Standard IA2A, GADA and ZnT8A Assays

Contents

Differences from IAA

1. We make the label ourselves with methionine we order in.
2. The radioisotope is ^{35}S, it emits **beta radiation** and is counted on a top count (beta counter).
3. We use **2µl** serum in the assay
4. Label is diluted to **20,000 cpm**
5. We incubate the serum and label for **20-24hrs**.
6. PAS is added at **2.5ml**/plate for IA2A/GADA or **2ml**/plate for ZnT8A
7. We incubate the PAS with the label/serum for **1hr**.
8. Assays use **Low Tween Salt** buffer (LT-TBST/Denver/Standard). Up until the wash step this buffer always includes 0.1% BSA.
9. We transfer into **optiplates**, add scintillant and count on the top count. It takes 1hr/1.5hrs per plate.
10. We label on Monday, Tuesday, Wednesday or Thursday.
11. We Assay on Tuesday, Wednesday, Thursday or Friday

Preparing Radiolabels for Standard Assays

N.B. 35-S Methionine and other reagents are stored in the -70°C freezer marked for storage of radioactive materials.

Before starting ensure that the water bath is at 30°C

Wear laboratory coat and pair of medical examination gloves during all steps (Double gloving is recommended). Also wear finger monitor under gloves

1. To prepare label, mix the following reagents in the following order in a sterile 2.0ml Sarstedt tube (the stock 35-S methionine must be opened and added to the reaction mixture in the fume cupboard with fan running and sash lowered):

Reagent		Single (ul)	Double (ul)	Quad (ul)
Lysate	Defrost rapidly in hand, store on ice	25	50	100
TNT buffer	Mix well, since salts tend to precipitate	2	4	8
SP6 polymerase	Keep on ice	1	2	4
Minus Met	Thaw, store on ice	1	2	4
35-S Methionine		4.5	9	18
RNAsin	Keep on ice	1	2	4
Plasmid DNA*	Thaw, store on ice	1	2	4
dH2O		14.5	29	58
Final volume of:		50ul	100ul	200ul

Defrost lysate rapidly in hand

* Plasmid DNA - the volume added is dependent on the concentration.
1ul is added when the conc. = 1mg/ml

Change the amount of water added depending on the volume of plasmid added.

Do not pipette less than 1ul of plasmid as it's not very accurate.

You need 1ul of ZnT8R for a single and 1.3ul of ZnT8W for a single.

GAD Std = PTh65   IA2 Std = Ezio

2. Spin tube at 1500rpm (4°C), then gently mix by tapping the tube. Place the tube in a floating holder and place that in the water bath at 30°C for 1hr 30.

3.   Fill in label worksheet in diabetes&metabolism/database/antibodies/radac/stock/label2011.xls.

4.   Approx. 20 mins before the end of incubation time (while the label is in the water bath), take a Nap5 column and leave to drain into a pot. When it has completely drained fill with Denver buffer and drain again – repeat this 2 more times.

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 the label type on each tube, (e.g. ZnT8), and add the date to tubes 1-4.

6.   After the radiolabel has been in the water bath 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 counts begin 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 label2011.xls. The spreadsheet will then give an estimate of the incorporation for that preparation- this should be 10% or greater for ZnT8 and IA2, and 30% or greater for GADPTh65. 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, either in: **ZnT8** folder, e.g. **ZnT8RBr1** file
OR **IA2** folder, e.g. **IA2DK01** file
OR **GAD** folder, e.g. **GADDK01** file
These can be found in **Antibodies** folder.

Each assay should include a standard curve and internal and external quality control samples. The standard curve goes on first, followed by a negative QC, 4 more QCs and 2 external QCs (e.g.Q1743) follow, these 6 are distributed randomly through the plate.
As the standard curve is usually split across 2 plates, **plates are paired and must be assayed together**.

ZnT8A Standards (for both R & W) (17/6/09) – ND571 (09/04/09) diluted with the negative pool.

Std Z1 = neat Std Z2 = 1/2   Std Z3 = 1/4   Std Z4 = 1/8
Std Z5 = 1/16 Std Z6 = 1/32 Std Z7 = 1/64 Std Z8 = 1/128

Z1, Z3, Z5, Z7 go on one plate and **Z2, Z4, Z6, Z8** go on the other plate**.**
These are always followed by the **negative pool (16/04/09)**

ZnT8-R QCs   - Negative = **JB QC**   - Low Positive = **Low R QC**
                      - Medium Positive = **RB ND570**   - High Positive =**RB ND572**

ZnT8-W QCs   - Negative = **JB QC**   - Low Positive = **RB ND624**
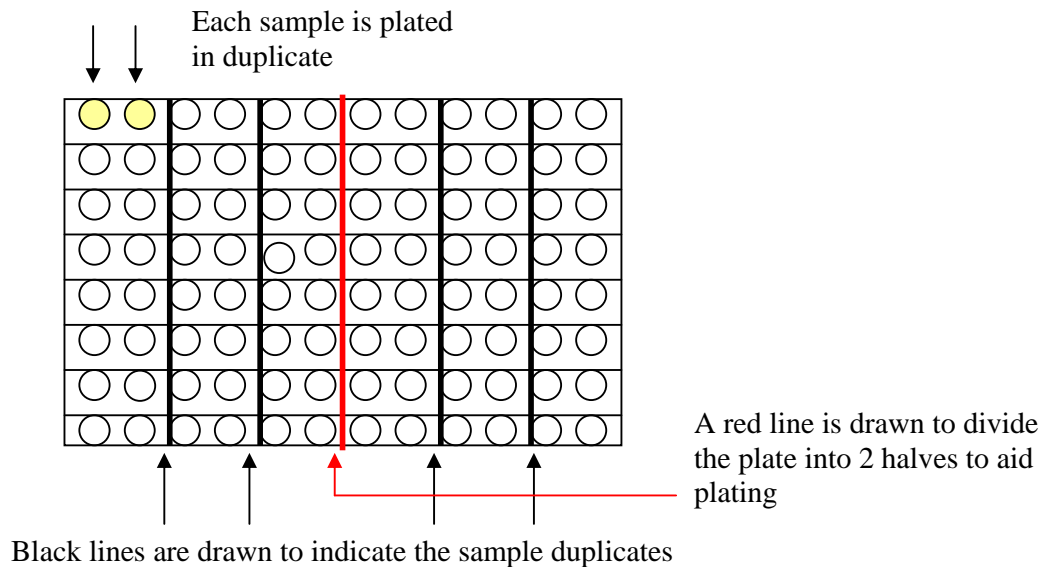   - Medium Positive = **RB ND570**   - High Positive =**RB ND542**

GADA/IA2A Standards - NIH TEDDY GAD/IA2 **Standards A-F** (yellow lids)

Standards **A, C, E,** go on one plate and **B, D, F** go on the other plate**.**
These are always followed by the NIH TEDDY GAD/IA2 **Negative Pool**

GAD QCs   - Negative = **AW** (11/10/99)   - Low Positive = **HW** (28/05/97
& 23/6/99)
                    - Medium Positive = **Med09**   - High Positive =**AO** (22/10/97)

IA2 QCs   - Negative = **CR** (26/11/09)   - Low Positive = **Low06**
(28/03/06)
                    - Medium Positive = **ME** (11/04/96)   - High Positive =**PR** (10/04/96)

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.

Each sample is plated in duplicate

A red line is drawn to divide the plate into 2 halves to aid plating

Black lines are drawn to indicate the sample duplicates

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

In the workbook copy the CODE column from the plate map and paste into **Plated Template** in **IA2, GAD or ZnT8** 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 **DK GAD Plated**, **DK IA2 Plated, ZnT8 R Plated,** or **ZnT8 W Plated** table in the **Plating Database** (Access), which can be found in the **Antibodies** folder.

Diluting Radiolabels

1. Make up label in a 20ml sterilin tube. Label tubes before starting with the date and the label name.
2. For each plate being assayed, 2.5mls of Denver buffer+ 0.1% BSA is required and the following volume of label:

ZnT8 and IA2:

Assuming that % incorporation is 10%, add ~ 40µl radiolabel per plate (double label)
(i.e. 40µl label to 2.5mls of buffer.)

GADPTh65:

Assuming that % incorporation is 30%, add ~ 15µl radiolabel per plate (double label)
(i.e. 15µl label to 2.5mls of buffer.)

If diluting a small amount of label (10ml or less) add an additional 500µl of buffer to your total volume as you may run short otherwise.

3. 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.

4. If you put plate into the **New** topcount, then:

- Click on the **Start Sample Mapping** icon. This opens up another window.
- Then go to **File**, **Total Count/unknown map**.
  - Clear map by selecting cells and hitting "delete" on the keyboard.
  - Double click to select the relevant cells.
  - Save, then close the Sample Map window down.
- On the main instrument Top Count window select **Total Count** protocol instead of **gada.** Then click on the **Manually Load Plates** icon.

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

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.

5. 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 differently (counts come out higher on the old one)

6.   If the label has a count between these values then it is ready to use:
using a stepper pipette, add 25µl of labelled antigen to each well, re-cover plate with parafilm and centrifuge. 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.

7.   If any adjustments have to made, the label will then need to be recounted following the same procedure as before.

8.   **Record the label making in the 'Counts' book.**

Washing/Adding PAS

- **For each plate** being assayed, take 2.0ml of 50% PAS suspension (for ZnT8 assays) or 2.5ml of 50% PAS suspension (for IA2/GAD assays) and transfer to a 20mL sterilin tube.

- Fill the tube with Denver+0.1% BSA buffer, centrifuge and pour off supernatant.

- Repeat this wash step 3 more times.

- For each plate you need 5mls of washed PAS/Denver buffer, i.e. fill the tube up to the **5**ml line with buffer for **1** plate. One sterilin tube can be used for up to 4 plates (final volume then being 20mls).

  Note: For each new batch of sterilin tubes, measure out 10ml of buffer using a pipette and assess the accuracy of the measurement lines on the tube- sometimes 10ml on the tube is actually closer to 11 or 9.

- Mix well and ensure that PAS stays in suspension during pipetting by frequent mixing. Using a stepper pipette (with the end cut off), pipette 50µl of PAS 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 hr.

Denver Buffer (1 Litre)

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

VERY IMPORTANT: Buffer must be kept ice-cold - at ALL stages of the assay.
Also, Denver buffer with BSA added shouldn't be kept for longer than 48hrs (make up fresh if older).

Washing and transferring the assay

- After plates have been on shaker for 1 hr, 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 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 ~10secs 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 is in suspension before transferring to the optiplate.

- Go back to wells to remove any residue, wiggle the pipette about whilst sucking up. Check the tips for residue before discarding in the ^{35}S 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. Re-shake plate every 6 columns (2 times a 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, if necessary adding a little buffer 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 an hour (label starts unbinding from PAS in excess buffer).**

- Add 200µl Microscint40 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 repeat count protocol plate top of the stack)
    - For the New Top Count also put metal weights on back and front stackers.
  - To count plates on the **New** beta counter:
    - On the main instrument Top Count window select **gada** protocol instead of **Top Count.** Then click on the **Load from Stacker** icon.
    - **Start Assay**- following a ten minute count delay plates will count for 5 minutes per well.
  - 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
    - F2 to load the plate
    - Following a ten minute count delay plates will count for 5 minutes per well.

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 Diabetes & Metabolism\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 Macros and run "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. "1.04.11. New". Counts should be saved in the **Counts2011** 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 **Counts2011.**

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.

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:**IA2A**.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:

1. Insert the floppy disk containing the saved file. Open the notepad file and **Save As** 'New Counts' file in Diabetes & Metabolism\Database\Antibodies\ IA2, GAD, TG and ZnT8 Calculating.   Click 'Yes' when asked if you want to replace the existing file.

2. Open "Old Top Count Macro" in the same folder.

3. Select Macros and run "Anna Old Top Count Macro to run from New counts file". Click 'Yes' when asked if you want to replace the existing file.

4. **Save As** the date the plates were put on the counter followed by "Old" to indicate which counter it was counted on, e.g. "1.04.11. Old". Counts should be saved in the **Counts2011** folder in the **IA2, GAD, TG and ZnT8 Calculating** folder.

5. 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 next to the first set saved in **Counts2011.**

The counts will now be in two columns and are ready to be copied and pasted into the appropriate work sheet. The first counts numbered 1 to 95 will be for the first plate in the stack, 97 to 191 will be for the second plate, and so on.

Calculating Results

These are guidelines not absolute rules.
Threshold for positivity for ZnT8A R&W = 1.8, GADA std = 33, IA2 std = 1.4

1. Take the counts out of the excel file saved in the **Counts2011** 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. ZnT8RBr1.
2. For GAD/IA2:
    - Check that the formula in the "Corr cpm" column is correct, i.e. that it is the DK Std negative which is being subtracted from the mean counts.
    - Check the correct counts have appeared for the standard curve - to the bottom left of the 'A' plate's map. Right click on the graph and **select data,** selecting **Corr cpm** (X-Value) and **Log2 DKU** (Y-Value).
    - Check the graph – the r^2 value should be **above 0.98**, if it is not identify any bad duplicates and delete them from the standard curve (from the columns where you pasted in the counts).
    - Once you are happy with the standard curve, change the plate ID in the title of the graph.
    - Take the equation for the trendline off the graph and put it in the plate map under the **DK Units** heading. Make sure you apply this to all of the standards/samples/QCs on both plates.
    For ZNT8R/W:
    - Check the correct counts have appeared for the standard curve- to the top right of the plate's map or between the pair of plates. Right click on the graph and **select data,** selecting **Cpm** (X-Value) and **Log value** (Y-Value).
    - 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 where the data is sourced from.
    - Once you are happy with the standard curve, change the plate ID in the title of the graph.
    - Take the equation for the trendline off the graph and put it in the plate map/pair of plate maps under the **Log2 units** heading. Make sure you apply this to all of the standards/samples/QCs.
3. Look at the %Error column. Investigate any errors over 30%.

- Errors on standards should have been dealt with 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 (repeat) 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 (reject) in the **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 **F or Z8**) ignore the error.
    ii. If one or both of the duplicates are above the bottom of the standard curve than mark RPT in the **Units** column.

4. Now go through the QCs and check that each is in range, ranges can be found for the QCs on the "tab" marked 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.
5. Mark on the worksheets: Total counts (from the Counts book)
   Assay date (day label was added)
   PAS Lot
   Label Prep (date label was made)

Now someone needs to check that 1-5 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 Units and CODE cells for all the assay results.
2. Paste these in the **Standard (DK) results template.xls/ ZnT8 Results template.xls** in the appropriate 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 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 **DK GAD Results**, **DK IA2 Results**, **ZnT8 R Results** or **ZnT8 W Results**, depending on the assay.
6. Now go into the **DK GAD Plated**/ **DK IA2 Plated**/ **ZnT8 R Plated**/ **ZnT8 W Plated** tables 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.