

TG Autoantibody Assays

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5. Take Eleven 2.0ml Sarstedt tubes and label them as follows:

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

Also write 'TG' on each tube, and add the date to tubes 1-4.

6. After the radiolabel has been in the waterbath for 1.5 hrs remove tube and place on ice.
7. Put 500ul of Denver buffer and 2ul of label into the first tube marked TC (for total count).
8. Load the full 50ul of label into the column, then add 50ul Denver buffer to the tube (to wash) and add this to the column, then load an additional 450ul Denver buffer to the column. Before each of these additions, check that the last addition has completely entered the column. This is made apparent by the top of the column giving a dull appearance as opposed to looking shiny when liquid is still resting upon the upper membrane.

(This assumes a single label has been made. If a double is made instead, the total volume of the reaction is 100µl and 100µl of buffer will be needed to wash out the tube. You will then only require 350µl of buffer to reach the total void-volume of 550µl. If a quadruple is made, the total volume of the reaction is 200µl and 200µl of buffer will be needed to wash out the tube. You will then only require 150µl of buffer to reach the total void-volume of 550µl.)

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

Also: As tubes labelled 1-4 should contain the products, put these on ice.

9. Collect the first fraction in the tube marked '0' (this should be clear), and avoid allowing any pink fluid from entering this tube.
10. The tube marked '1' will collect the first pink fraction when a further 300ul of buffer is added to the column.
11. From now until tube '5', each time you collect a new fraction; add 100ul of buffer to the column (for Quadruple, fraction 3 and 4 are divided into four 50ul fractions).
12. After collecting a fraction in tube '5' add 250ul of buffer collecting a fraction in tube '6'.
13. After collecting a fraction in tube '6' add 500ul of buffer collecting fraction in tube '7'.
14. After collecting a fraction in tube '7' add 750ul of buffer, collecting fraction in tube '8'.

15. After collecting a fraction in tube '8' add 1000ul of buffer then collect the last fraction in tube '9'.

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

The table below shows how much buffer is added to the column and which tube it will be collected into, note that **the TC is not put through the column**, and the **550ul collected into tube 0 is the void volume (i.e. the buffer that remains in the column until the label and additional buffer is loaded)**.

Vol added (ul)	Fraction
500	TC
550	0
300	1
100	2
100	3
100	4
100	5
250	6
500	7
750	8
1000	9

When all 11 fractions have been collected, take 2ul from each tube and transfer to an OptiPlate. To each well add 200ul of microscint 40, vortex plate for 1 min, then load into the topcount. Count using total count protocol (see page 8).

Ideally, there will be two peaks in the resulting data. The first peak relates to incorporated methionine and the second peak relates to free methionine. The incorporated methionine (i.e. the labelled antigen) fractions are what are required. To reduce background radioactivity, it is important to prevent free methionine from contaminating the incorporated methionine. To do this, aliquots from the first peak are only selected for use while the radioactive count is still falling. As soon as the count begins to rise (the start of the second peak) that aliquot and the one previous are disregarded.

16. Transfer the counts for the different fractions to the worksheet in label2010.xls. The spreadsheet will then give an estimate of the incorporation- this should be 10% or greater. This estimate will then be used to calculate the approximate volume of label needed to label each plate. For example 10% incorporation in a single reaction should provide sufficient label for at least 4 plates at 20,000cpm (approx. 125ul stock/plate).
17. Pool the fractions containing the labelled antigen (usually fractions 1-3), mix and then divide between the same fraction tubes (approximately 170ul per tube). Store these at -70°C until use.
18. Discard all the other fractions in the radioactive sink, washing to waste with copious quantities of running water. Clear the working area and perform environmental and personal radioactive contamination monitoring with the appropriate mini-monitor. Record the monitoring results on the logsheet.

Plating

Enter plate map in the appropriate workbook in the **TG** folder, e.g. **TGBr13** file.
This can be found in **Antibodies** folder on Database drive (//smh-srv2/Database\$).

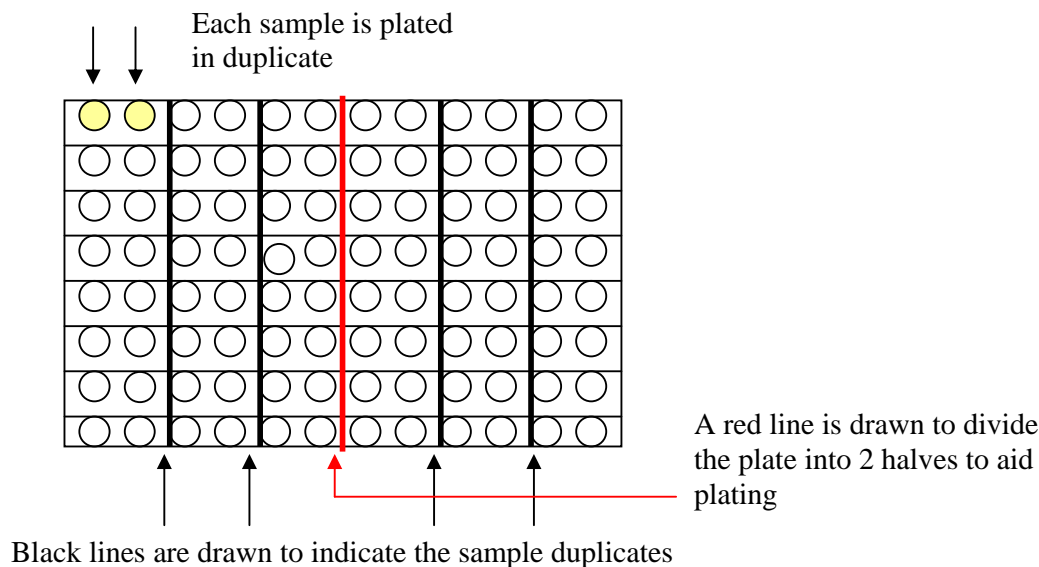
Each assay should include a standard curve and internal and external quality control samples.
The standard curve goes on first, followed by 4 internal QCs and 2 external QCs (e.g. Q0265),
which are distributed randomly through the plate.

TG Standards (1/05/98) – 9 dilutions of PJB (20/4/98) in SG (28/04/98):

S100 = 1/2 S50 = 1/4 S25 = 1/8 S12.5 = 1/16 S6.25 = 1/32 S3.1 = 1/64
S1.6 = 1/128 S0.8 = 1/256 S0.4 = 1/512 (low Standard)

TG QCs - Negative = **SG** (28/04/98) - Low Positive = **LA** (18/12/98)
 - Medium Positive = **MH** (4/12/98) - High Positive = **High** (PJB
10/11/98)

Divide plate into halves, then sub-divide into 2 well segments and place in ice tray. Pipette 2µl of each sample into bottom of each pair of wells, using a clean pipette tip for every aliquot, thus allowing the tip box to become a guide for where the next aliquot should be dispensed.



Put plate in freezer if labelling in the future, or in the fridge if it will be labelled that day.

In workbook copy the cells with the Codes for the Standards, QCs and Samples. Paste into **Plated Template** in **TG** folder.

Insert correct Plate ID, e.g. 20/03/2008 A AL, copy and paste next to all CODES.

Select and Copy the cells by selecting the relevant rows. Paste into the **TG Plated** table in the **Plating Database** (Access), which can be found in the **Antibodies** folder on the **Database** drive.

Diluting Radiolabel for TG Assays

1. Measure 0.372g of EDTA in a 20ml sterilin tube and add 10mls of Denver buffer+0.1% BSA. This will make a 0.1M solution. The EDTA does not dissolve easily and will require about 30mins of gentle shaking (put on the vortex shaker). Once it has dissolved add ~200ul of 5M NaOH to bring the pH to 7.4 (check).
2. Label a 20ml sterilin tube with the date and label name (aliquots can be frozen at -20°C).
3. For each plate being assayed, use 2.375mls of Denver buffer+ 0.1% BSA and 125ul of the 0.1M EDTA solution. This equates to 50ul of 0.1M EDTA solution in each ml of dilution buffer, i.e.0.005M EDTA per ml of dilution buffer.
4. Assuming that % incorporation is 10%, add ~ 40µl radiolabel per plate (double label)
(i.e. 40µl label to 2.5mls of buffer.)

If diluting a small amount of label (10ml or less) add an additional 300-500µl of buffer to your total volume as you may run short otherwise (remember to add ~ 25ul of EDTA solution too).

5. When the label has been diluted, measure the total count (TC) by putting 25µl of the diluted label into a well of an OptiPlate, add 200µl of Microscint 40, cover with a sticky plate cover and shake for approximately 1 minute.
If you put plate into the **OLD** topcount, then:
Press **F1** - this brings up the count directory
Press **F4** - this brings up the plate map
Press **F4** - this brings up the plate window
Press **F2** - this clears the current cell
- then go into the appropriate cell (position of where the label has been added in the plate)
Press **F9** - this selects that cell to be read by the counter
Press **F1** - this exits the plate window and returns to the plate map
Press **F3** - this accepts the current plate map
Press **Alt F2** - this brings up the count control
Press **F4** - this brings up the protocol window
- type '4' in the box to select protocol 4 (this is the total count protocol)
Press **F1** - this exits back to the count control screen
Press **F10** - this turns the stacker on/off (should be off as only one plate is being loaded)
Press **F2** - this loads and begins the count protocol
Press **F1** - this exits back to the directory
Press **F1** - this exits the directory

If you put plate into the **New** topcount, then:
(there are short cut icons you can use which is quicker but this is the fool-proof route!)

- Go to **Application** then click on the **Sample Mapping** icon. This opens up another window.
- Then go to **File, Open, Total Count** file.
 - Clear map by selecting cells and hitting “delete” on the keyboard.
 - Identify the required wells by selecting the relevant cells, go to **Edit**, and select **Fill Wells**.
 - Close the Sample Map window down.
- On the main instrument Top Count window select **Total Count** protocol instead of **gada**. The go to **Control, Plate source** and select **Manual**.

N.B. There is a count delay of 1 minute in the total count protocol, and then it will count for a further 1 minute and then display the total count.

6. The ideal total count for all labels is 20,000 counts. However, there is a range that is acceptable:

Lowest acceptable count: 19,000

Highest acceptable count: 21,000

Note: the New and Old Top Counts read slightly differently (counts come out higher on the old one)

7. If the label has a count between these values then it is ready to use, therefore:
Using a stepper pipette, add 25µl of labelled antigen to each well and centrifuge.
Cover plate with parafilm, mix on a shaking platform, put into ice tray and leave in the fridge to incubate for ~20hrs.

If it is outside of these values it will need adjusting. The calculations required to work out how to increase / decrease the count can be found on the ‘Count Calculations’ protocol.

8. If any adjustments have to made, the label will then need to be recounted following the same procedure as before.
9. **Record the label making in the ‘Counts’ book.**

Washing/Adding PAS

- **For each plate** being assayed, take 1.2ml of 50% PAS suspension and transfer to a 20ml sterilin tube. Fill the tube with Denver+0.1% BSA buffer, centrifuge and pour off supernatant.
- After the first wash, add 1ml of 50% IgA-Agarose suspension to the tube and repeat the wash step 3 more times.
- For each plate you need 5mls of washed PAS/Agarose/Denver buffer, i.e. fill the tube to just above the 5ml line with buffer for 1 plate.

Note: One sterilin tube can be used for up to 4 plates (final volume then being just over 20mls).

- Mix well and ensure that PAS/Agarose stays in suspension during pipetting by frequent mixing. Using a stepper pipette (with the end cut off), pipette 50µl of PAS/Agarose suspension into each well of the plate containing incubated serum and radiolabelled antigen.
- Centrifuge plates: prog 0 (1500 rpm, 3 mins @ 4°C).
- Place plate back into the fridge on a shaking platform (at 750rpm) for ~ 1.5 hrs.

Washing and transferring the assay

- After plates have been on shaker for 1.5 hrs, remove from fridge and using the multi-dispenser fill the wells with Denver buffer (WITHOUT BSA).
- Centrifuge plates: prog 0 (1500 rpm, 3 mins @ 4°C)
- Aspirate with 'long' aspirator, leaving a volume of ~50µl in each well.
- Repeat wash step 4 more times. Aspirate plates and leave in the fridge until ready to transfer.
- Add 100µl of ice-cold Denver buffer (+/-BSA doesn't matter) to each well using a stepper pipette. **Only add buffer to the plate you are immediately going to transfer.**
- Mix plate for 10-20secs on the shaking platform.
- Using a multi-channel pipette (yellow tips) transfer the samples to an optiplate. Mix samples with the pipette to ensure the PAS/Agarose is in suspension before transferring to the optiplate. Aim to re-shake plate every 3 columns (4 times a plate) to start off with. You can go for twice a plate when you're up to speed!

- Go back to wells to remove any residue, wiggle the pipette about whilst sucking up. Check the tips for residue before discarding in the ³⁵S waste bin. If there appears to be residue in the tips, shake pipette (over the sink) to bring it down to the end of the tip and transfer it to the plate.
- After the transfer is complete, check the wells for residue by standing beneath the light and looking up through the bottom of the plate. If any PAS or liquid can be seen, mark the bottom with a red marker and transfer each well individually, adding 50µl buffer if necessary to the appropriate well.
- Leave Optiplates in the fridge while transferring any remaining plates. Then centrifuge Optiplates and aspirate excess buffer using the 'short' aspirator. **Do not leave un-aspirated plates in the fridge for longer than ~ 1hour (label starts unbinding from PAS/Agarose in excess buffer).**
- Add 200µl Microscint 40 to each well and seal securely with a sticky lid. Vortex for ~3 mins to form an emulsion, then place in the topcount for counting.
- To load plates on the beta counter:
 - Place the stacker rack at the front of the counter and lock into place
 - Stack plates in the rack (plate 1 first and stop plate last)
 - For the New Top Count also put metal weights on back and front stackers.
- To count plates on the **Old** beta counter press:
 - Alt F2 to get into the count window
 - F4 to bring up protocols and select Protocol 1
 - F10 to switch on the stacker (only required if counting more than one plate)
 - F2 to load the plate
 - Remember to put repeat count protocol plate as last plate in the stack.
 - Following a ten minute count delay plates will count for 5 minutes per well.
- To count plates on the **New** beta counter:
 - On the main instrument Top Count window select **gada** protocol instead of **Top Count**. Then go to **Control, Plate source** and select **Stacker**.
 - Start Assay

Transferring Results from Old Beta counter to the worksheets

- A. To get data from Topcount to computer:

Insert floppy disk.

Press F7 - this takes you to DOS.

At prompt type 'd' then press return.

At prompt type "copy" & then name of the last but one file followed by a:\.....
.txt

E.g. copy gada05.036 a:\TGA.txt

A message should appear to say that the file has been copied. It may also ask if you want to overwrite, if it does then type 'y' and press enter.

To get back to the original main screen type 'x' then press enter.

B. To get the counter data into the computer:

Insert the floppy disk containing the saved file. Open the notepad file and **Save As** 'New Counts' file in:

Database\$
↓
Antibodies
↓
IA2, GAD, TG and ZnT8 Calculating

Click 'Yes' when asked if you want to replace the existing file.

Open the Access database named "Old Top Count Macro" in the same folder.

Select "Macros" then select "Anna Old Top Count Macro to run from New counts file".

Click 'Yes' when asked if you want to replace the existing file.

You should then be automatically taken into results\ABRES.XLS

Save As the date the plates were put on the counter in **Antibodies \ IA2, GAD, TG and ZnT8 Calculating \Counts2010**, e.g. 1.4.10.xls.

Repeat to obtain the second set of counts, which will be in the **last** results file on the Beta counter.

Instead of saving a new file, copy and paste the second set of counts in ABRES.XLS next to the first set saved in **Counts2010**.

Transferring Results from New Beta counter to the worksheets

1. **Open** the one but last file in the **gada** folder in the **Data** folder on the counter's hard disk (there is a shortcut to **Data** on the desk top) e.g. **gada1.160**, use "**Notepad**" to open this file.
2. Use the **Edit-Replace** function to replace "unk" with "0" (this is a Zero!), **Replace All**. This should change the information in the Sample Type column from the format "UNK001" to "0001"
3. Save onto floppy disk. Repeat for the last file (second set of counts).

4. Insert floppy disk into computer, open the first file with “Notepad” and **Save As** ‘New Counts’ file in Database\$\Antibodies \ IA2, GAD, TG and ZnT8 Calculating. Click ‘Yes’ when asked if you want to replace the existing file.
5. Open “Anna’s New Top-Count Macro” in the same folder.
6. Select Macro and open “Top Count Macro (New)”. When asked if you want to replace the existing file “ABRES2.xls” select yes.
7. **Save As** the date the plates were put on the counter, followed by “New” to indicate which counter it was counted on, e.g. “1.04.10. New.xls”. Counts should be saved in the **Counts2010** folder in the **IA2, GAD, TG and ZnT8 Calculating** folder.
8. Repeat steps 4-6 for the second set of counts, but copy and paste the information in the new **Abres2** file next to the first set of counts just saved in **Counts2010**.

Note.

- The macro will not work if the “Sample Type” column of the Notepad file includes any letters.
- Make sure you save the notepad file over the “New counts.txt” file otherwise you will run the macro on the old data.
- The Macro requires the **Database** to occupy the **X drive** on the computer.

Additional information

The results for all plates counted will be in the same file and need to be transferred separately from ABRES to the correct worksheets. There are 48 lines of data for each plate. Transfer is follows:

	Start on number	Finish on number
Plate 1:	1	95
Plate 2:	97	191
Plate 3:	193	287
Plate 4:	289	383

Calculating Results

These are guidelines not absolute rules.

Threshold for positivity for TG= 1.31

1. Take the counts out of the excel file saved in the **Counts2010** folder, in the **IA2, GAD, TG and ZnT8 Calculating** folder in the **Antibodies** folder. **Paste values** into the correct space in the appropriate workbook e.g. TGBr13.
2. Check the correct counts have appeared (in the top left of that plate's worksheet) for the standard curve. Right click on the graph and **select data**, selecting the counts (X-Value) and the Log2 units (Y-Value).
3. Check the graph – the r^2 value should be above 0.95, if it is not identify any bad duplicates and delete them from the standard curve (on the top left of the worksheet's where the data is sourced from).
4. Once you are happy with the standard curve, change the Plate ID in the title of the graph.
5. Take the equation for the trendline off the graph and put it in the worksheet under the column **Log 2 TGA**. Make sure you apply this to all of the standards/samples/QCs.
6. Look at the %Error column. Investigate any errors over 30% further.
 - If the error is on a standard ignore it because you should have dealt with it while drawing the graph.
 - If the error is on a QC, check whether the QC is “in”
 - a. If it's “in” ignore the duplicate.
 - b. If it's “out” but deleting one of the duplicates brings it into range then mark as a RPT in the **Units** column.
 - c. If it's “out” and deleting one of the duplicates doesn't bring it into range then mark as a RJT in the **TGA Units** column and RJT any samples in that range.
 - If the error is on a Sample/ExQC look at the counts.
 - i. If both of the duplicates are below the bottom of the standard curve (the counts for standard **S0.4**) ignore the error.
 - ii. If one or both of the duplicates above the bottom of the standard curve than mark RPT in the **TGA Units** column.
7. Now go through the QCs and check that each is in range, ranges can be found for the QCs on the “tab” marked **New TGA QC**; they should tell you what to do if the QC is out. QCs that are out of range and any samples this affects are rejected by marking RJT in place of the units.
8. Mark on the worksheets
 - Total Counts (from the Counts book)
 - Assay Date (Day label was added)
 - PAS lot
 - IgA lot
 - Label Date (Date Label was made)

Now someone needs to check that 1-8 has all been done correctly. Then transfer the results into the database.

Transferring Results

Once the Results have been calculated by someone else and you have checked they are done correctly:

- 1 Copy the TGA Units and CODE cells for all the assay results.
- 2 Paste these in the **TGA results template.xls** in the **TG** folder.
- 3 Identify the assay date from the worksheet and paste this into the results template under the “date of assay” heading and copy down for all the results.
- 4 Now select the three columns of information and sort by **TGA Units**. Any RPT or RJT values should sort to the bottom.
- 5 Copy the results (not RPT or RJT) by selecting the rows; paste these results into the **Plating Database** (Access) in the **Antibodies** folder, in the table named **TG Results**.
- 6 Now go into the **TG Plated** table and delete the plates you have just obtained results for. If you shut down Access it will automatically save changes.
- 7 Once you’ve transferred the results from a plate, highlight it blue in the workbook.

Denver Buffer (1 Litre)

Trizma Base	2.422g	
Sodium Chloride	8.70g	
BSA	1g (not present in wash buffer)	
dH2O	1 litres	
HCl	~3.5mls	(This should bring pH to 7.4 - check!)
Tween 20	1.5ml	