

# **Insulin Autoantibody Assays**

## Contents

<b>Initial dilution of Insulin Label.....</b>	<b>3</b>
<b>IAA Plating.....</b>	<b>4</b>
<b>cIAA Plating.....</b>	<b>5</b>
<b>Diluting IAA Label.....</b>	<b>6</b>
<b>Diluting cIAA Label.....</b>	<b>7</b>
<b>Washing PAS, Adding PAS.....</b>	<b>8</b>
<b>Washing Plates and Transferring.....</b>	<b>9</b>
<b>Getting Counts off the Gamma Counter.....</b>	<b>10</b>
<b>Calculating Results for IAA assays.....</b>	<b>11</b>
<b>Calculating Results for CIAA assays.....</b>	<b>12</b>
<b>Transferring Results.....</b>	<b>13</b>

### Differences from <sup>35</sup>S assays

1. We order in label ready made.
2. The radioisotope is <sup>125</sup>I, it emits **gamma radiation** and is counted on a gamma counter.
3. We dilute the stock label in **TBT** (Tris) buffer + **1% BSA**; we wash the PAS and plates in **TBT** buffer.
4. We use **5µl** serum in the assay
5. We incubate the serum and label for **72hrs**.
6. We incubate the PAS with the label/serum for **1hr 45 mins**.
7. We transfer into **microtubes** and count on the gamma counter. It takes 2hr30mins per plate.
8. We label on Monday, Tuesday or Friday.
9. We assay on Monday, Thursday or Friday.

### **Initial Dilution of Insulin Label**

- When the <sup>125</sup>I- insulin label (50µCi) first arrives it is put inside a lead pot or in the lead impregnated perspex box and stored in the fridge.
- On the same day it arrives label is dissolved in 2ml TBT buffer.
- Wear a finger monitor and:
  - Loosen the lid a little to release pressure
  - Use a 1ml syringe to inject 1ml of TBT through the rubber septum into the vial
  - Use a second 1ml syringe to inject another 1ml of TBT into the vial**(Inject the buffer SLOWLY and take care due to risk of needlestick injury and do not re-sheath syringe)**
  - Dispose of the syringes in the <sup>125</sup>I radioactive waste bin.
- Leave the vial on the bench for 30 minutes for the insulin to dissolve, take the lid off and then mix by very gently pipetting up and down.
- Aliquot 5 X 400 µl into 2ml sarstedt tubes (red lids) labelled with “<sup>125</sup>I- insulin label”, the aliquoting date and Lot number. Rinse out the vial with an additional 400µl of buffer and add to one of the tubes. Label this as ‘1/2 strength’.
- The aliquots are stored in a lead pot in the -70°C freezer until use.

# IAA Plating

Enter plate map in the appropriate workbook in **IAA** folder, e.g. **IAAbr32** file. This can be found in the **Antibodies** folder.

For IAA the standard curve is split across two plates (5 on one, 4 on the other). Each plate should also include internal and external quality control samples. You should fit 75 samples per pair of plates.

The standard curve goes on first, followed by 4 internal QCs and 2 External QCs (e.g.Q1923), these 6 are distributed randomly through the plate.

IAA Standards (11/10/10) – 9 dilutions of J.W. (10/08/09) in negative pool (16/04/09)

**JWA, JWC, JWE, JWG, JWI** go on one plate.

**JWB, JWD, JWF, JWH** go on the other plate.

IAA QCs  
(19/03/09)

- Negative = **AW** (04/02/09)

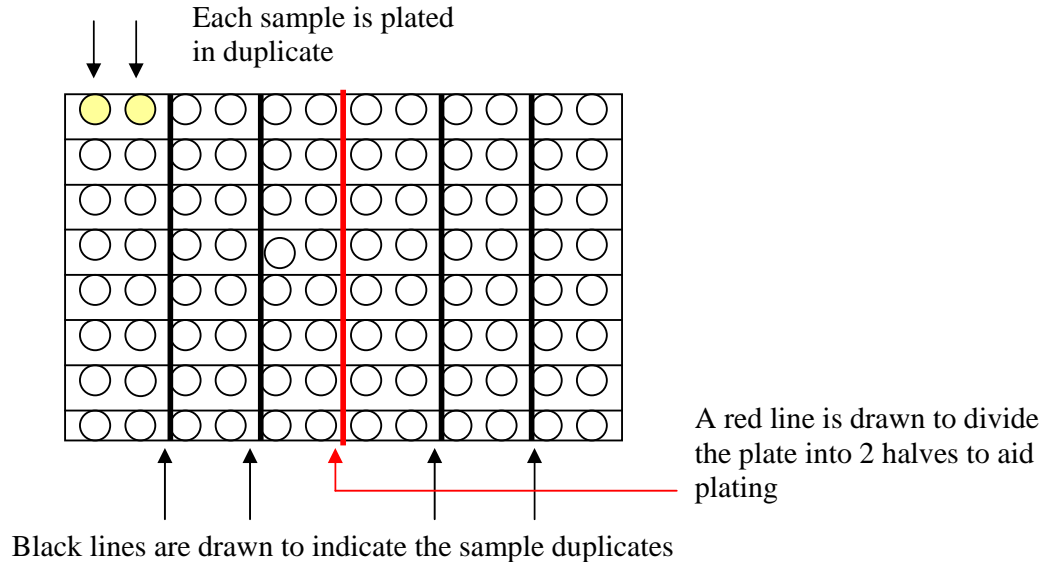
- Low Positive = **Low09**

- Medium Positive = **NM10** (24/11/09)

- High Positive = **High10**

(17/11/09)

Divide plate into halves, then sub-divide into 2 well segments and place in ice tray. Pipette 5µl 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 the workbook copy the CODE column from the plate map and paste into **Plated Template** in **IAA** 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 **IAA plated table** in the **Plating Database** (Access), which can be found in the **Antibodies** folder.



In the workbook copy the CODE column from the plate map and paste into **Plated Template** in **IAA** 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 **cIAA plated table** in the **Plating Database** (Access), which can be found in the **Antibodies** folder.

## Diluting IAA Labels

**Wear laboratory coat, finger monitor and pair of medical examination gloves during all steps. Keep insulin label in lead pots when possible to reduce exposure to radiation. All tips should be discarded into the appropriate radioactive waste sharps bin**

1. Remove the stock  $^{125}\text{I}$ - insulin label from the freezer and leave in a lead pot to defrost.
2. Using a black marker label a 20ml sterilin tube with today's date and "IAA label".
3. For each plate add 2.5mls of TBT+1% BSA buffer and ~ 25 $\mu\text{l}$   $^{125}\text{I}$ -labelled insulin to the sterilin tube. Mix well. If diluting a small amount of label (10ml or less) add an additional 500 $\mu\text{l}$  of buffer to your total volume as you may run short otherwise.

(Ideally label should be prepared fresh each time, but label remnants can be used for up to 4 days after preparation if stored in the fridge. Counts must be adjusted on the day of use).

4. Pipette 25 $\mu\text{l}$  of the diluted insulin label into a microtube (avoid getting any up the sides of the tube- try to get it all at the bottom) and count on gamma counter for ~ 3 mins.
5. Adjust the counts obtained to 14-15,500 by adding more stock label or TBT+1% BSA buffer as appropriate. Record the final count, lot number, number of plates and assay date in the "Counts" book.
6. When the label gives satisfactory counts, use a stepper pipette to add 25 $\mu\text{l}$  to each well, re-cover plate with parafilm and centrifuge. Mix on a shaking platform and leave in the fridge to incubate for ~72hrs.
7. Return remaining stock label to the -70 °C freezer and store diluted label in the radiation fridge.

## Diluting IAA Competition Labels

1. Remove the stock  $^{125}\text{I}$  insulin label from the freezer and leave to defrost.
2. Date label two 20ml sterilin tubes using a black marker. Using a red marker label one tube as IAA 'HOT' label, then using a blue marker label the other tube as IAA 'COLD' label.
3. Into the 'HOT' tube, pour 2.5mls of TBT+1% BSA buffer and add  $\sim 25\mu\text{l}$   $^{125}\text{I}$ -labelled insulin (volumes per plate). Mix well.
4. Transfer  $25\mu\text{l}$  of 'HOT' label to a microtube and count on gamma counter for  $\sim 3$  mins.

**The ideal count for the 'HOT' label at this stage is  $\sim 16,500$  counts ( $\pm 500$  counts is acceptable).**

5. When the 'HOT' label is ready, pour it into the 'COLD' label tube, swirl around (with lid on!), and then pipette half back into the 'HOT' label tube.

**N.B. Take care not to cross contaminate the labels from now on**

6. Using a syringe (avoid any air bubbles) add  $80\mu\text{l}/\text{ml}$  cold insulin (i.e.  $200\mu\text{l}$  for 2.5ml) to the 'COLD' label and mix well.
7. Add an equal volume of TBT+1% BSA buffer (i.e.  $80\mu\text{l}/\text{ml}$ ) to the 'HOT' label and mix well.
8. Transfer  $25\mu\text{l}$  of 'HOT' label to a microtube labelled with 'H' and  $25\mu\text{l}$  of 'COLD' label to a microtube labelled with 'C'. Count on gamma counter for  $\sim 3$  mins.

**Both labels should fall within the range of 14,000 and 15,500 counts, and within 500 counts of each other. Ideally the 'COLD' counts will be slightly higher than the 'HOT'.**

9. When both labels are giving satisfactory counts, use them to label a competition plate/s.  $25\mu\text{l}$  of Hot label is added to the first 4 rows using a  $25\mu\text{l}$  stepper syringe. The same tip can be used to add  $25\mu\text{l}$  cold label to the bottom 4 rows.

\

## Washing/Adding PAS

- **For each plate** being assayed, take 2.0ml of glycine blocked PAS suspension and transfer to a 20ml sterilin tube.
- Fill the tube with TBT buffer, centrifuge and pour off supernatant.
- Repeat this wash step 3 more times.
- For each plate you need 5mls of washed PAS/TBT Buffer, i.e. fill the tube up to the 5ml 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 ~ 1hrs 45 mins.

## TBT Buffer (1 Litre)

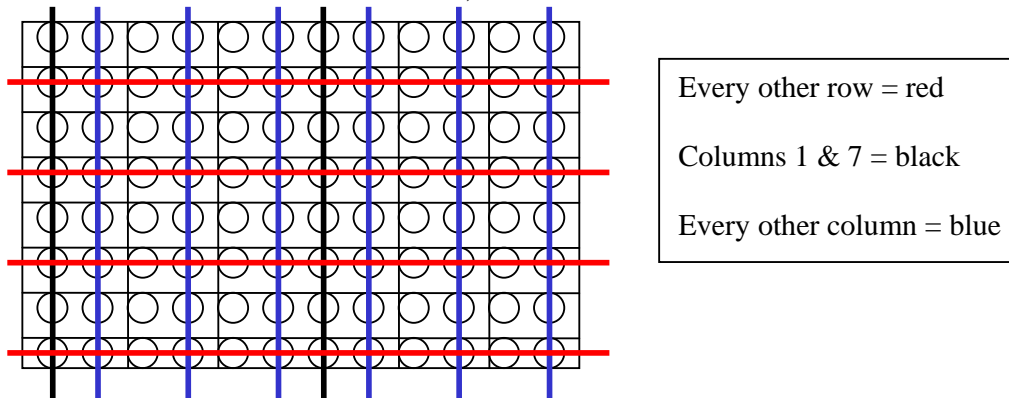
Trizma Base	6.06g
dH <sub>2</sub> O	1 litre
HCl	~4-5ml (This should bring pH to 8.0 - check!)
Tween 20	10ml

**VERY IMPORTANT: Buffer must be ice-cold before being used- at ALL stages of the assay.**



## Washing and transferring the assay

- After plates have been on shaker for 1hr 45 mins, remove plates from fridge and using the multi-dispenser fill the wells with TBT buffer.
- 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.
- Label the micro-tube box with
  - number, i.e. first one transferred is "1"
  - Plate details
  - Label the top left microtube (1A) with the number used above
  - Also mark the microtubes in black, blue and red as shown below



- Add 100µl of TBT buffer to each well using a stepper pipette.
- Mix plate for ~10secs on the shaking platform.
- Using a multi-channel pipette (yellow tips) transfer the samples into micro-tubes. Mix samples with the pipette to ensure the PAS is in suspension before transferring to the micro-tubes.
- Go back to wells to remove any residue, wiggle the pipette about whilst sucking up. Check the tips for residue before discarding in the  $^{125}\text{I}$  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 micro-tubes. 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 micro-tubes in the fridge while you transfer remaining plates, when you are finished, centrifuge the micro-tubes.
- Load micro-tubes on the gamma counter:

- First place rack “1B” (15min count) on old counter / “1 Long” (15 min count) on new counter. Arrange more racks in numerical order. The counters take up to 100 racks (or 10 plates) each.
- Use tweezers to load the tubes into the racks.
- Place the empty “Stop” rack on the end.
- Make sure there is a disk in the machine when using the old counter.
- Press “Start” on the old counter/ press the arrow button on the touch-screen of the new counter.

**Note: There is a shorter 10 minute counting protocol (“TC” on old counter/ “1 Short” on new counter) but usually the 15 minute protocol is used.**


## **Transferring Results from Gamma Counter**

### **Old Counter**

- Take the floppy disk from the gamma counter and open it up on the computer.
- Open the latest file and save as the date the plates went on the counter in the **Counts 2011** folder, in **IAA calculating** folder in **Antibodies**.
- In the same folder also save the file as the **IAA counts** notepad file (it is fine to overwrite the previous data).
- Open the **IAAmacro** (Excel File) in the **IAA calculating** folder
- On the tool bar go to **View > Macros > View Macros> Run**
- For IAA go back to the IAA folder and open the latest worksheet (it should begin Iaabr with the highest number at the end). Copy the data (the two columns of IAA counts in the IAAmacro file), then paste into the appropriate plate map.
- For cIAA take the first half of the plate and paste into the space for hot counts, take the second half of the plate and paste into the space for cold counts.

Note: The macro will only do 3 plates at a time. If you have more than 300 counts in your **IAA counts** notepad file you’ll need to delete the first 300 lines of data from the notepad file, **save**, then re-run the macro on the next 300 set of counts.

### **New Counter**

- Press the ‘’ button on the keyboard to bring up the start bar on the touchscreen
- Locate new counts in **My Documents >My Assays >Results >IAA and cIAA Results**
- Save the most recent file to the USB drive as a ‘.csv’ file
- Open file on networked computer and save the new counts file in the **Counts 2011** folder, in **IAA Calculating** folder in **Antibodies**.
- In the same folder also save as the **Counts** csv file (overwriting previous data).
- **Open the New Counter Macro (Excel File) in the IAA calculating folder**
- **On the tool bar go to View > Macros > View Macros> Run**
- Data can be copied and pasted into worksheets as above.

Note: The macro will only do 4 plates at a time. If you have more than 400 counts in your **Counts** csv file you’ll need to delete the first 400 lines of data from the csv file and re-run the macro on the next 400 set of counts.

## Additional information

After the Gamma counter has finished counting the micro-tubes should be loaded back into their corresponding boxes. The radioactive waste left in the micro-tubes can then be aspirated using the vacuum pump and discarded down the radiation sink. The empty micro-tubes can then be discarded into the I<sup>125</sup> solid waste bin, and the boxes checked to make sure they are not contaminated before discarding into the clinical waste bin.

## Calculating Results for IAA Assays

These are guidelines not absolute rules. Threshold for competing IAAs is 0.4 Units.

1. Check the correct counts have appeared for the standard curve- to the top left of the plate map, under the plate ID. Right click on the graph and **select data**, selecting the counts (X-Value) and the Log2 units (Y-Value).
2. Check the graph – the  $r^2$  value should be above 0.99, if it is not identify any bad duplicates and delete them from the standard curve where the data is sourced from.
3. Once you are happy with the standard curve, change the plate ID in the title of the graph.
4. Take the equation for the trendline off the graph and put it in the plate map under the **Log2 Units** heading. Make sure you apply this to all of the standards/samples/QCs on both plates.
5. 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 **IAA 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 **IAA Units** column and RJT any samples in that range.
  - If the error is on a Sample/ExQC look at the units column.
    - i. If both of the duplicates are below the competition limit (0.4) ignore the error.
    - ii. If the duplicates are either side of the 0.4 boundary then mark RPT in the **IAA Units** column. However we often ignore the error if the average cpm means the IAA units are over 0.4 as the result will be competed anyway.
    - iii. If both the duplicates are above the 0.4 boundary ignore the error (unless it’s very high).
6. 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.
7. Mark on the worksheets:
  - Total counts (from the Counts book)
  - Assay date (day label was added)
  - PAS lot
  - Date PAS was blocked
  - Label lot

Now someone needs to check that 1-7 has all been done correctly. Then transfer the results into the database.

## Calculating Results for cIAA Assays.

These are guidelines not absolute rules. Threshold for positivity for cIAAs is 0.2 units but we confirm anything over 0.6 for the purposes of TEDDY.

1. Check the correct counts have appeared for the standard curve- to the right of the plate map. The standard curve is made up of standards from two plates. Right click on the graph and **select data**, selecting the counts (X-Value) and the Log2 units (Y-Value).
2. Check the graph – the  $r^2$  value should be above 0.99, if it is not identify any bad duplicates and delete them from the standard curve where the data is sourced from.
3. Once you are happy with the standard curve, change the plate ID in the title of the graph.
4. Take the equation for the trendline off the graph and put it in the plate map under the **Log2 Units** heading. Make sure you apply this to all of the standards/samples/QCs on both plates.
5. Look at the %Error columns. Investigate any errors over 30%.
  - Remember you have both Hot and Cold counts to play with! The highest Hot and lowest Cold cpms will give you the highest units. The lowest Hot and highest Cold cpms will give you the lowest units. If #NUM! appears in any cells it just means the Cold has cpms higher than the Hot.
  - 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”
    - i. If it’s “in” ignore the duplicate.
    - ii. If it’s “out” but deleting one of the bad duplicates brings it into range then mark as a RPT (repeat) in the **IAA Units** column.
    - iii. If it’s “out” and deleting one of the duplicates doesn’t bring it into range then mark as a RJT (reject) in the **IAA Units** column and RJT any samples in that range.
  - If the error is on a Sample/ExQC look at the units column.
    - iv. If both of the duplicates are below 0.3 ignore the error.
    - v. If the duplicates are either side of the 0.3 boundary then mark RPT in the **IAA Units** column.
    - vi. If both the duplicates are above the 0.3 boundary ignore the error (unless very high).
6. 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.
7. Mark on the worksheets:
  - Hot and Cold counts (from the Counts book)
  - Assay date (day label was added)
  - PAS lot
  - Date PAS was blocked
  - Label lot

Now someone needs to check that 1-7 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 IAA Units and CODE cells for all the assay results.
- 2 Paste these in the **IAA/CIAA results template.xls** in the **IAA** 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 **IAA Results** or **CIAA Results**, depending on the assay.
- 6 Now go into the **IAA Plated/CIAA 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.