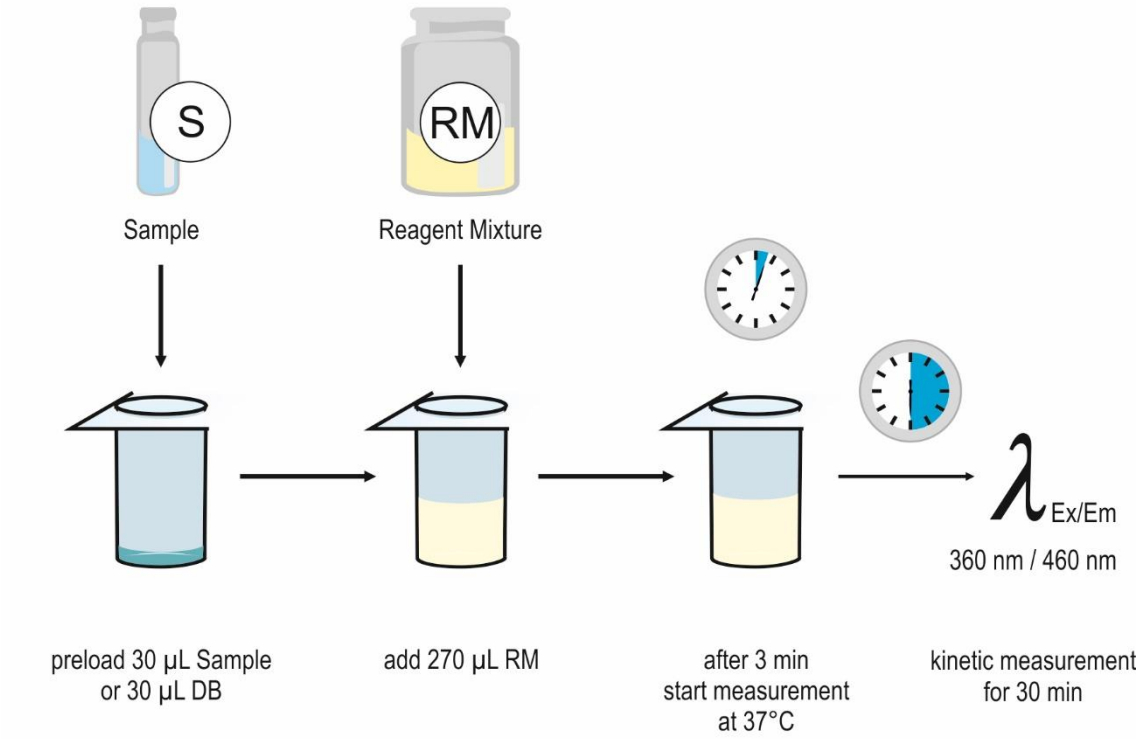
Table 3: Components optionally available at Zedira (not included in the kit)

Component	Preparation	Storage
T022 hTG2 REFERENCE	Dissolve as indicated in the respective CoA Serial dilution 100-6.25 nM (7.80-0.05 µg/mL) in **DB** Final assay concentration will be one-tenth	Prepare enzyme diluent directly before use Do not vortex! Store diluent on ice
T040 mTG2 REFERENCE	Dissolve as indicated in the respective CoA Serial dilution 100-6.25 nM (7.80-0.05 µg/mL) in **DB** Final assay concentration will be one-tenth	Prepare enzyme diluent directly before use Do not vortex! Store diluent on ice
T038 rTG2 REFERENCE	Dissolve as indicated in the respective CoA Serial dilution 100-6.25 nM (7.80-0.05 µg/mL) in **DB** Final assay concentration will be one-tenth	Prepare enzyme diluent directly before use Do not vortex! Store diluent on ice
A171 N-ME-ABZ-PEPTIDE-CALIBRATOR reaction product formed for calibration purposes	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
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

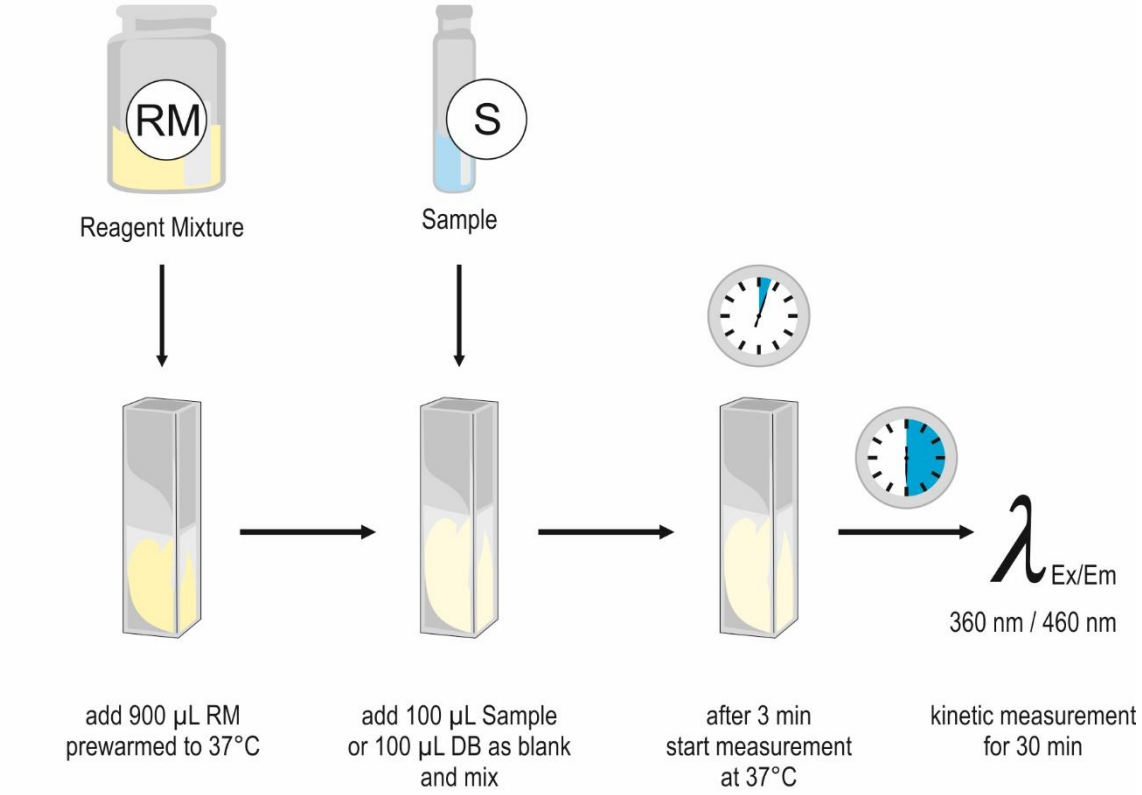
6. Schematic assay overview



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. However, the kit may also be run at ambient temperature.

The ZEDI*XCITE* 360/460 FLUOROGENIC TG2-ASSAY KIT (F016) 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.

Optionally: In case of measuring undiluted biological samples, consider the addition of dithiothreitol (DTT) to the REAGENT MIXTURE (**RM**). Briefly, add 90 µL of DTT (1 M in water) to 18 mL of **RM** and mix thoroughly.

Blank: Use DILUTION BUFFER (**DB**) instead of SAMPLE (**S**) to generate a blank. Measurement of samples in duplicate or triplicate is recommended.

Start the kinetic measurement 3 minutes after starting the reaction using the instrument settings shown in table 4.

Table 4: Instrument settings for fluorescence spectrophotometer.

Excitation wavelength	360 nm
Emission wavelength	460 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 [3].

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: A TG2 REFERENCE is optionally available (see section 4) for quantification. You may also consider using Zedira product A171 ("N-ME-ABZ-PEPTIDE-CALIBRATOR"), the reaction product formed, for calibration purposes.

8. Number of sample measurements

The assay reagents per kit are sufficient for 130 measurements in microtiter plates and 38 measurements in cuvettes.

9. Results

Fluorescence increase is proportional (2nd polynomial fit) to the TISSUE TRANSGLUTAMINASE activity. The correlating TG2 amount can be calculated with a reference curve. Figure 2 shows a typical plot.

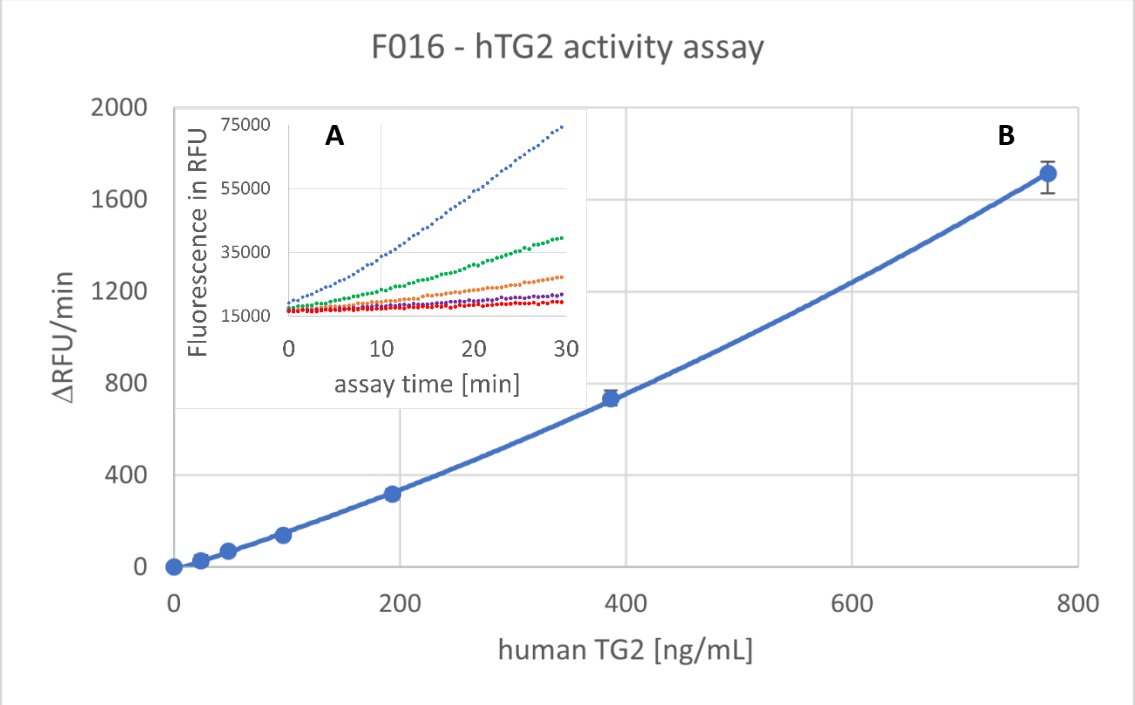


Figure 2A: **Recombinant human Tissue Transglutaminase** (T022, hTG2 REFERENCE) dependent increase in fluorescence emission (RFU: relative fluorescence units) for a serial dilution from 780 to 50 ng/mL (10 to 0.63 nM) over 30 min. The increase in fluorescence was found to be linear between 20 and 30 min. Each concentration was determined in quadruplicate. The control without hTG2 did not yield any increase in signal intensity (not shown). The graph represents the average curves of quadruplicate measurements (error bars are not shown due to readability).

Figure 2B: Plotting ΔRFU/min (taken from figure 2A, assessed between 20 and 30 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 100 ng/mL (1.25 nM) hTG2.

10.Calibration

Since the determination of a hTG2 concentration (in ng/mL or nM) might be not applicable, the enzyme activity can be calculated to **Enzyme Units** using Zedira product A171("N-ME-ABZ-PEPTIDE-CALIBRATOR").

The standard definition for 1 unit (U) is the amount of enzyme that catalyzes the reaction of 1 µmol of substrate per minute. However, in most applications, the conversion of 1 µmol of substrate is not feasible and other definitions may be preferred.

In this assay the common non-standard definition of 1 U = 1 nmol/min is used.

The conversion of enzyme concentration to units requires the extinction coefficient, determined by the measurement of the fluorescence (RFU) of a serial dilution of A171 ("N-Me-ABZ-PEPTIDE-CALIBRATOR") as shown in figure 3. The micromolar extinction coefficient ε $[\mu M]^{300\mu L}$, measured with 300 µL assay volume in a microtiter plate, equals to the slope of the linear regression and was determined to

$$\varepsilon\ [\mu M]^{300\ \mu L} = 373{,}590 \quad \left[\frac{RFU \times mL}{nmol}\right]$$

Note: Only valid for the individual Fluorescence Microplate Reader; cannot be translated to any other reader. Determination of ε is required on each individual reader.

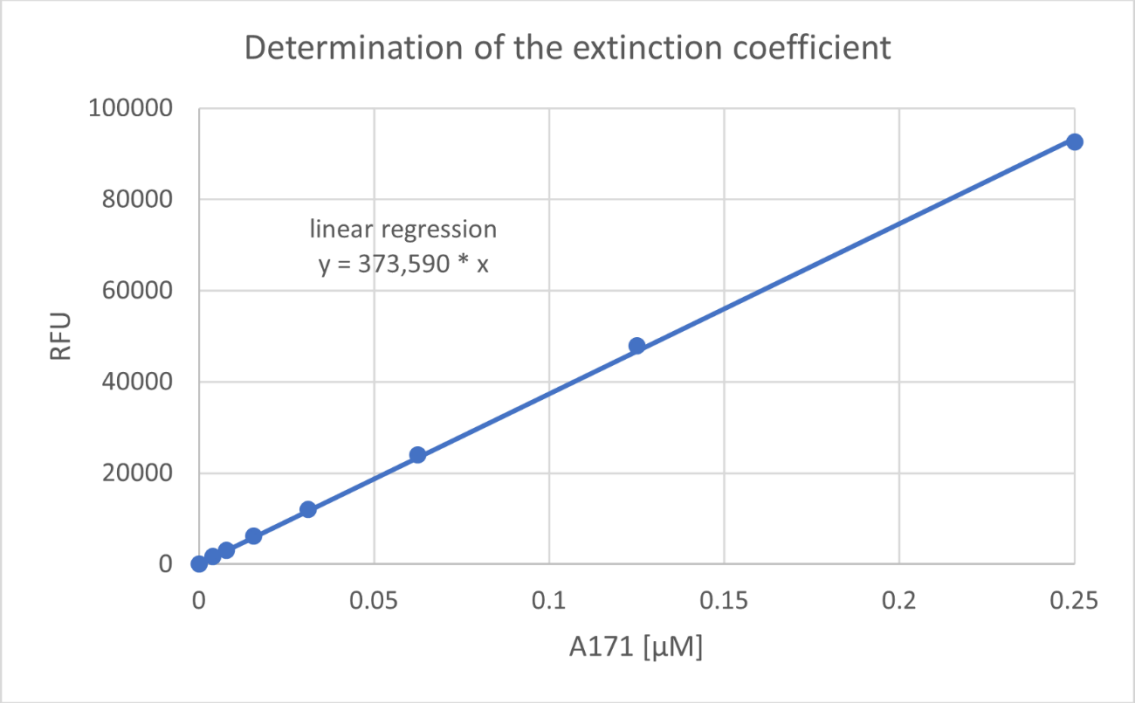


Figure 3: Determination of the extinction coefficient ε $[\mu M]^{300\mu L}$ by plotting the fluorescence (RFU) of a serial dilution of A171 ("N-ME-ABZ-PEPTIDE-CALIBRATOR"). The extinction coefficient equals to the slope of the linear regression.

In figure 2B, the y-axis (ΔRFU/min) represents the increase of fluorescence corresponding to the reaction product formed, plotted against the indicated hTG2 concentration.

The x-axis in figure 2B is converted to **Enzyme Activity** by dividing the increase of fluorescence (ΔRFU/min) by the extinction coefficient resulting in figure 4.

$$\textbf{\textit{Enzyme Activity}} = x = \frac{y}{\varepsilon^{300\ \mu L}} \times DF \quad \left[\frac{RFU \times nmol}{min \times RFU \times mL} = \frac{nmol}{min \times mL} = \frac{U}{mL}\right]$$

$$with \quad y = \frac{\Delta RFU}{t} \quad and \quad dilution\ factor\ DF = \frac{V}{v} = \frac{total\ assay\ volume}{volume\ of\ sample}$$

In this example, e.g. 1,714 ΔRFU/min correspond to an **Enzyme Activity** of 0.05 U/mL or 46 U/L. This also corresponds to 10 nM hTG2.

However, in case there is no reference TRANSGLUTAMINASE available, measurement of the extinction coefficient (figure 3) is sufficient to determine the **Enzyme Activity** of biological samples like tissue extracts.

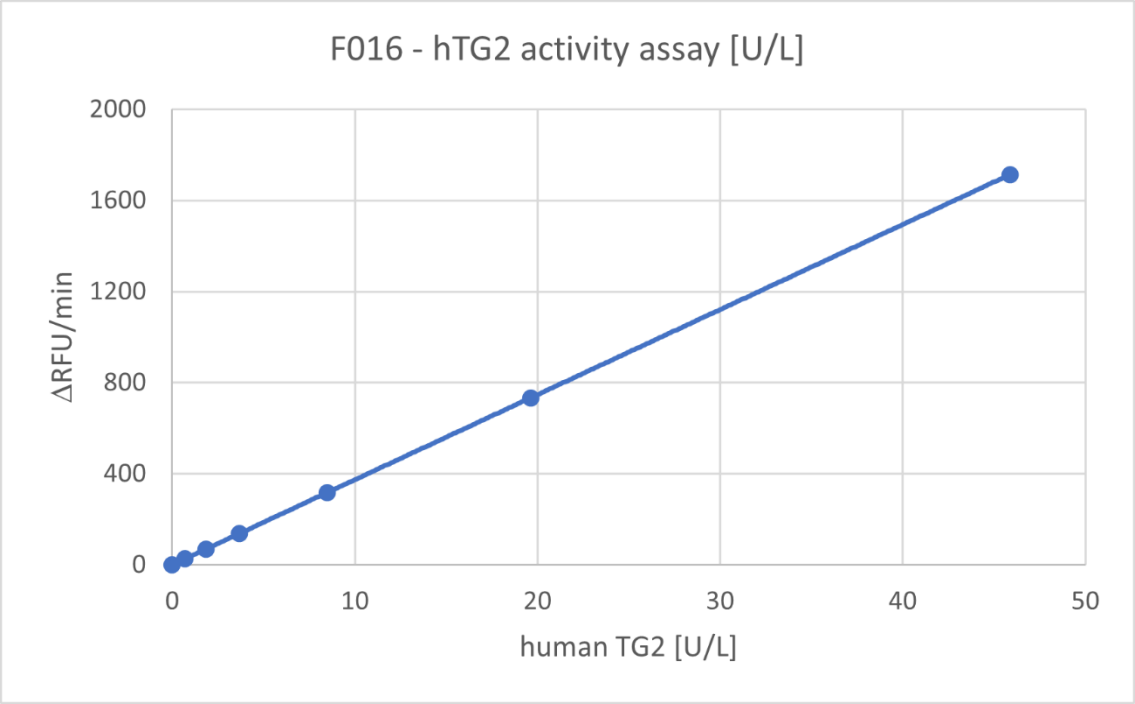


Figure 4: Enzyme Activity of a hTG2 REFERENCE in U/L, determined by dividing the increase of fluorescence (ΔRFU/min) by the extinction coefficient.

NOTE: All examples shown are only valid for the individual Fluorescence Microplate Reader used for measurement and cannot be translated to any other reader.

Reference measurements or calibration is required on each individual reader. Suitable references and a calibrator are optionally available at Zedira, but not included in the kit.

11.Reference Range

The ZEDI*XCITE* 360/460 FLUOROGENIC TG2-ASSAY KIT (F016) is suitable for measurements of 1.25 to 10 nM (100 to 780 ng/mL or 3.7 to 46 U/L) of TG2.

Please note: ZEDI*XCITE* 360/460 FLUOROGENIC TG2-ASSAY KIT is able to detect - but not to quantify - TG2 concentrations lower than 100 ng/mL in e.g. biological samples.

12.Kinetic parameters

The determination of TISSUE TRANSGLUTAMINASE activity is based on the ability of TG2 to cleave isopeptide bonds. The kinetic parameters obtained for TG2 specific peptidic substrate in ZEDI*XCITE* 360/460 FLUOROGENIC TG2-ASSAY are summarized in table 5. For comparison, the non-specific fluorogenic substrate A101 [2] is presented. In summary, the ZEDI*XCITE* substrate peptide performs about 20-fold better than A101.

Table 5: Kinetic parameters of TG2 substrates determined by ZEDI*XCITE* 360/460 FLUOROGENIC TG2-ASSAY KIT (F016) with hTG2.

	K_M [µM]	k_{cat} [s^{-1}]	k_{cat}/K_M (M^{-1} s^{-1})
ZEDI*XCITE* N-METHYL-ABZ-substrate peptide A170	5.5	0.03	5,188
A101	8.6	0.002	252

13.Limitations

The ZEDI*XCITE* 360/460 FLUOROGENIC TG2-ASSAY KIT (F016) is meant for research and development only. The kit has been optimized for the measurement of purified TG2 in buffer. In case of determination of TG2 in biological samples, suitable controls are recommended (e.g. spiking with purified TG2 as positive control or blocking TG2 with Z006, a TG2 inhibitor, as negative control).

14.Precision

The coefficient of variation determined during kit development was 3.9% for 10 nM (780 ng/mL) recombinant human TISSUE TRANSGLUTAMINASE (hTG2).

References

[1] Parameswaran, K.N. *et al. J. Biol. Chem.* **1997**, *272*, 10311.

[2] Oertel, K. *et al. Anal. Biochem.* **2007**, *367*, 152.

[3] BMG Labtech "How to optimise fluorescence gain"