Human Recombinant TG In Vitro Translation & Autoantibody Immuno-Radioassay

PROTOCOL

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Principle:

- *In vitro* transcription and translation (in one step, using rabbit recticulocytes) of labelled antigen (^{35}S-Methionine - human tTG)
- Incubation of serum with labelled antigen overnight
- Precipitation of antibody-bound labelled antigen with anti-human IgA Agarose in a 96-well plate format, with each serum tested in duplicate
- Washing of the 96-well plates to remove unbound labelled antigen
- Counting of each well with a 96-well plate β counter
- Results expressed as an index that adjusts the cpm of the test serum for the cpm of positive and negative control sera in a particular assay

Plan for performing the assay:

In vitro transcription/translation of antigen (see Part III of this protocol): Labeled antigen can then be stored at -70^{o}C for at least one month and used in multiple assays.

Assay sera:

Day 1: mid-day: retrieve and thaw sera to be tested

afternoon: (1) set up incubation of sera in washing buffer (Part IV)
(2) prepare 96-well plates (Part V)

Day 2: morning: (1) add anti-human IgA agarose into plates
(2) add incubate to anti-IgA agarose in plates (Part VI)
(3) wash plates
(4) dry plates

afternoon: (5) count
(6) analyse data (Part VII)

QA/QC for TgIgA Assay

Establish of lab-cut-off

The cutoff index of tTG was established as the 3X100th percentile in the control group (184 subjects, an index of 0.05). We constructed a receiver operating characteristic (ROC) curve using EMA positivity and negativity among the patients with diabetes (n=859) as a standard for comparison of the IgA transglutaminase antibodies. The cutoff index of 0.05 corresponded to 100% sensitivity among EMA^+ diabetic patients and 94% specificity among EMA^- patients with diabetes.

Intra-assay CV: 4.8% (n=8)

Inter-assay CV: 10% (n=10)

QA: 1) All assays are run in duplicate, along with two standard samples (one positive and one negative sample). Two-well duplicates for each sample will be aliquoted with two separated events (one-well aliquoting per sample for each process). Upon finishing sample aliquoting on the full plate, the plate should be hold against the light and checked from bottom for the wells missing samples. Every positive sample with index above 99^{th} percentile of upper limit of normal control must be repeated in a separated assay. The 3^{rd} will be run if 2^{nd} disagree with 1^{st}. The result will be reported as the mean value of two agreement (+,+ or +,-,+).

2) The positive must show positive, and negative control must be negative in each assay.

3) Shewart chart is plotted over time.

4) The lab should attend any national or international workshops or efficient evaluations if available.

Part I: Reagents, Supplies, and Equipments

Reagents and Supplies

	Suggested suppliers (cat. no.)	
• - Trizma Base	Fisher (BP152-5);	
• - NaCl	Fisher (BP358-212);	
• - Tween 20	Sigma (P-1379)	
• - Bovine Serum Albumin	Sigma (A-7906)	
• - Anti-human IgA Agarose (α-chain specific)	Sigma (A-2691)	
• - In Vitro TNT Kit	Promega	L4600
• - RNasin	Promega	N251A
• - ^{35}S-Methionine	Perkin Elmer (NEG 009T)	
• - NAP Column	GE HealthCare (17-0853-02 or 17-0854-02 if separating multiple labeling reactions)	
▪ - pGEM-TG plasmid	full length	
▪ - 5N HCl		
• - Parafilm	Sigma (P7793-1EA)	
• - 96-well round bottom plate	Fisher (08408220)	
• - 96-well filtration plates	Fisher (07200754)	
• - Bottle-Top 500 ml-Filter Units	Fisher (0974064A or B)	
• - TopSeal	Perkin-Elmer (6005185)	
• - Sealing Foil	USA Scientific (2923-0100)	
• - Microscint-20	Perkin-Elmer (6013621)	
• - Aluminum foil		

Equipments

• - TopCount β-counter (or similar)	Perkin-Elmer
• - 96-well Plate Shaker	Wallac - Delfi
• - Water Bath Incubator or thermal block	
• - Fume Hood	
• - Biological & radiation safety cabinets	
• - –80 and -20 ^{0}C freezers	
• - 4 ^{0}C refrigerator	
• - Pipette-Aid	
• - Water purification system	
• - Ice maker	
• - Radioactive contamination monitor	
• - Radiation sink	
• - pH meter	

- - Vortex mixer
- - Stepper pipette
- - Pipettes/tips
- - Ice trays
- - Vacuum-operated 96-well plate washer Millipore (MAVM0960R)

Part II: Buffers

(1) Plain buffer (150 mM NaCl, 20 mM Tris-HCl, 0.1%Sodium Azide pH 7.4)

30 ml 5M NaCl
10 ml 2M Tris-HCl pH 7.4
1 gm Sodium Azide (essential, to prevent bacterial contamination)
up to 1000 ml

(2) Washing buffer (0.15% Tween-20, 0.1% BSA in plain buffer)

1.5 ml Tween-20
1 gm BSA
plain buffer to 1000 ml

Important Points:

- Buffer should be filtered (0.45 micron filter) to prevent any particles blocking the membrane in bottom of the wells of the 96 well plate (which would decrease washing efficiency and increase the assay background)
- Store buffers at 4^{o}C in a sterile bottle for up to 3 months

Part III: In Vitro Transcription/Translation

(1) Reaction

- In a given reaction the full-length human tTG cDNA is labelled with ^{35}S-Methionine
- All reagents and tubes must be sterile, otherwise RNAse may destroy RNA.
- Keep all reagents and tubes on ice while on the bench.
- Set up approximately 10 reaction tubes (depending on the amount of ^{35}S available) that will contain DNA, plus one control tube that will contain no DNA and should theoretically not precipitate any detectable radioactivity)
- Store reagents at -20^{o}C, except for the Reticulocyte Lysate which is stored at -70^{o}C.
- **Important: the Reticulocyte Lysate must be thawed rapidly just before use.** To do this roll the tube between hands. Do not use a hot water bath. Each tube can only be thawed twice, after which there will be a significant decrease in the amount of product. Reticulocytes have ribosomes but no nucleus (normoblasts have nucleus).

Add in following order:

	Reaction tubes with DNA (50ul)	**Control tube without DNA**
Water (double distilled, sterile)	13.5 ul	6.5 ul
*"TNT" Reaction Buffer	2 ul	1.0 ul
Rnasin (inhibits RNAse)	1 ul	1.0 ul
*Amino Acid Mixture (with no Methionine for tTG)	1.5 ul	1.0 ul
DNA plasmid (tTG cDNA	1-2 ug	-------
^{35}S-Methionine	4 ul(40uCi)	2.0 ul
*"TNT" SP6 RNA Polymerase	1 ul	1.0 ul
"TNT" Rabbit Reticulocyte Lysate	25 ul	12.5 ul

(Items marked with an asterisk (*) are included in the TNT kit.)

- Mix the reagents in each reaction tube by pipetting up and down. Do not vortex as this will create bubbles that interfere with the reaction.
- Incubate the reaction at 30^{o}C for 90 minutes.

(2) Purification

- Open the top cover of the column and then bottom cover and let it dry.
- Add 1 ml of washing buffer to the column and let it go through.
- Add reaction mix onto the column, let it go into the column, add small amount of washing buffer to wash the wall of column and then add more washing buffer.
- Collect the whole red part from the column (labeled protein product will come out together with hemoglobin present in reticulocyte).

(3) Analysis the activity of labeled protein

- Remove 2 µl from collection tube of purification and add 98 ul of buffer.
- Take out 5 ul of above dilution and add to a well containing 50 ul of MicroScin-20
- Place on a plate shaker for 5 minutes
- Count on a TopCount.
- Calculate percentage incorporation for each reaction tube. This will be needed to determine how much volume to use in the assay.
- Labeled GAD65 can be stored at -80^{o}C for at least one month.

Part IV: Incubation of Serum Samples with 35S -tTG

Each 96-well plate is sufficient for testing 48 samples in duplicate (44 test sera plus two positive and two negative control sera). Usually, four plates can easily be run at one time (total of 188 samples).

(1) Spin down sera to remove fibrin clots (otherwise these may partially block membrane in bottom of wells)

(2) Calculate how much ^{35}S -tTG is required.

6 ml of Washing Buffer for one plate:

48 x 2.5 x 50 = 6 ml (48 samples, with 50 ul/well; in duplicate but multiply by 2.5 rather than 2 to allow for some extra)

20,000 cpm of TCA precipitate is used for labeled antigen for each well.

48 x 2.5 x 20,000 = 2.4×10^6 cpm of ^{S}35-tTG for one plate.

In this example, say the ^{35}S-tTG tube being used contains 0.64×10^6 cpm/2 ul (or **0.32×10^6 cpm/ul**), as determined by the calculation at the end of Part III, then: 2.4×10^6 cpm/ 1 plate requires the volume of 0.32×10^6 cpm/ul of ^{35}S-tTG = 7.5 ul from the ^{35}S-tTG tube.

Therefore, add 7.5 ul from the ^{35}S-tTG reaction tube to 6 ml washing Buffer for one plate.

Keep the Buffer-labelled antigen mixture on ice.

(3) Mix each serum sample with Buffer-antigen mixture in a PCR tube (or similar tube).

Serum: 2.5 ul

Buffer-antigen mixture: 60 ul

(4) Using the same control samples for every plate. One positive control and one negative control are used for tTG.

(5) Vortex and incubate overnight at 4^{o}C.

Part V: Preparation of Filtration Plate

(1) Coat the plate with BSA by adding 200 ul of Washing Buffer to each well.

Incubate overnight at room temperature, after placing the plate on aluminium foil.

(3) Remove the washing buffer.

(4) The plates are now ready for runing the assay, but can be stored at 4^{o}C if necessay.

Part VI: Immunoprecipitation with Anti-human IgA Agarose

(1) Add 20 ul of 25% anti-human IgA agarose to each well. Use Eppendorf multipipettor and **resuspend the anti-human IgA agarose after each row of the plate is done.** (Will need 2ml of 25% anti-human IgA agarose per plate.)

(2) Add 50 ul of overnight incubate to each wells (i.e., each serum will be tested in duplicate).

(3) Shake the plate on a Plate Shaker for 1 hour at 4^{o}C. Accurate timing important.

(4) Place the plate on Millipore plate washer device (with vacuum set low).

(5) Wash the plate three times in this way with 200 ul of Washing Buffer per well.

(6) Add 120 ul of Washing Buffer to each well. Shake for at least 5 minutes at 4^{o}C.

(7) Wash the plate four times with 200 ul of washing buffer per well (change the plate direction after two times of washing at this stage).

(8) Place the plate under a lamp for approximately 10 minutes to dry. Rotate the plate several times to ensure even drying and check the appearance. Drying is complete when deep fissures appear in the Agarose visible in the bottom of the wells. Do not over-dry and be careful not to melt the plastic parts of the plate.

(9) Add 40 ul of scintillation cocktail (Microscint-20) to each well.

(10) Count on Top Count 96-well plate β counter.

Part VII: Data Analysis

(1) CPM Index for each sample:

Index for tTG:

$$\frac{\text{unknown sample cpm - negative control cpm}}{\text{positive control cpm - negative control cpm}}$$

(2) Coefficient of Variation

For Duplicates:

$$\frac{\text{(High CPM - Low CPM)/1.128}}{\text{Mean CPM}} \times 100$$