ab204700

Transglutaminase Activity Assay Kit (Colorimetric)

Instructions for Use

For rapid, sensitive and accurate measuring of Transglutaminase activity.

This product is for research use only and is not intended for diagnostic use.
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**INTRODUCTION**

1. **BACKGROUND**

Transglutaminase Activity Assay Kit (ab204700) uses the deamidation reaction of the transglutaminase enzyme with a donor and acceptor substrate resulting in the formation of a hydroxamate product. The hydroxamate product reacts with the Stop Solution forming a purple complex that can be measured colorimetrically at OD = 525 nm. The limit of quantification of this assay is ~10 µU or 80 ng of recombinant hTG2 enzyme.

Transglutaminases (EC 2.3.2.13) are calcium dependent enzymes that catalyze the post-translational modification of proteins by formation of isopeptide bonds. This occurs either through protein cross-linking via formation of γ-glutamyl-ε-lysine bonds or through incorporation of primary amines at selected peptide-bound glutamine residues. The transglutaminase enzyme family comprises the intracellular forms (TG1, TG3 and TG5) expressed mostly in the epithelial tissue; TG2 which is both intracellular and extracellular and expressed in various tissue types; TG4 which is expressed in the prostate gland; factor XIII which is expressed in blood; TG6 and TG7, whose tissue distribution is unknown and band 4.2 (lacking enzymatic activity) which is present on erythrocyte membranes. Transglutaminases also exhibit GTPase, phosphodiesterase and protein kinase activity. Transglutaminases are associated with certain neurological and autoimmune disorders and also cancer.
2. ASSAY SUMMARY

- Standard curve preparation

  ↓

- Sample preparation

  ↓

- Add reaction mix and incubate for 2 hours

  ↓

- Measure optical density (OD525 nm)
3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit at -20°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature. Reconstituted components are stable for 2 months.

5. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.
6. MATERIALS SUPPLIED

<table>
<thead>
<tr>
<th>Item</th>
<th>Amount</th>
<th>Storage Condition (Before Preparation)</th>
<th>Storage Condition (After Preparation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG Assay Buffer</td>
<td>12 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Homogenization Buffer (10x)</td>
<td>10 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>DTT</td>
<td>125 µL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Donor Substrate</td>
<td>1 Vial</td>
<td>-20°C</td>
<td>-80°C</td>
</tr>
<tr>
<td>Acceptor Substrate</td>
<td>2 Vials</td>
<td>-20°C</td>
<td>-80°C</td>
</tr>
<tr>
<td>Hydroxamate Standard</td>
<td>1 Vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Stop Solution</td>
<td>5 mL</td>
<td>-20°C</td>
<td>-20°C / +4°C</td>
</tr>
<tr>
<td>Positive Control</td>
<td>1 Vial</td>
<td>-20°C</td>
<td>-80°C</td>
</tr>
<tr>
<td>Plate Sealer</td>
<td>1 each</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
</tbody>
</table>

7. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully perform this assay:

- MilliQ water or other type of double distilled water (ddH₂O)
- PBS
- Microcentrifuge
- Pipettes and pipette tips
- Colorimetric microplate reader – equipped with filter for OD 525 nm
- 96 well plate: clear plates
- Heat block or water bath
- Dounce homogenizer or pestle (if using tissue)
- Glycerol – prepare a 20% glycerol solution in ddH₂O
8. **TECHNICAL HINTS**

- This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.

- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.

- Keep enzymes, heat labile components and samples on ice during the assay.

- Make sure all buffers and solutions are at room temperature before starting the experiment.

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.

- Avoid foaming or bubbles when mixing or reconstituting components.

- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.

- Ensure plates are properly sealed or covered during incubation steps.

- Make sure you have the right type of plate for your detection method of choice.

- Make sure the heat block/water bath and microplate reader are switched on.
9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening.

9.1 TG Assay Buffer:
Ready to use as supplied. Equilibrate to 37°C before use. Store at -20°C.

9.2 Homogenization Buffer (10X):
Make 1X Homogenization buffer by diluting 1:10 in ddH₂O. Store at -20°C. Keep on ice while in use.

9.3 1 M DTT:
Ready to use as supplied. Aliquot DTT so that you have enough volume to perform the desired number of assays. Store at -20°C. Keep on ice while in use.

9.4 Donor Substrate:
Reconstitute the Donor Substrate in 1.1 mL of ddH₂O. Aliquot substrate so that you have enough volume to perform the desired number of assays. Store at -80°C protected from light. Once the probe is thawed, use within two months.

9.5 Acceptor Substrate:
Reconstitute each vial of the Acceptor Substrate in 550 µL of ddH₂O as needed. Aliquot substrate so that you have enough volume to perform the desired number of assays. Store at -80°C protected from light. Once the probe is thawed, use within two months.

9.6 Hydroxamate Standard:
Reconstitute the Hydroxamate Standard in 330 µL of ddH₂O to generate a 10 mM standard stock solution. Aliquot standard so that you have enough volume to perform the desired number of assays. Store at -20°C. Keep on ice while in use.

9.7 Stop Solution:
Ready to use as supplied. Store at -4°C or -20°C. Keep on ice while in use.

9.8 **Positive Control:**
Reconstitute the Positive Control in 30 μL of 20% glycerol in ddH₂O. Aliquot substrate so that you have enough volume to perform the desired number of assays. Store at -80°C protected from light. Thaw and mix gently before use.

9.9 **Plate Sealer:**
Ready to use as supplied. Equilibrate to room temperature before use.
10. STANDARD PREPARATION

- Always prepare a fresh set of standards for every use.
- Diluted standard solution is unstable and cannot be stored for future use.

10.1 Dilute TG Assay Buffer 1:1 with ddH₂O.

10.2 Using 10 mM Hydroxamate standard, prepare standard curve dilution as described in the table in a separate microplate or microcentrifuge tubes:

<table>
<thead>
<tr>
<th>Standard #</th>
<th>Volume of Standard (µL)</th>
<th>Diluted TG Assay Buffer (µL)</th>
<th>Final volume standard in well (µL)</th>
<th>End Conc. Hydroxamate (nmol in well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>150</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>144</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>138</td>
<td>50</td>
<td>40</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>132</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>126</td>
<td>50</td>
<td>80</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>120</td>
<td>50</td>
<td>100</td>
</tr>
</tbody>
</table>

Each dilution has enough amount of standard to set up duplicate readings (2 x 50 µL).
11. SAMPLE PREPARATION

General Sample information:

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you complete the Sample Preparation step before storing the samples. Alternatively, if that is not possible, we suggest that you snap freeze cells or tissue in liquid nitrogen upon extraction and store the samples immediately at -80°C. When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

11.1 Cell (adherent or suspension) samples:

11.1.1 Add DTT to 1X Homogenization Buffer at a final concentration of 0.2mM.

11.1.2 Harvest the amount of cells necessary for each assay (initial recommendation = 2-5 x 10^6 cells).

11.1.3 Wash cells with cold PBS.

11.1.4 Add 150 – 300 μL cold Homogenization Buffer (with DTT) containing protease inhibitor cocktail.

11.1.5 Disrupt cells by five cycles of freezing and thawing.

11.1.6 Transfer cells to cold microcentrifuge tube.

11.1.7 Centrifuge sample for 20 minutes at 4°C at 16,000 x g using a cold microcentrifuge.

11.1.8 Collect supernatant and transfer to a clean pre-chilled tube and keep on ice.

11.2 Tissue samples:

11.2.1 Add DTT to 1X Homogenization Buffer at a final concentration of 0.2mM.
11.2.2 Harvest the amount of tissue necessary for each assay (initial recommendation = 100 mg).

11.2.3 Rinse tissue with PBS and transfer tissue to pre-chilled homogenizer.

11.2.4 Add 500 μL cold Homogenization Buffer (with DTT) containing protease inhibitor cocktail.

11.2.5 Homogenize tissue with a Dounce homogenizer sitting on ice, with 10 – 15 passes.

11.2.6 Transfer tissue to cold microcentrifuge tube.

11.2.7 Centrifuge sample for 20 minutes at 4°C at 16,000 x g using a cold microcentrifuge.

11.2.8 Collect supernatant and transfer to a clean pre-chilled tube and keep on ice.

**NOTE:** We suggest using different volumes of sample to ensure readings are within the Standard Curve range.

To check the Transglutaminase activity, protein concentration of lysates should be at least 5 mg/mL.

We recommend using the lysates immediately to assay the Transglutaminase Activity.
ASSAY PROCEDURE and DETECTION

12. ASSAY PROCEDURE and DETECTION

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- It is recommended to assay all standards, controls and samples in duplicate.

12.1 Set up Reaction wells:
- Sample wells = 25 – 50 µL samples (adjust volume to 50 µL/well with ddH₂O).
- Background control sample wells= 50 µL ddH₂O.
- Positive control = 2 µL Positive control + 48 µL ddH₂O. **NOTE:** add positive control to plate just before adding Transglutaminase Reaction Mix.

12.2 Reaction Mix:
Prepare 50 µL of Transglutamate Reaction Mix for each reaction:

<table>
<thead>
<tr>
<th>Component</th>
<th>Reaction Mix (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG Assay Buffer</td>
<td>25</td>
</tr>
<tr>
<td>Donor Substrate</td>
<td>10</td>
</tr>
<tr>
<td>Acceptor Substrate</td>
<td>10</td>
</tr>
<tr>
<td>1M DTT</td>
<td>1</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>4</td>
</tr>
</tbody>
</table>

Mix enough reagents for the number of assays (samples and controls) to be performed. Prepare a master mix of the Reaction Mix to ensure consistency. We recommend the following calculation: X µL component x (Number reactions +1).

12.3 Add 50 µL of Reaction Mix into each sample, positive control and background control sample wells.

12.4 Mix and incubate at 37°C for 2 hours protected from light.

12.5 Set up Standard wells in a separate 96-well plate towards the end of the 37°C 2 hours incubation of the Sample plate = add 50 µL standard dilutions.
12.6 Add 50 μL Stop solution to all Standards and sample wells.

12.7 Seal the sample plate, and centrifuge at 1800 x g for 15 minutes to pellet the precipitate formed. **NOTE:** Standard will not precipitate after adding stop solution.

12.8 Carefully transfer 100 μL of supernatant from all sample and background control wells into desired wells in the 96-well clear plate containing the standards.

12.9 Measure output at OD = 525 nm on a colorimetric microplate reader.
**13. CALCULATIONS**

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.

- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).

13.1 Average the duplicate reading for each standard and sample.

13.2 If the sample background control is significant, then subtract the sample background control from sample reading.

13.3 Subtract the mean absorbance value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance.

13.4 Plot the corrected absorbance values for each standard as a function of the final concentration of hydroxamate.

13.5 Draw the best smooth curve through these points to construct the standard curve. Most plate reader software or Excel can plot these values and curve fit. Calculate the trendline equation based on your standard curve data (use the equation that provides the most accurate fit).

13.6 Activity of Transglutamate (nmol/T/mg) in the test samples is calculated as:

\[
\text{Transglutaminase activity} = \frac{((B \times 1.5)/T)}{mg}
\]

Where:

- B = Amount of hydroxamate from the Standard Curve (nmol).
- 1.5 = nmoles of hydroxamate product generated in 150 μL reaction volume.
- T = Incubation time (minutes).
- mg = Amount of protein/reaction in mg
Unit Definition:

1 Unit Transglutaminase activity = amount of enzyme which generates 1.0 µmol of hydroxamate per minute under the assay conditions.
14. **TYPICAL DATA**

**TYPICAL STANDARD CURVE** – Data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.

Figure 1. Typical Hydroxamate Standard calibration curve.
Figure 2. Transglutaminase activity in HepG2 cells (human hepatoblastoma cell line) and rat liver lysate: HepG2 cells were stimulated with vehicle (DMSO), IL6 (1 µM), Dexamethasone (DXM -1 µM), or with IL6 (1µM) and DXM (1 µM). Approximately 250 µg protein was used for determining transglutaminase activity in cells and tissue lysate. Activity is expressed as nmoles of product formed in 2h and is normalized to the protein amount.

*NOTE: HepG2 cells have similar intrinsic Transglutaminase activity in the presence or absence of vehicle control (DMSO).
15. QUICK ASSAY PROCEDURE

**NOTE:** This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.

- Prepare standard, donor substrate, acceptor substrate, and positive control (aliquot if necessary); get equipment ready.
- Prepare standard curve.
- Prepare samples in duplicate (find optimal dilutions to fit standard curve readings).
- Set up 2 plates: one for standard (50 µL) and one for samples (50 µL), positive control (50 µL) and background wells (50 µL).
- Prepare Transglutaminase Reaction Mix (Number samples + positive control + background control + 1).

<table>
<thead>
<tr>
<th>Component</th>
<th>Colorimetric Reaction Mix (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG Assay Buffer</td>
<td>25</td>
</tr>
<tr>
<td>Donor Substrate</td>
<td>10</td>
</tr>
<tr>
<td>Acceptor Substrate</td>
<td>10</td>
</tr>
<tr>
<td>1M DTT</td>
<td>1</td>
</tr>
<tr>
<td>ddH2O</td>
<td>4</td>
</tr>
</tbody>
</table>

- Add 50 µL of Transglutaminase Reaction Mix to the samples, positive control and background wells.
- Incubate plate at 37°C 2 hours protected from light.
- Add 50 µL of Stop Solution to the samples, positive control and background wells and to all standards.
- Centrifuge at 1800 x g for 15 minutes to pellet the precipitate formed.
- Transfer 100 µL of supernatant from all sample, positive control and background control wells into the 2nd 96-well clear plate containing the standards.
- Measure plate at OD = 525 nm.
## 16. TROUBLESHOOTING

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay not working</td>
<td>Use of ice-cold buffer</td>
<td>Buffers must be at room temperature</td>
</tr>
<tr>
<td></td>
<td>Plate read at incorrect wavelength</td>
<td>Check the wavelength and filter settings of instrument</td>
</tr>
<tr>
<td></td>
<td>Use of a different 96-well plate</td>
<td>Colorimetric: Clear plates</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fluorometric: black wells/clear bottom plate</td>
</tr>
<tr>
<td>Sample with erratic readings</td>
<td>Samples not deproteinized (if indicated on protocol)</td>
<td>Use PCA precipitation protocol for deproteinization</td>
</tr>
<tr>
<td></td>
<td>Cells/tissue samples not homogenized completely</td>
<td>Use Dounce homogenizer, increase number of strokes</td>
</tr>
<tr>
<td></td>
<td>Samples used after multiple free/thaw cycles</td>
<td>Aliquot and freeze samples if needed to use multiple times</td>
</tr>
<tr>
<td></td>
<td>Use of old or inappropriately stored samples</td>
<td>Use fresh samples or store at -80°C (after snap freeze in liquid nitrogen) till use</td>
</tr>
<tr>
<td></td>
<td>Presence of interfering substance in the sample</td>
<td>Check protocol for interfering substances; deproteinize samples</td>
</tr>
<tr>
<td>Lower/Higher readings in samples and Standards</td>
<td>Improperly thawed components</td>
<td>Thaw all components completely and mix gently before use</td>
</tr>
<tr>
<td></td>
<td>Allowing reagents to sit for extended times on ice</td>
<td>Always thaw and prepare fresh reaction mix before use</td>
</tr>
<tr>
<td></td>
<td>Incorrect incubation times or temperatures</td>
<td>Verify correct incubation times and temperatures in protocol</td>
</tr>
<tr>
<td>Problem</td>
<td>Cause</td>
<td>Solution</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>--------------------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Standard readings do not follow a linear pattern</td>
<td>Pipetting errors in standard or reaction mix</td>
<td>Avoid pipetting small volumes (&lt; 5 µL) and prepare a master mix whenever possible</td>
</tr>
<tr>
<td></td>
<td>Air bubbles formed in well</td>
<td>Pipette gently against the wall of the tubes</td>
</tr>
<tr>
<td></td>
<td>Standard stock is at incorrect concentration</td>
<td>Always refer to dilutions on protocol</td>
</tr>
<tr>
<td>Unanticipated results</td>
<td>Measured at incorrect wavelength</td>
<td>Check equipment and filter setting</td>
</tr>
<tr>
<td></td>
<td>Samples contain interfering substances</td>
<td>Troubleshoot if it interferes with the kit</td>
</tr>
<tr>
<td></td>
<td>Sample readings above/ below the linear range</td>
<td>Concentrate/ Dilute sample so it is within the linear range</td>
</tr>
</tbody>
</table>
17. FAQ
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