The Newcastle upon Tyne Hospitals NHS Trust
Department of Clinical Biochemistry
Freeman Hospital, Newcastle General Hospital
and
Royal Victoria Infirmary

Standard Operating Procedure for

HbA1c USING THE TOSOH A1c2.2

MEASUREMENT OF HbA1c USING THE TOSOH A1c2.2

PERSONNEL

All appropriately trained staff and trainees under supervision.

PRINCIPLE

Diabetes Mellitus is defined as the absolute or relative deficiency of insulin that may progress to hyperglycaemia, and is often associated with specific micro and macrovascular complications. Therapy for diabetes requires the long-term maintenance of a blood glucose level as close as possible to a normal level, minimising the risk of long-term vascular consequences. An accurate index of the mean blood glucose concentration over a period of approx. 60 days may be established by the measurement of haemoglobin A_{1c} (HbA_{1c}) every two to three months.

HbA_{1c}, the glycoprotein of interest, is formed in two steps by the non enzymatic glycation of HbA. The first step is the formation of an unstable aldimine (Schiff base, labile or pre-A_{1c}), a reversible reaction between glucose and the □-chain of haemoglobin. Schiff base formation is directly proportional to the blood glucose concentration.

During red blood cell circulation, some of the Schiff base is converted to form a stable ketoamine, HbA_{1c}.

The level of HbA_{1c} is proportional to both the average glucose concentration and the life span of the haemoglobin in the circulation. The measurement of HbA_{1c} has, therefore been accepted for the clinical management of diabetes.

The Tosoh Haemoglobin A_{1c} method utilises the principles of ion exchange high performance liquid chromatography (HPLC) for the automatic separation of Haemoglobin A_{1c} (HbA_{1c}). Whole blood is sampled directly from the primary tube, diluted in haemolysing buffer and pumped via a pre-filter to the ion-exchange column. A series of three buffers are used to elute the haemoglobin from the column and the peak measurement achieved by dual wavelength detection. Quantitation is by use of two point calibrators. Each blood sample takes 3 minutes to elute and there is no pre-treatment of the sample to remove the labile A1c as this is eluted as a separate peak. Results are printed out with sample identity (where barcodes are in use), peak identities, retention times, peak areas, %Hb and with a full chromatogram.

SAMPLE

No specific preparation of the patient is required. There are no special precautions with respect to the patient except that if patients are known to have a haemoglobinopathy this should be indicated on the request form.

Blood should be collected into EDTA blood collection tubes. However, while lithium heparin and fluoride oxalate samples are not the samples of choice, they may be suitable if fresh.

Samples are stable for at least 1 week when stored at 4°C (although the labile HbA1c peak will decrease during this time). Storage, if required, is at 4°C prior to analysis and at 4°C for 1 week after analysis is complete.

Interferences - There is no evidence that lipaemia and icterus significantly interfere with the method. There are no reports of any drugs interfering with the method. Samples from patients with haemoglobinopathies will give abnormal patterns, see below for instructions on reporting. Uraemia is not known to interfere with this method.
Minimum sample volume required is 5□l, but 1ml in EDTA is preferred.

IMPRECISION

Within batch
Derived using patient samples, n = 15

Sample	1	2	3	4
Mean HbA1c%	5.42	6.58	10.67	12.85
SD	0.04	0.04	0.05	0.07
CV%	0.76	0.63	0.42	0.58

Data from Tosoh A1c2.2 evaluation, 1997

Between batch
Derived using quality control material on 9 days over a 15 day period

Sample	1	2
Mean HbA1c%	5.14	9.22
SD	0.10	0.18
CV%	1.99	1.99
n	21	21

Data from Tosoh A1c2.2 evaluation, 1997

HEALTH AND SAFETY

Refer to the Departmental Safety manual, Index code: **SAFETY1.DOC.**

There are no COSHH implications for this analysis.

RISK ASSESSMENT

Provided that the departmental safety procedures described in the laboratory manual **SAFETY1.DOC** are observed and protocols, manufacturers' instructions and good laboratory practice are adhered to throughout this procedure, the following assessments apply:

Stage	Risk assessment
Preparation	Low risk
Electrical	Low risk
Mechanical	Moderate risk; moving parts may be exposed during unscheduled maintenance
Physical	Low risk
Chemical	Low risk
Sample	High risk; danger of infection. Treat all samples as potentially high risk.
Disposal	High risk. Used samples must be treated as biological waste and disposed of accordingly. Liquid reagent waste to be washed away in sink with copious volumes of water.

PREPARATION OF STANDARDS

Calibrators are supplied by Tosoh Eurogenetics, stored in the clinic fridge and should be reconstituted by the addition of 4mls of deionised water. Mix gently and allow to stand until completely dissolved before use. Calibrator values vary with lot number and **must** be assigned DCCT values before use, see below.

Before reconstitution calibrators are stable at 4^{o}C until the expiry date on the bottle, and for 7 days when reconstituted and stored at 4^{o}C.

Calibration of this system for routine use is by comparison of blood samples with the HbA1c method used at the RVI. Tosoh calibrators are run intermittently as an additional check. Refer any possible need to recalibrate to Angela Parnham.

PREPARATION OF REAGENTS

Reagents required are supplied as follows:

1. **Buffer 1** - use as supplied. Stored under bench. Stable until expiry date on bottle.
2. **Buffer 2** - use as supplied. Stored under bench. Stable until expiry date on bottle.
3. **Buffer 3** - use as supplied. Stored under bench. Stable until expiry date on bottle. DO NOT POOL

4. **Haemolysis & Wash Solution** - use as supplied. Stored under bench. Stable until expiry date on bottle.
5. **Analytical column** - use as supplied. Store at 4^{o}C prior to use. Each column should be usable for at least 1500 injections.
6. **Calibrator** - supplied lyophilised, reconstituted with deionised water (see above). Before reconstitution stable until expiry date on bottle, after reconstitution stable for 7 days. Use undiluted.

N.B. Buffers 1 & 2 and the haemolysis/wash solution can be pooled within a lot number. When installing a new pouch, ensure surplus air is expelled from the pouch by squeezing gently whilst tightening the screw cap. Similarly, pouches taken from the instrument should have the air expelled before capping. Pouches containing pooled buffer should be labelled as such.
Buffer 3 **must not** be pooled.

QUALITY CONTROL

Internal QC and External QA must be performed as defined for this chemistry in QC1.DOC.

Refer to manufacturer's instructions of quality control material for information on any storage, reconstitution, stability etc.

PREPARATION OF SAMPLE

1. Mix patient samples by gentle inversion 2 or 3 times and ensure that it is not clotted.
2. Samples with at least 7mm depth of blood in the primary tube require no preparation other than attaching the bar code to the tube and removal of the cap.
3. Small blood samples, paediatric capillary samples and QC should be pre-diluted 5µl blood in 1 ml of haemolysis & wash solution (i.e. 1:200 dilution) in a 1.5ml conical micro tube without cap (Sarstedt Cat no 72.696). Use a variable 1 to 25µl Microman pipette with yellow tips for the blood and a 1ml Gilson Pipetman for the haemolysis & wash reagent. Mix well. The micro tube should then be placed in a plastic carrier tube complete with metal collar (as supplied by Tosoh Eurogenetics), ensuring the collar is clean and seated properly on the carrier. Diluted samples are stable for 7 hrs at room temp (although some decrease in the labile peak area may be seen over time).
4. Whole blood samples can be left on the sample table for at least 5 hours without needing to be mixed again before sampling.

EQUIPMENT USED

A1c 2.2 Glycohemoglobin Analyser HLC-723GHb.

Start Up Procedure

1. Check the reagent pouches contain sufficient reagent for the expected number of injections to be run. If not, change the buffer pouch, taking care to exclude as much air as possible from the new pouch before tightening the screw cap.
2. Check from the instrument log and by examination of recent chromatograms that the pre filter and column have sufficient injections left for the expected run length. The TP (theoretical plates) number printed on each chromatogram should be greater than 250 for any patient blood sample. If lower than this, check chromatograms carefully and consider changing column. If necessary follow instructions in the manual to replace column or pre-filter as appropriate.
3. Check the waste container and empty if necessary.
4. Check there is sufficient paper.
5. Complete the instrument maintenance log sheet.
6. The instrument is pre-programmed to start up automatically at 8am every morning when it performs the warm-up. If it is to be used at another time (for example In the Diabetes Clinic for a Thursday afternoon) follow point 7 below.
7. If used outside the automatic start-up times press POWER. The system performs a 22 minute warm up program, which includes priming through buffers, after which the system enters STANDBY status. The analyser will remain in standby for 2 hours before automatically shutting down.
8. In the case where an automatic start-up is to be used, any buffer, filter and column replacements should be completed at the end of the previous working day. (In the clinic the instrument is set to switch on at 8am. If no morning clinic is run, the analyser will switch off again after 2 hours and will need to be switched on manually at least 25 minutes before the afternoon clinic is due to start.)

Shut Down Procedure

After the run is complete the Tosoh returns automatically to wash mode, and after 15 minutes to STANDBY mode. After a pre-set period (currently 2 hours) the analyser will shut down completely. It must not be turned off at the mains as this would necessitate rebooting and loading of programme data from a floppy disk (see below).

1. Dispose of haemolysates as for clinical waste.
2. Remove and cap sample tubes.
3. Empty the waste container at appropriate times and as for clinical waste.
4. Clean the path taken by the sample needle.

Following Mains Shutdown

If the power has been off, when the analyser is first turned on, after a few seconds it will display a blank blue screen. Insert the backup program disk, which should automatically load. Follow any further instructions displayed until the normal status screen is displayed. Check the calibration status. If necessary, manually enter the current calibration factors. (Refer to senior staff for guidance and method). Remove and store safely the backup disk.

CALIBRATION

Calibration is performed only on change of reagent lot, new column or as indicated by QC values. **No calibration should be performed without first discussing with Angela Parnham or Ian Gibb**.

To prompt calibration using Tosoh calibrators ensure the analyser is in STAND-BY,

1. Press [SET] on the status screen
2. Press [DAILY CALIB] on the SET screen
3. Press [↵] on the set screen, when the Daily Calib button will darken
4. Press diagonal up arrow in bottom right hand corner of screen to return to STATUS screen
5. Check status screen says " CALIB. : YES", indicating that calibration will occur.

Once calibration is requested as above place the undiluted calibrator samples in micro tubes in carrier tubes with metal collars (as for small samples) in positions 1 & 2 of the first rack. Calibrator 1 requires at least 800µl and calibrator 2 at least 600µl in the tubes as multiple aliquots will be taken from each cup.

QC and patient samples can be loaded into the rack immediately after the calibrators.

Calibration performs analysis on three samplings from position 1 and analysis of two samplings from position 2. The calibration equation will be printed out and, if calibration is successful, analysis of unknowns will proceed.

Once an acceptable calibration has been achieved, calibration should be turned off by following points 1 to 5 above, this time ending with the Daily Calib button light and the STATUS screen indicating calibration complete. If calibration is not turned off, the instrument will be set to do a calibration the next time it is turned on.

PROCEDURE

1. Load QC and blood samples as required. Pre-diluted and primary samples can be mixed within a single rack and spaces can be left in a rack, the rack will feed through until the next sample is located.
2. The racks are loaded onto the analyser with the fully open side facing the rear and barcodes aligned with the rear aperture. A maximum of five racks can be loaded at any one time.
3. Press the START key. The analyser will enter a 2 * 2.2 minute mini-prime cycle and will then proceed to pick up the first sample.
4. If the run length is known, place the run stop clip on the end of the last rack. If the run length is undetermined (e.g. a clinic run), do not use the run stop clip, but ensure that there is always a completely empty rack behind any waiting for analysis. This allows samples to be easily added without the risk of dislodging the rack in the sampling position, and acts as a stop signal if it feeds through empty.

Ensure that samples are removed from the sample table as they are completed to avoid their analysis being repeated.

5. Note that the racks are not positively identified. Results are identified with a sample ID which is either the sample barcode when available, or an index number which indicates the rack sequence number and the position within the rack (e.g. 03 - 08 is the third rack from the most recent “Start”, sample position 8). There is also a number that is the run and sequence number (e.g. No. 075 00048 which is run 75, sequence no 48) and is not reset by returning to wash or standby.
6. Once the last available sample has been analysed the analyser will enter WASH for 15 minutes, and then STANDBY. Analysis can be restarted from WASH by pressing “START”. (This will reset the rack index back to 1.) The wash cycle can be interrupted at any time by pressing the STOP key once, causing the analyser to return to STANDBY immediately. Pressing the START key will always initiate a new mini prime sequence. There is no requirement to recalibrate the analyser from standby.

CALCULATION AND VERIFICATION OF DATA

Refer to the QC document, Index Code: QC1.DOC, for the current QC ranges and Westgard rules to determine the acceptability of the quality control results. If in doubt consult the QC officer.

Examine chromatograms for correct separation and visualisation of abnormal Hb fractions. **All** traces showing deviation from the usual pattern **must** be referred to a senior member of staff for the section and the result withheld. Repeat analysis should be performed if the chromatogram shows improper separation. Note that any indication of the identity of an abnormal Hb variant on the chromatogram is not reliable and should not be conveyed to the physician.

Occasionally, a Hb variant may “stick” to the column and then elute off with one or more subsequent samples, not necessarily those immediately following the variant. After a variant peak has been seen, be extra vigilant on following traces and if there is any indication of carryover, allow the analyser to go into a full 15 minute WASH cycle. Once the wash is complete, reanalyse all samples which followed the variant sample.

Samples with HbF < 5% are reported without comment or recalculation. Samples with HbF between 5 and 10% are recalculated to adjust for the HbF (see below). Samples with HbF >10% should have the result withheld and the chromatogram referred to a senior member of staff for the section.

To correct for HbF >5 to <10%

$$\left[\left(\frac{\text{SA1c}\cdot\text{Area}}{(\text{Total}\cdot\text{Area}-\text{F}\cdot\text{Area})}\text{X100}\right)\text{X}\cdot\text{Factor}\cdot\text{A}\right]\pm\text{Factor}\cdot\text{B}$$

where factors A and B are the slope and intercept used in the calibration equation.

Any sample giving a result less than 3.5% or greater than 18% should be repeated immediately and, if the result is confirmed, referred to a senior member of staff for the section. The result should be withheld (see below for comment wording).

The total area for any chromatogram should fall between 800and 4500, any sample falling outside this range should be repeated, if necessary making a suitable manual dilution to give an appropriate total area. (The HbA1c result is calculated as a ratio to total haemoglobin, so neither the dilution ratio nor dilution accuracy is critical for valid quantitation, no adjustment needs to be made to the result for non-standard dilutions).

If in any doubt refer to the Senior MLSO / Duty Biochemist.

REPORTING OF RESULTS

The value to be reported is identified on the printout as SA1C.

When in use in the clinic, results are recorded on the worksheet, along with the lab number, patient name, and timing information. The result is reported to the requesting doctor as soon as it is available (and the trace has been scrutinised) on the clinic result form for the patient. Results are also entered into the laboratory computer system.

Results are reported to one decimal place and the units are %.

If there is any abnormality about the trace (confirmed by repeat analysis), or the results falls outside the extremes (3.5 to 18%) the result must be withheld and the form returned with the comment "**Abnormal trace – result withheld for further investigation**". Sample and trace should be retained and referred at the earliest opportunity.

Once a patient has been identified as having a haemoglobinopathy (or other abnormality), a card should be started with patient identification details and notes about how the sample has been reported. A copy of the trace should be stapled to the back of the card for future reference. Further details and copies of traces should be added as the patient re-attends. This card should be sent to the senior member of staff who is to report the abnormality for comparative purposes, and then returned to the clinic.

REFERENCE RANGES

Non - diabetic subjects	Less than 6.1%
Inadequate control of diabetics	Greater than 7.5%

The above ranges are for guidance only, all patients should have an individual target balancing risk of long term complications with quality of life and risk of hypoglycaemic attacks.

CLINICAL SIGNIFICANCE

It has been demonstrated that the level of HbA1c is related to the risk of developing long term complications in diabetes, for example retinopathy, nephropathy and neuropathy (the DCCT trial). There is no actual cut-off point below which the risk becomes zero. In attempting to improve diabetic control to minimise these risks, the day to day quality of life for the patient must be considered, and in particular the problems and dangers of hypoglycaemic attacks must not be increased to unacceptable levels. For this reason each patient should be assessed in the light of his or her personal circumstances.

Patients who have haemolytic anaemia may exhibit decreased glycated haemoglobin values due to the shortened life span of the red cells. This effect depends upon the severity of the anaemia. Samples from patients with polycythaemia or post splenectomy may exhibit increased glycated haemoglobin values due to the extended life span of the red cells.

Haemoglobin variants may lead to quantitation of the HbA1c being impossible. Even where quantitation is feasible, the interpretation guidelines may be inappropriate and it may only be possible to monitor trends in glycation, not assess long term risk. In rare cases the individual may have no HbA and, therefore, no HbA1c.

REFERENCES

Tosoh A1c2.2 Analyser Instruction manual.
Tosoh A1c2.2 Evaluation Document.
Diabetes Control and Complications Trial Research Group. The effect of intensive treatment of diabetes on the development and progression of long term complications in insulin-dependent diabetes mellitus. New Eng J Med 1993; 329: 977 - 86

Assignment of DCCT values to TOSOH Calibrators

The calibrators supplied by Tosoh are assigned values, but these values may not be aligned with DCCT results as produced in the trial and currently in use in USA. Laboratories in the Northern Region (in conjunction with local diabetologists) have a policy of standardising all results to DCCT, regardless of analyser. This is achieved by calibration to the RVI Tosoh, which is in turn calibrated to the DCCT Central Laboratory in Minnesota. In order to maintain this link for results from the Tosoh, it is **essential** that changes in lot number of calibrators be accompanied by confirmation of DCCT values.

As soon as a new lot number of calibrators is received, before it is required for routine use, a senior member of staff should perform the following protocol.

1. Select from the most recent RVI Tosoh run 10 fresh blood samples with values evenly covering a wide range of values (at least one below 6% and at least one greater than 12%). Check that none of the selected samples is from a patient with an elevated urea (if necessary, get the urea measured on EDTA plasma on the Olympus to confirm).
2. Input the clinic Tosoh assigned values for the new calibrators, perform a calibration with the new calibrators and run the selected samples.
3. Calculate the regression between the DCCT Tosoh results and the clinic Tosoh results, using X for the RVI Tosoh and Y for the clinic Tosoh.

   Clinic Tosoh = (M * DCCT RVI Tosoh) + C

4. Assign DCCT values to the calibrators by calculation;

$$\text{DCCT} \cdot \text{Tosoh} = \frac{(\text{Quoted} \cdot \text{Tosoh} - \text{C})}{\text{M}}$$

5. Input the calculated DCCT Tosoh values for the standards. Recalibrate using the new calibrators and the calculated values. Run a further 10 well spread samples from the RVI Tosoh and QC samples again.
6. Confirm that the measured values for the blood samples are now within ± 0.2% for values below 8% and ± 0.3% for values greater than 8% of those from the RVI Tosoh. The QC values should be within the target ranges.
7. Clearly document all data and calculations for standard assignment and file one copy with the instrument logs. Place another copy with the new batch of calibrators.
8. **Remember to re-enter the DCCT calibrator values for the current batch of calibrators and re-enter the current calibration factors into the analyser ready for the next routine batch of analyses.**

DECONTAMINATION PROTOCOL FOR TOSOH A1c 2.2 HPLC

This procedure should only be carried out when instructed so to do by Eurogenetics or after consultation with Angela Parnham or Gordon Pulman.

1. Remove the tubing from buffers 1, 2 and 3 and place in a solution of 70% ethanol.
2. Go to the maintenance screen (press menu then mainte).
3. Unscrew the brown connector on the right hand side of the column and position a bottle underneath to catch anything that emerges.
4. Open valve three only by pressing the SV3 key. Press pump and allow to pump for 20 minutes. Close valve three by pressing the SV3 key again. Open valve two. Pump for 20 minutes, then close valve two, open valve one and pump for 20 minutes.

5. Repeat step 4 three times.
6. Switch off the pump by pressing pump key.
7. Replace the pre filter with a new one.
8. Remove the column and replace it so that the flow is inverted.
9. Open valve 1 and press the pump key. Allow to pump for 20 minutes with the column reversed. The pressure will increase and may rise above 200 giving a high-pressure error message. Monitor the pressure and press the E reset key several times as the pressure reaches 200. If the pressure rises to 300 switch off the pump and allow the pressure to drop. Continue by pressing the pump key again.
10. Replace leads into buffer solutions.
11. Remove the column and replace it so that the flow is in the correct direction (flow from right to left).
12. Switch the instrument off and then on again using the power key on the keypad (not the mains power switch.).
13. Allow the instrument to carry out its warm up procedure.
14. Repeat steps 12 and 13 so that the instrument has carried out two full warm up cycles.
15. Check quality control before resuming sampling.