

**Human Recombinant GAD-65 In Vitro Translation
& Autoantibody Immuno-Radioassay**

PROTOCOL

Liping Yu and George S. Eisenbarth

Barbara Davis Center for Childhood Diabetes

QA/QC for GAD65Ab Assays

Establish of lab-cut-off

The cutoff of 20 DK units/ml of GAD65Ab was established as the 98th percentile of 500 healthy control subjects. We constructed a receiver operating characteristic (ROC) curve among the newly diagnosed (within 2 weeks) patients with diabetes (n=50). The cutoff of 20 DK units corresponded to 86% sensitivity and 98% specificity.

Intra-assay CV: GAD65Ab 5% (n=10)

Inter-assay CV: GAD65Ab 10% (n=35)

- 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 +,-,+).
 - 2) The low positives 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.

Principle:

- *In vitro* transcription and translation (in one step, using rabbit reticulocytes) of labeled antigen (^{35}S -Methionine-GAD65)
- 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 NIDDK units/ml using reference calibrators.

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:

<u>Day 1:</u>	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)
		(3) prepare protein-A Sepharose (Part V)
<u>Day 2:</u>	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, and Equipments

Reagents and Supplies

	<u>Suggested suppliers (cat. no.)</u>
• - 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)
▪ - pTh-GAD65 plasmid	Original from Åke Lernmark (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 °C freezers	
• - 4 °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

- Wash Buffer: 20 mmol TBS Buffer pH 7.4 containing 0.15% Tween-20 (TBST)
 - 2.424 g Tris-Base
 - 8.70 g Sodium chloride
 - 980 ml distilled water ($\geq 15 \text{ M}\Omega\cdot\text{cm}$)
- *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 3 months

Part III: In Vitro Transcription/Translation

(1) Reaction

- In a given reaction GAD65 is labeled 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 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 ($\sim 50\%$) and consequently there is need to purify the labeled product to remove free radio-labeled methionine 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 (with no Methionine)	1 ul	1.0 ul
DNA plasmid (GAD65)	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 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 red part from the column except for end red part (labeled protein product will come out together with hemoglobin present in reticulocyte).

(3) Analysis the activity of labeled protein

- Remove 2 μ l from collection tube of purification and add 98 μ l of buffer.
- Take out 5 μ l of above dilution and add to a well containing 50 μ l 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°C for at least one month.

Part IV: Incubation of Serum Samples with ^{35}S -GAD65

Each 96-well plate is sufficient for testing 48 samples in duplicate. A control set including 7 NIDDK calibrators and local high and low positive and negative controls must be included in every assay. The control set is suggested being used every 4 plates if multiple plates will be performed. Usually, 4-8 plates can easily be run at one time.

(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 -GAD65 is required.

12 ml of antigen Buffer for two plates:

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

20,000 cpm of each antigen for each well.

$96 \times 2.5 \times 20,000 = 4.8 \times 10^6$ cpm of GAD65 for two plates.

In this example, say the GAD65 reaction tube being used contains 1.2×10^6 cpm/2 ul (or **0.6×10^6 cpm/ul**), as determined by the calculation at the end of Part III, then:

4.8×10^6 cpm required / 0.6×10^6 cpm per ul in the reaction tube = 8 ul required from the tube

Therefore, add 8 ul from the GAD65 reaction tube to 12 ml antigen Buffer for two plates.
Keep the Buffer-labeled antigen mixture on ice.

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

Total volume: $50 \text{ ul/well} \times 2.5 = 125 \text{ ul}$ (to test each serum in duplicate; the factor of 2.5 allows for some extra)

Serum: $2 \text{ ul/well} \times 2.5 = 5 \text{ ul}$

Buffer-antigen mixture: $125 \text{ ul} - 5 \text{ ul} = 120 \text{ ul}$

Therefore mix 5 ul of serum with 120 ul of buffer-antigen mixture.

(4) Seal the plate with aluminum sealing foil

(5) Vortex, simply spin down, and incubate overnight at 4°C .

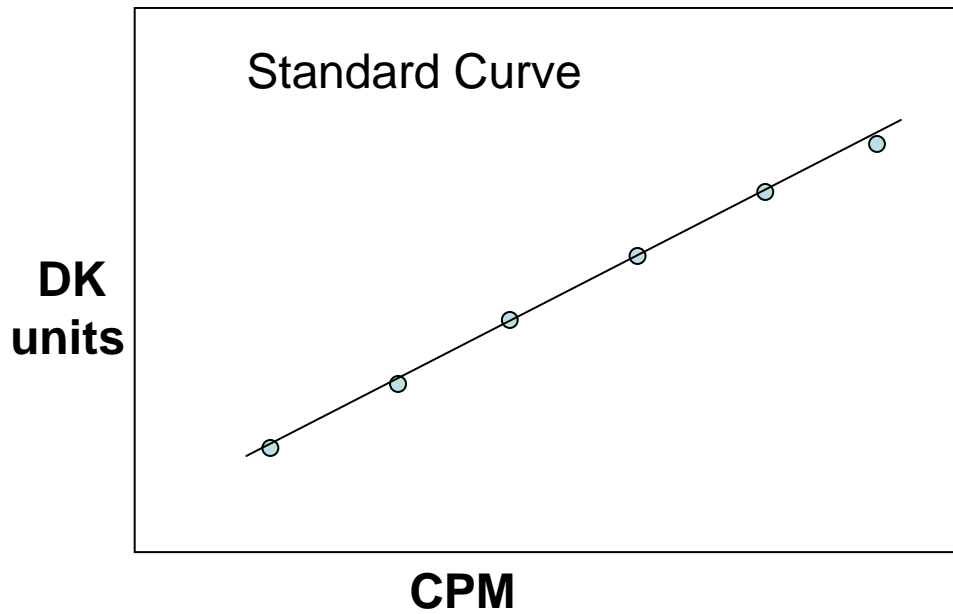
**Part V: Preparation of MultiScreen Filtration Plates
and Protein A-Sepharose**

- (1) Coat the plate with BSA by adding 200 ul of antigen buffer to each well.
- (2) Incubate overnight at room temperature, after placing the plate on aluminum foil.
- (3) Remove the antigen buffer.
- (4) The plates are now ready for running the assay, but 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.

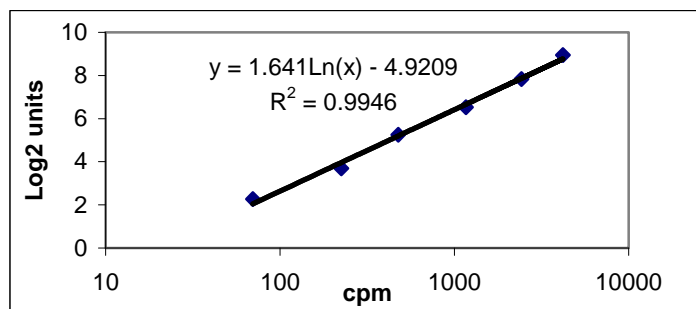
Part VI: Immunoprecipitation with Protein A-Sepharose

- (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 50 ul of overnight incubate to each of 2 wells (i.e., each serum will be tested in duplicate).
- (3) Shake the plate on a Plate Shaker for 45 minutes at 4°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 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 in 37 C incubator for 15 minutes. 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



Dilution	Standard Curve			Original		index	DK units
	cpm	bkg-N	Log2 unit	DK unit	index		
A	4497	4190	8.945444	493	0.635	0.564	435
B	2732	2425	7.83289	228	0.318	0.330	234
C	1471	1164	6.523562	92	0.156	0.163	101
D	783	476	5.247928	38	0.071	0.072	37
E	532	225	3.70044	13	0.036	0.038	16
F	377	70	2.263034	4.8	0.017	0.018	4
N	307	0			0.008	0.008	0



All unknown samples will be calculated for their DK units with the equation created by the standard curve in the same assay.