Human Recombinant ZnT8 In Vitro Translation & Autoantibody Immuno-Radioassay

PROTOCOL

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QA/QC for ZnT8Ab Assay

Establish of lab-cut-off

The cutoff index, 0.020, of ZnT8Ab was established as the 99th percentile in the 100 control group. We constructed a receiver operating characteristic (ROC) curve among the newly diagnosed (within 2 weeks) patients with diabetes (n=50). The cutoff index of 0.020 corresponded to 60% sensitivity and 99% specificity.

Intra-assay CV: 3.2% (n=8)

Inter-assay CV: 10.4% (n=15)

QA: 1) All assays are run in duplicate, along with three standard samples (one high positive, one low positive, and a negative control serum samples). 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 99th percentile of upper limit of normal control must be repeated in a separated assay. The 3rd will be run if 2nd disagree with 1st. The result will be reported as the mean value of two agreement (+,+ or +,-,+). The lab does not re-test negative samples since 1) it is rare to see negative results re-test as positive - the frequency is extremely low; 2) most of screening samples are negative so it would be very costly to re-test them.

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

3) Shewart chart is plotted over time.

4) All raw data and their analysis of QC control sets in each assay must be passed through lab director or supervisor.

5) All final reporting results must be double-checked by lab director or supervisor before reported or uploaded.

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

Principle:

- *In vitro* transcription and translation (in one step, using rabbit reticulocytes) of labeled antigen ^{35}S-Methionine –ZnT8.
- Incubation of serum with both labeled antigens together overnight
- Precipitation of antibody-bound labeled antigens with protein-A Sepharose in a 96-well plate format, with each serum tested in duplicate
- Washing of the 96-well plates to remove unbound labeled antigens
- 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 antigens (see Part III of this protocol): Labeled antigens can then be stored at -80°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 antigen buffer (Part IV)
		(2) prepare 96-well plates (Part V)
		(3) prepare protein-A Sepharose (Part V)
Day 2:	morning:	(1) add incubate to protein-A Sepharose in plates (Part VI)
		(2) wash plates
		(3) dry plates
	afternoon	(4) count
		(5) analyse data (Part VII)

Part I: Reagents & Supplies

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)
• - Protein A-Sepharose	GE HealthCare (17528003)
• - 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)
▪ - pcDNA3.1 JH5.2 plasmid	Original from John Hutton (R/W dimmer, a.a. 268-369).
▪ - 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

(3) Antigen Buffer: TBST containing 0.1% BSA.

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°C in a sterile bottle for up to 2 weeks

Part III: In Vitro Transcription/Translation

(1) Reaction

- 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 a reaction tube (the reaction volume 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°C, except for the Reticulocyte Lysate which is stored at -80°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).

- The TNT kit gives high incorporation (~50%) and consequently there is need to purify the labeled product to remove free radio-labeled methionine or leucine with a sizing column.

Add in following order:

	Reaction tubes with DNA	**Control tube without DNA**
Water (double distilled, sterile)	14-15 ul	6.5 ul
*"TNT" Reaction Buffer	2 ul	1.0 ul
Rnasin (inhibits RNAse)	1 ul	1.0 ul
*Amino Acid Mixture	1-2 ul	1.0 ul
DNA plasmid (ZnT8)	1-2 ug	-------
^{35}S-Methionine	4 ul	2.0 ul
*"TNT" 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°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 antigen 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 antigen buffer to wash the wall of column and then add more antigen 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 antigen 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 ZnT8 can be stored at -80°C for at least one month.

Part IV: Incubation of Serum Samples with ^{35}S –ZnT8

Each 96-well plate is sufficient for testing 48 samples in duplicate (42 test samples sera plus two positive, two negative controls. Usually, 4-8 plates can easily be run at one time (200 to 400 samples).

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

(2) Calculate how much ^{35}S –ZnT8 is required.

12 ml of Antigen Buffer for two plates:

96 x 2.5 x 50 = 12 ml (96 samples, with 50 ul/well; in duplicate but multiply by 2.5 rather than 2 to allow for some extra)

20,000 cpm is used for each well.

96 x 2.5 x 20,000 = $4.8x10^6$ cpm of ZnT8 for two plates.

In this example, say the ZnT8 reaction tube being used contains $1.2x10^6$ cpm/2 ul (or **$0.6x10^6$ cpm/ul**), as determined by the calculation at the end of Part III, then:$4.8x10^6$ cpm required/$0.6x10^6$ cpm per ul in the reaction tube = 8 ul required from the tube

Therefore, add 8 ul from the ZnT8 reaction tube to 12 ml Antigen Buffer for two plates. **Keep the Buffer-labelled antigen mixture on ice.**

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

Serum: 2.5 ul/well

Buffer-antigen mixture: 60 ul /well

(4) Vortex and incubate overnight at 4°C.

Part V: Preparation of MultiScreen Filtration Plates and Protein A-Sepharose

(1) Coat the plate with BSA by adding 150 ul of Antigen Buffer to each well.

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

(3) Remove the antigen buffer.

(4) The plates are now ready to run the assay, but they can be stored at 4°C if necessary.

(5) Prepare Protein-A Sepharose:

- Use only plastic tubes because Protein-A sticks to glass
- Washing Protein-A Sepharose with antigen buffer in a 50 ml tube three times. Spin down and remove the fluid phase.
- Finally add antigen buffer to give 50% concentration of Protein-A Sepharose by volume.

Part VI: Immunoprecipitation with Protein A-Sepharose

(1) Add 25 ul of 50% Protein A-Sepharose to each well. Use Stepper pipettor and **resuspend the Protein-A Sepharose after each row of the plate is done.** (Will need 2.5 ml of 50% Protein-A Sepharose per plate.)

(2) Add 55 ul of overnight incubate to each well.

(3) Shake the plate on a Plate Shaker for 45 minutes at 4°C.

(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 100 ul of Washing Buffer to each well. Shake for at least 5 minutes at 4°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 or 37 C incubator for approximately 15 minutes to dry. Do not over-dry.

(9) Add 30 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 ZnT8Ab:

$$\frac{\text{Sample CPM of 35-S - NC1}}{\text{Total PC1 - NC1}}$$

(2) Coefficient of Variation

For Duplicates:

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

For Triplicates:

$$\frac{\text{(High CPM - Low CPM)/1.693}}{\text{Mean CPM}} \quad \text{x 100}$$