Standard Method for GAD and IA-2
A. Assay Procedure

General Principle

- *In vitro* transcription and translation (in one step, using rabbit reticulocytes) of labeled antigen (^{35}S-Methionine-GAD65 and ^{35}S-Methionine - IA-2ic$_{604-979}$)
- Incubation of serum with either labeled antigen 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 antigen
- Counting of each well with a 96-well plate β counter.
- Results expressed as NIDDK units/ml using reference calibrators.

Materials

	Suggested suppliers (cat. no.)
• - Trizma Base	Fisher (BP152-5);
• - NaCl	Fisher (BP358-212);
• - Tween 20	Sigma (P-1379)
• - Bovine Serum Albumin	Sigma (A-7906)
• - nProtein A-Sepharose FF	Amersham (17528003)
• - In Vitro TNT Kit	Promega L4600
• - RNasin	Promega N2511
• - ^{35}S-Methionine	MP Biomedicals (51001H at 10 mCi/ml) or Perkin-Elmer (NEG009T001MC at 11 mCi/ml)
• - NAP Column	Amersham (17-0853-02 or 17-0854-02 if separating multiple labeling reactions)
▪ - pTh-GAD65 plasmid	Original preparation from Prof Ake Lernmark. Batch prepared by Denver.
▪ - pSP64-PolyA-IA-2ic plasmid	Original preparation from Ezio Bonifacio. Batch prepared by Denver.
• - 5N HCl	VWR International (30018.360)
• - Parafilm	Sigma (P7793-1EA)

Materials specifically required if using the filtration method for washing Protein-A Sepharose

• - 96-well round bottom plate	Fisher (08408220)
• - 96-well filtration plates	Fisher (07200754)
• - Bottle-Top 500 ml-Filter Units	Fisher (09-740-22J)
• - TopSeal	Perkin-Elmer (6005185)
• - Microscint-20	Perkin-Elmer (6013621)

Materials specifically required if using the Deep Well method for washing Protein-A Sepharose

• - 96-Polystyrene deep-well plate	SLS (MOL2178)
• - Optiplate	Perkin Elmer (6005299)

- - TopSeal Perkin-Elmer (6005185)
- - Microscint 40 Perkin Elmer (6013641)
- - 30 ml Universal container SLS (SLS7502)

Equipment

- - 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
- - –70 ^{0}C freezer
- - 4 ^{0}C refrigerator
- - Pipette-Aid
- - Water purification system
- - Ice maker
- - Radioactive contamination monitor Thermo Electron Corporation
- - Radiation sink
- - pH meter
- - Vortex mixer
- - Stepper pipette
- - Pipettes/tips
- - Ice trays

Equipment specifically required if using the filtration method for washing Protein-A Sepharose

- - Vacuum-operated 96-well plate washer Millipore

Equipment specifically required if using the Deep Well method for washing Protein-A Sepharose

- - Refrigerated centrifuge with 96 well trays for deep-well plates. (this would be desirable for earlier steps)
- - plate washer Dynatech Laboratories
- - 8-way washer/aspirator Nunc (Immunowash 8)
- - 8-way washer/dispenser manifold Sigma (M2531-1EA)
  Prongs modified to be 34mm long (long) and 8mm long (short)
- - Water pump

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 (≥ 15 MΩ.cm)

5N HCl
1.5 ml Tween-20

Dissolve the Tris base and NaCl in 980 ml distilled water. Adjust the pH to 7.4 using 5N HCl (approximately 3ml) and then add 1.5 ml Tween-20, mix well. Chill to 4ºC before use.
Care should be taken to avoid contamination (e.g. wash bottles with Decon 90 and/or sterile filter the buffer).

Antigen Buffer: TBST containing 0.1% BSA. The antigen buffer should be prepared fresh whenever required by adding 0.1% w/v of BSA to TBST (e.g. add 1 mg of BSA per ml of TBST).

Protocol

a) Biosynthesis of radiolabelled human recombinant GAD_{65} or $IA\text{-}2_{ic}$. Note that the following procedure is for a single 50 µl reaction. Multiple reactions can be performed for the same antigen and volumes changed accordingly.

^{35}S labelled GAD or IA-2 is produced by incubating the GAD or IA-2 cDNA with components of the rabbit reticulocyte/SP6 polymerase kit using the amino acid mix minus methionine (Promega Corporation, Madison, WI), RNasin Ribonuclease inhibitor (Promega), and ^{35}S methionine (>1000Ci/mmol, MP Biomedicals or Perkin Elmer).

1. Reagents are stored at -70ºC until use. Immediately place the TnT RNA polymerase on ice. Rapidly thaw the rabbit reticulocyte lysate by hand warming and then place on ice. The other components can be thawed at room temperature and then stored on ice. (Stock 35-S methionine should be opened in a fume cupboard).

2. Add the components listed below to a sterile 1.5 ml microcentrifuge tube. Mix after each addition by gently flicking the vial. After addition of all the components, mix gently by briefly pipetting the reaction up and down. Centrifuge briefly to collect the reaction mixture at the bottom of the tube. Green tops should be used for GAD and blue tops for IA-2.

Rabbit reticulocyte lysate reaction mixture for GAD or IA-2.

25 µl Rabbit reticulocyte lysate (Red fluid)
2 µl TnT reaction buffer
1 µl TnT RNA polymerase SP6
1 µl Amino acid mixture minus methionine, 1 mmol/l
4.5 µl (45 µCi) ^{35}S methionine (1,000 Ci/mmol) at 10 mCi/ml (MP Biomedicals 51001H)*
1 µl RNasin Ribonuclease inhibitor, 40 Units/µl
1 µg DNA Template

make up to final volume of 50 µl with ribonuclease-free water

NB. RNasin Ribonuclease inhibitor and nuclease free water are not supplied with the kit.

* If using NEG009T001MC (Perkin Elmer) add 4.1 µl (45 µCi) ^{35}S methionine (1,000 Ci/mmol) at 11 mCi/ml

3. Incubate the reaction at **30ºC** (thermal block or water bath) for 90 minutes.

4. Meanwhile, return the stock reagents to -70°C freezer as soon as possible, after filling in the labeling worksheet, including lot numbers and activity date of methionine used.

5. Close to the end of incubation time (while the label is in the water bath), take a Nap-5 column and leave to drain into a pot. When it has completely drained, fill to top with Antigen buffer and drain again – repeat this 2 more times.

6. Take eleven 2.0ml tubes and label them as follows:

TC, 0, 1, 2, 3, 4, 5, 6, 7, 8, 9

Also write the label type on each tube, (e.g. pThGAD65/S-35), and add the date to tubes 1-3.

7. After the radiolabel has incubated for 90 minutes remove tube and place on ice.

8. Put 500 µl of Antigen buffer and 2 µl of label into the first tube marked TC (for total count) and mix.

9. Put the 2ml tube labelled 0 underneath the Nap-5 column, ensuring that all drips from the column will be collected in the tube. Load the remaining 48 µl of label onto the column and then wash out the reaction tube with 100 µl Antigen buffer and add this to the column. Once this has loaded add an additional 400 µl Antigen buffer to the column. At this point the peak containing protein should be close to the bottom of the column (as seen by red haemoglobin band). Add a little more Antigen buffer (approximately 25-50 µl) until the red band is about to elute (avoid allowing any pink fluid from entering the 0 tube). Before each of these additions, check that the last addition has completely entered the column. This is indicated by the top of the column appearing dull as opposed to looking shiny when liquid is still resting upon the upper membrane.

N.B. When collecting fractions, remember to make sure that the last drop at the end of the column is caught in the tube by touching the tube to the column before removing it. Always swap tubes BEFORE adding the buffer for the next fraction.

Also: As tubes labelled 1-3 should contain the labelled protein, put these on ice.

10. The tube marked '**1**' will collect the first pink fraction when a further 300 µl of Antigen buffer has been added to the column.

11. Fractions **2-5**, are collected in the same way after addition of 100 µl of Antigen buffer to the column. (Fractions **2-4** will all contain red haemoglobin and even fraction **5** may be slightly pink.)

12. After collecting a 100 µl fraction in tube '**5',** add 250µl of Antigen buffer and collect the fraction in tube '**6**'.

13. Add 500 µl of Antigen buffer and collect the fraction in tube '**7'**.

14. Add 750 µl Antigen buffer and collect the fraction in tube '**8**'.

15. Add 1000 µl of Antigen buffer and collect the last fraction in tube '**9**'.

NB: the volume of Antigen buffer/labeled protein that is added each time is equal to that which will be collected

Volume added (µl)	Fraction
500	TC
*600	0
300	1
100	2
100	3
100	4
100	5
250	6
500	7
750	8
1000	9

*****This volume varies slightly between different columns. Add to the column the remaining 48 µl of labeled protein and 100 µl of antigen buffer. Then add 400 to 500 µl of buffer to give a final volume of 550 to 650 µl (the void volume).

The table above shows how much salt buffer/label is added to the column and which tube it will be collected into. **Note that the TC is not put through the column, and that the 550-650 µl collected into tube 0 is the void volume (i.e. the buffer that remains in the column until the label and additional Antigen buffer is loaded). When multiple reactions are added to the column the volume of antigen buffer used to wash the label onto the column (Step 9) should be correspondingly reduced (ie. For a double reaction, 350 to 450 µl of buffer is added to give a final volume of 550 to 650 µl). No more than a 4X reaction should be added to a Nap5 column. If larger reactions are used they should be split between different Nap5 columns or loaded onto a Nap10 column (in this case the elution volumes would need to be adjusted to account for the larger column volume).**

16. When the 10 fractions and total count dilution have been collected, mix each tube, take 2 µl from each tube and transfer to an optiplate for counting. To each well add 50 µl of distilled water and 150 µl of scintillant, mix, and count on the appropriate protocol.

17. Fractions 1-3 should contain the majority of the labeled antigen and these should be pooled, mixed and frozen at -70°C as 125 µl aliquots until use. Ideally, the labeled antigen should be used within one month, following storage at -70°C.

18. Transfer the counts for the different fractions and diluted total count to the labeling worksheet. This worksheet adjusts the counts found in each fraction by the volume of each fraction and then divides this result by the volume of the original reaction mixture to give the cpm per µl of the original reaction mixture (corrected counts). The proportion of the corrected counts in the pooled fractions is then expressed as a percentage of the sum of the corrected counts in all fractions to give an estimate of the eluted 35-S incorporated into labeled protein for use in the assays *(ideally the sum of the eluted corrected counts in all fractions divided by the corrected total counts (% through) should be close to 100%).* The estimated incorporation can then be used to calculate the approximate volume of label needed for each plate. For example 10% incorporation in a single reaction (approximately 4.5 µCi of labeled antigen) should provide sufficient label for about 4 plates at 20,000 cpm per well (approx. 125 µl stock/plate).

Incorporation is normally >30% for GAD65 and >10% for IA-2ic. If less than this, the label should be discarded since these labels are usually of poor quality. The column is discarded as radioactive waste.

b) Autoantibody measurements.

Preparation of working radiolabelled antigen solution.

Day 1. GAD or IA-2 stock label is diluted in ice-cold Antigen buffer, to give 20,000 cpm/25 µl. Sufficient volume for the whole assay should be made. The working antigen solution should be kept on wet ice.

Two techniques distinguished by the washing procedure can be followed. The first uses filtration washing and the second centrifugation washing in deep well plates.

b1) Filtration washing technique

Day 1. Standards and unknowns are thawed and well mixed. 2.4 µl of each serum is pipetted in duplicate, into the wells of a standard 96-well plate which is placed on ice. Pipette tips are changed after each well and care is taken to dispense the samples in the bottom of the wells. 30 µl of GAD or IA-2 label (24,000 cpm) is added to each well and the plate covered with parafilm. The plates are centrifuged briefly (approx 1000 rpm/250g) in order to bring label down to bottom of well. After mixing for 30 seconds on an orbital shaker (1000 rpm) the plate is incubated for 20-24 hours at 4ºC on wet ice.

Day 1. On the same day as setting up the serum-antigen mix, take an empty 96-well filtration plate and coat it with BSA by adding 200 µl of Antigen Buffer to each well. Incubate overnight at room temperature, after placing the plate on aluminum foil (the plate can be wrapped in foil for the overnight incubation).

Day 2. Remove the Antigen buffer. The plates are now ready for running the assay, but can be stored at 4ºC if necessary. Immune complexes are precipitated by addition of 12.5 µl/well protein A sepharose gel (PAS) (Pharmacia Biotech AB, Uppsala, Sweden). 25 ml protein A sepharose gel slurry (Total volume = 35 ml with ethanol) is made up to 50ml with 15ml sterile saline (This is the 50% slurry). After completely re-suspending the gel by inverting the tube several times, 2.5 ml/plate of the 50% slurry is transferred to a universal container (the slurry should be kept re-suspended while pipetting by occasional mixing), and washed 3 times by addition of 5-10 ml of ice-cold Antigen buffer per ml of gel, centrifuging at 500g for 3 minutes at 4ºC and pouring off the supernatant. The washed gel is then re-suspended in ice-cold Antigen buffer to twice the volume added before the washing (to make a 25% slurry), ready for addition to the plates. Since the slurry is now 25%, 5 ml/plate will be needed.

Add 50 µl of 25% Protein A-Sepharose to each well on ice. Use Eppendorf multistep pipettor and resuspend the Protein-A Sepharose after each row of the plate is done. (You will need 5 ml of 25% Protein-A Sepharose per plate.)
Transfer 25 µl of overnight serum-antigen mix from each well to the corresponding well on the 96-well filtration plate.
Wrap the plate in Al foil and shake the plate on a Plate Shaker for 60 minutes at 4ºC. Accurate timing is important.

Place the plate on Millipore plate washer device (with vacuum set low – about 15 Hg).
Wash the plate three times under vacuum with 200 µl of Washing Buffer per well. Stop vacuum.
Add 120 µl of Washing Buffer to each well. Transfer to a plate shaker. Shake for at least 5 minutes at 4ºC. Transfer back to the Millipore plate washer.
Wash the plate four times with 200 µl of washing buffer per well (change the plate direction after two times of washing at this stage).
Place the plate under a lamp for approximately 10 minutes to dry. Rotate the plate several times to ensure even drying and check its appearance. Drying is complete when deep fissures appear in the Sepharose visible in the bottom of the wells. Do not over-dry and be careful not to melt the plastic parts of the plate.
Add 30 µl of scintillation cocktail (Microscint-20) to each well. Apply the transparent sheet of top seal to seal the top of the plate and tape the non-transparent sheet (leftover from a top seal) to the bottom of the plate to prevent leaking immediately after adding scintillation liquid (leaking should be minimal, so it should not necessary to use another top seal to seal the bottom of the plate).
Count on Top Count 96-well plate β counter – 300 seconds/well.

b2) Centrifuge washing technique

Day 1. Standards and unknowns are thawed and well mixed. 2 µl of each serum is pipetted in duplicate, into the wells of a 96 deep-well plate which is placed on ice. Pipette tips are changed after each well and care is taken to dispense the samples in the bottom of the wells. 25 µl of GAD or IA-2 label (20,000 cpm) is added to each well and the plate covered with parafilm. The plates are centrifuged briefly (approx 1000 rpm/250g) in order to bring label down to bottom of well. After mixing for 30 seconds on an orbital shaker (1000 rpm) the plate is incubated for 20-24 hours at 4ºC on wet ice.

Day 2. Immune complexes are precipitated by addition of 12.5 µl/well protein A sepharose gel (PAS) (Pharmacia Biotech AB, Uppsala, Sweden). 25 ml protein A sepharose gel slurry (Total volume = 35 ml) is made up to 50 ml with 15 ml sterile saline. After completely re-suspending the gel by inverting the tube several times, 2.5 ml/plate of the 50% slurry is transferred to a universal container (the slurry should be kept re-suspended while pipetting by occasional mixing), and washed 3 times by addition of 5-10ml of ice-cold Antigen buffer per ml of gel, centrifuging at 500g for 3 minutes at 4ºC and pouring off the supernatant. The washed gel is re-suspended in ice-cold Antigen buffer (to give a 25% slurry) before use (5ml/plate).

50 µl of resuspended Protein A (25%) is added to each well using an eppendorf multi-pipette, care being taken not to allow the gel to settle in the pipette. The plate is then spun briefly at 1000 rpm, covered with parafilm, and left on a shaker (1000 rpm) at 4ºC for 60 minutes (accurate timing is important). 800 µl of cold TBST (without BSA) is then added to each well with a plate washer (Dynatech Laboratories Ltd., Billingshurst, West Sussex, UK) using an 8-well washer/aspirator and the plate centrifuged at 500g and 4ºC for 3 minutes.

The supernatant is aspirated using a long multi-aspirator, leaving approximately 100 µl in the bottom of the wells and then another 800 µl TBST is added to each well. The plate is centrifuged at

500g and 4ºC for 3 minutes and aspirated. This process is repeated 3 more times. 100 µl TBST is then added to each well using a stepper repeat pipette (Eppendorf). The pellet is resuspended by pipetting up and down using a multichannel pipette and transferred to a white 96 (300 µl) well microtitre plate (Optiplate, Packard Instruments, Meriden, CT). (Shaking the deep-well plate briefly during transfer can help to keep the PAS is in suspension and avoid bubbles). The optiplate is centrifuged at 500g for 3 minutes at 4ºC and the supernatant aspirated using a short multi-aspirator to leave approximately 50 µl in each well, care being taken not to disturb the sediment. 200 µl Microscint 40 scintillant (Packard) is added to each well and the plate covered with sticky plastic film (Topseal). The plate is shaken until the scintillant is mixed, and then placed in a counting cassette and counted for 300 seconds/well (Topcount, Packard Instruments, Meriden, CT).