Proportion of patients who retain insulin positive islets at >5y duration of disease subdivided according to their age at diagnosis in years.
Absolute values for circulating proinsulin in subjects diagnosed with type 1 diabetes at increasing ages. Median values are indicated with black lines.
### Supplementary Table 1: Donor information for T1D cases where immunophenotype was characterised.

| Case ID | Biobank | Age at Diagnosis (y) | Duration of disease (y) | Gender | Cause of death |
|---------|---------|----------------------|-------------------------|--------|----------------|
| E308    | EADB    | 3                    | 0.02                    | F      | DKA            |
| E207    | EADB    | 3                    | 0.06                    | F      | N/A            |
| SC41    | EADB    | 4                    | 0.06                    | F      | DKA            |
| E428    | EADB    | 5                    | 0.02                    | M      | DKA            |
| E235    | EADB    | 6                    | 0.27                    | M      | DKA            |
| E181    | EADB    | 7                    | 0.01                    | F      | DKA            |
| E265    | EADB    | 7                    | 0.14                    | F      | DKA            |
| E405    | EADB    | 8                    | 0.01                    | F      | DKA            |
| E581    | EADB    | 10                   | 0.02                    | F      | DKA            |
| E375    | EADB    | 11                   | 0.01                    | F      | DKA            |
| E567    | EADB    | 11                   | 0                      | F      | DKA            |
| E336    | EADB    | 12                   | 0.16                    | F      | DKA            |
| E261    | EADB    | 18                   | 0.06                    | F      | DKA            |
| SC57    | EADB    | 18                   | 0.02                    | F      | DKA            |
| E168    | EADB    | 18                   | 0.16                    | M      | Hypoglycaemia  |
| E556    | EADB    | 18                   | 0.33                    | M      | Hypoglycaemia  |
| E385    | EADB    | 19                   | 1.5                     | F      | DKA and pneumonia |
| SC76    | EADB    | 20                   | 0.06                    | M      | DKA            |
| E560    | EADB    | 42                   | 1.5                     | F      | Glioma         |

N/A – not available; DKA – Diabetic ketoacidosis

### Supplementary Table 2: Donor information for Control cases used for the recent onset (<2y) proinsulin study

| Case ID | Biobank | Age at Diagnosis (y) | Gender | Cause of death                      |
|---------|---------|----------------------|--------|------------------------------------|
| 240/90  | EADB    | 2                    | M      | Haemophilus influenza meningitis    |
| 242/89  | EADB    | 3                    | M      | Meningococcal septicaemia          |
| 184/90  | EADB    | 5                    | M      | Unknown                            |
| 245/90  | EADB    | 6                    | M      | Pneumonia                          |
| 315/89  | EADB    | 9                    | M      | Intussusception                    |
| 333/66  | EADB    | 16                   | M      | Thymomae haemorrhage               |
| 191/67  | EADB    | 25                   | M      | Post-op death (colectomy)          |
| 65/71   | EADB    | 40                   | M      | Stroke (CVA)                       |
### Supplementary Table 3: Donor information for T1D cases used for the long duration (>5y) proinsulin study

| Case ID | Biobank | Age at diagnosis (y) | Duration of disease (y) | Gender | Cause of death |
|---------|---------|----------------------|-------------------------|--------|----------------|
| 6049    | nPOD    | 5                    | 10.0                    | F      | Anoxia         |
| 10382   | EADB    | 5                    | 6.0                     | M      | DKA            |
| 6302    | nPOD    | 6                    | 32.5                    | M      | Anoxia         |
| E558    | EADB    | 13                   | 12.0                    | F      | N/A            |
| 6306    | nPOD    | 14                   | 5.0                     | M      | Head trauma    |
| 6195    | nPOD    | 14.2                 | 5.0                     | M      | Head trauma    |
| 6070    | nPOD    | 15                   | 7.0                     | F      | Anoxia         |
| 6081    | nPOD    | 16.4                 | 15.0                    | M      | Cerebrovascular/stroke |
| SC95    | EADB    | 17                   | 5.0                     | F      | DKA            |
| 6038    | nPOD    | 17.2                 | 20.0                    | F      | Anoxia         |
| 6328    | nPOD    | 19                   | 20.0                    | M      | Anoxia         |
| 6040    | nPOD    | 30                   | 20.0                    | F      | Cerebrovascular/stroke |
| 6307    | nPOD    | 35                   | 10.0                    | F      | Anoxia         |

### Supplementary Table 4. Available clinical characteristics of patients in the EADB recent onset cohort (<2y since diagnosis) analysed for proinsulin:insulin localisation. Data presented as n (%) or median (inter-quartile range)

| Disease duration <2y (grouped by age at diagnosis) | <7y (n=5) | 7-12y (n=7) | >13y (n=6) |
|---------------------------------------------------|-----------|-------------|------------|
| Male                                              | 2 (40%)   | 0           | 3 (50%)    |
| Age at diagnosis (y)                              | 4 (3, 5)  | 10 (7.5, 11)| 18 (18, 19.5)|
| Duration of diabetes (y)                          | 0.06 (0.06, 0.08) | 0.02 (0.01, 0.06) | 0.06 (0.02, 0.17) |
**Supplementary Table 5.** Available clinical characteristics of patients in the EADB longer duration cohort (>5y since diagnosis). Data presented as n (%) or median (inter-quartile range).

| Disease duration longer >5y (grouped by age at diagnosis) | <7y (n=3) | >13y (n=10) |
|------------------------------------------------------------|-----------|-------------|
| Male                                                       | 3 (100%)  | 4 (44%)     |
| Age at diagnosis (y)                                       | 5 (5, 5.5)| 16.5 (14.25, 18.5) |
| Age at study (y)                                           | 15.0 (13, 26.75)| 28 (22, 38.5) |
| Duration of diabetes (y)                                  | 10 (8, 21.25)| 11 (5.5, 18.75) |

**Supplementary Table 6.** Antibody details.

| Antibody   | Company | Cat No | Clone | Species         | [Stock]   | Dilution          | Incubation time |
|------------|---------|--------|-------|-----------------|-----------|-------------------|-----------------|
| Insulin    | Dako    | A0564  | Polyclonal | Guinea-Pig | 14.4mg/ml | 1/343 (HRP and IF) | 1h (RT)        |
| Proinsulin | Abcam   | ab8301 | 3A1   | Mouse          | 2mg/ml    | 1/500 (IF)       | 2h (RT)        |
| CD20cy     | Dako    | M0755  | L26   | Mouse          | 0.2mg/ml  | 1/1500 (HRP); 1/800 (TSA IF) | 1h (RT) ; Overnight 4C |
| CD4        | Abcam   | ab846  | BC/1F6| Mouse          | 25mg/ml   | 1/25 (HRP)       | Overnight 4C |
| CD4        | Abcam   | ab133616 | EPR6855 | Rabbit | 0.054mg/ml | 1/100 (TSA IF) | 2 (RT)         |
| CD8        | Abcam   | ab4055 | Polyclonal | Rabbit | 0.2mg/ml | 1/400 (HRP) | Overnight 4C |
| CD8        | Dako    | M7103  | (C8/144B) | Mouse | 0.157mg/ml | 1/70 (2h or ON TSA IF) | 2h or TSA |

**Supplementary Table 7.** Clinical characteristics of patients in the TIGI cohort. Data presented as n (%) or median (inter-quartile range).

| Age at diagnosis subgroup | <7y (n=87) | >13y (n=84) |
|---------------------------|-----------|-------------|
| Male                      | 54 (62%)  | 37 (44%)    |
| Age at diagnosis (y)      | 3.1 (2.1, 4.9)| 18.5 (14.8, 22.0) |
| Age at study (y)          | 16.0 (12.2, 22.3)| 35.5 (28.8, 46.2) |
| Duration of diabetes (y)  | 12.4 (8.8, 18.6)| 15.8 (9.6, 27.6) |
| BMI age specific z score  | 1.1 (0.02, 1.6) | 0.75 (0.23, 1.41) |
| HbA1c (mmol/mol)          | 67 (58, 76) | 67 (58, 78) |
| HbA1c (%)                 | 8.3 (7.5, 9.1) | 8.3 (7.5, 9.3) |
| Insulin Dose (u/kg/day)   | 0.8 (0.7, 1.0) | 0.7 (0.5, 0.9) |
| C-peptide pmol/l          | <3 (<3, <3) | 34.5 (<3, 151) |
| Detectable C-peptide n (%)| 13 (15%)   | 62 (74%)    |
| Proinsulin pmol/l         | 0.45 (<0.3, 0.8) | 0.5 (<0.3, 1.6) |
| Detectable proinsulin n (%)| 63 (72%)  | 56 (67%)    |
Proinsulin Assay Workup and Validation

AIM

To assess the performance characteristics of the TECO Medical Intact Proinsulin ELISA kit in use on the Dynex DS2 analyser, by use of various precision studies and recovery experiments.

VALIDATION PLAN

The manufacturer provided a thorough assessment of the assay performance so the plan aimed to replicate these tests, as far as practicable. Alongside these tests some further performance characteristics were determined, such as the Limit of Quantification, the effect of repeated Freeze/Thaw cycles and possible interference from Haemolysis. The studies were performed by analysis of a combination of plasma, serum, supplied calibration material and International Standard reference material. The results are given below. It is recommended that each ELISA plate is read at 2 wavelengths, 405nm and 450nm, the latter showing greater sensitivity at the lower range, so each test has been performed and reported from readings in both wavelengths for comparison.

Quality Control

Quality Control material provided with the supplied kit was analysed in each assay run to assess the validity of the assay run. The concentrations of proinsulin obtained were compared with those of the supplied data.

| CONTROL 1 | Plate 2 | Plate 3 | Target Mean | Acceptable Range |
|-----------|---------|---------|-------------|------------------|
| 405nm     | 450nm   | 405nm   | 450nm       |                  |
| 11.635    | 11.474  | 11.221  | 11.208      | 10.6             |
| 11.976    | 11.830  | 11.417  | 11.419      | 6.9-14.3         |

Table 1. Proinsulin concentration obtained of Control 1 (pmol/L).

| CONTROL 2 | Plate 2 | Plate 3 | Target Mean | Acceptable Range |
|-----------|---------|---------|-------------|------------------|
| 405nm     | 450nm   | 405nm   | 450nm       |                  |
| 21.811    | 21.766  | 21.982  | 21.986      | 21.6             |
| 21.702    | 21.615  | 22.269  | 22.234      | 16.2-27.0        |

Table 2. Proinsulin concentration obtained of Control 2 (pmol/L).

All Quality control was within the acceptable ranges of the manufacturers.

Interassay precision

Due to only 2 plates being run in this validation, there is not sufficient data to calculate the Interassay performance. This is a performance characteristic that will require revisiting once further plates have been run, for example examining the values of the quality control material analysed.
**Intraassay precision**

Human Proinsulin WHO International Standard (NIBSC code 83/500) was reconstituted made up using a protein matrix solution (MultiAssay Diluent, Roche) in order to replicate human plasma. The International Standard was made up to three levels across the given performance range. Analysis of each homogenous mix was repeated 10 times. Given these values precision statistics could then be calculated.

| Sample 1 (approx. 5 pmol/L) | Mean value (pmol/L) | Standard Deviation | CV (%) |
|-----------------------------|---------------------|--------------------|--------|
| Sample 2 (approx. 25 pmol/L) | 4.910               | 0.369              | 7.51   |
| Sample 3 (approx. 100 pmol/L) | 21.450              | 0.584              | 2.72   |
|                               | 85.275              | 3.102              | 3.64   |

**Table 3. Intraassay Precision statistics, read at 405 nm.**

| Sample 1 (approx. 5 pmol/L) | Mean value (pmol/L) | Standard Deviation | CV (%) |
|-----------------------------|---------------------|--------------------|--------|
| Sample 2 (approx. 25 pmol/L) | 21.424              | 0.560              | 2.62   |
|                               |                     |                    |        |

**Table 4. Intraassay Precision statistics, read at 450nm. Only 2 samples are given for 450nm read as sample 3 is greater than the measuring range at this wavelength.**

From this study it can be seen that at all 3 tested levels all of the Coefficient of Variations (CV) are within the generally accepted limit of 10%. All levels, except Sample 1 at 405nm, perform well with CVs at less than 5%. From these data it can be noted that when the plate is read at 450nm the precision is greater at the lower end of the range than when read at 405nm. The precision of Sample 2 is comparable between the two different read wavelengths.

**Limit of Detection**

The zero standard provided with the assay kit was analysed with 10 repetitions, to calculate the potential noise, below the detection limit. The quoted limit of detection at plus 2 standard deviations is given as mL.

| Calibrator A 0 pmol/L | Mean OD | Standard Deviation | CV (%) |
|-----------------------|---------|--------------------|--------|
| 0.038                 | 0.003   | 7.28               |

**Table 5 Limit of Detection Data read at 405nm.**

| Calibrator A 0 pmol/L | Mean OD | Standard Deviation | CV (%) |
|-----------------------|---------|--------------------|--------|
| 0.053                 | 0.005   | 9.58               |

**Table 6. Limit of Detection Data read at 450nm.**

Both of the CVs are less than 10%.
Limit of Quantification

The first standard with a non zero value is run for 10 repetitions and statistics calculated to validate the precision at the lowest end of the assay range, indicating what can be a reliable range for clinical reporting. Standard B, with the given value of 2.5 pmol/L was analysed on the first experimental run.

| N=10 | Mean OD | Standard Deviation | CV (%) | N=8 | Mean concentration (pmol/L) | Standard Deviation | CV (%) |
|------|---------|-------------------|--------|-----|-----------------------------|-------------------|--------|
| Calibrator B 2.5 pmol/L | 0.069 | 0.004 | 5.58 | Calibrator B 2.5 pmol/L | 2.263 | 0.330 | 14.58 |

Table 7. Limit of Quantification, Calibrator B (2.5 pmol/L), both ODs and concentrations given, read at 405nm.

At this level, with the plate read at both wavelengths both CVs are greater than the acceptable limits of 10%. The experiment was repeated using calibrator B, but also moving up to the next calibrator C at 8 pmol/L.

| N=10 | Mean OD | Standard Deviation | CV (%) | N=8 | Mean concentration (pmol/L) | Standard Deviation | CV (%) |
|------|---------|-------------------|--------|-----|-----------------------------|-------------------|--------|
| Calibrator B 2.5 pmol/L | 0.151 | 0.011 | 7.35 | Calibrator B 2.5 pmol/L | 2.597 | 0.323 | 12.43 |

Table 8. Limit of Quantification, Calibrator B (2.5 pmol/L), both ODs and concentrations given, read at 450nm.

From these data it can be seen that the lower Limit of Quantification is unacceptable at the level of Calibrator B and would suggest that to have confidence in a reliable reporting range the lower limit should be from Calibrator C level, with these specific calibrator values 8 pmol/L.

To extend the reporting range at the lower end it is possible to use the data from the Intraassay Precision test to apply to the Limit of Quantification, specifically at 450nm, where at 5pmol/L the measured CV was 3.66%.
This could be further reinforced in the future by analysis of calibration material made up to a concentration of 5 pmol/L.

When using both wavelengths in combination this would give a measuring range of 5-40 pmol/L at 450nm and 8-100 pmol/L at 405nm.

**Upper limit of Detection**

To determine the upper limit of detection and consequently an upper reporting limit the highest calibrator was analysed repeatedly, and precision statistics calculated.

| N=10 | Mean OD | Standard Deviation | CV (%) |
|------|---------|--------------------|--------|
| Calibrator F 100 pmol/L | 1.242 | 0.023 | 1.815 |

| N=8 | Mean Proinsulin concentration (pmol/L) | Standard Deviation | CV (%) |
|-----|--------------------------------------|-------------------|--------|
| Calibrator F 100 pmol/L | 101.665 | 2.311 | 2.27 |

*Table 13. Upper Limit of Detection, Calibrator F (100 pmol/L), both ODs and concentrations given, read at 405nm*

| N=10 | Mean OD | Standard Deviation | CV (%) |
|------|---------|--------------------|--------|
| Calibrator E 40 pmol/L | 1.740 | 0.054 | 3.116 |

| N=8 | Mean Proinsulin concentration (pmol/L) | Standard Deviation | CV (%) |
|-----|--------------------------------------|-------------------|--------|
| Calibrator E 40 pmol/L | 38.696 | 1.054 | 2.72 |

*Table 14. Limit of Detection, Calibrator E (40 pmol/L), both ODs and concentrations given, read at 450nm*

At both wavelengths the CV’s are good at 2.27% and 2.72%. This indicates that results can confidently be reported up to 40 pmol/L at 450nm and 100 pmol/L at 405nm.
Recovery Test

Plasma and serum patient samples were spiked with known concentration of proinsulin (10pmol/L proinsulin), with the projected concentration calculated and compared to the observed values.

| RECOVERY PLASMA | NEAT Conc (pmol/L) | PROJECTED Conc (pmol/L) | OBSERVED Conc (pmol/L) | Recovery (%) |
|-----------------|--------------------|-------------------------|------------------------|--------------|
| A               | 12.216             | 22.216                  | 20.364                 | 91.7         |
| B               | 12.343             | 22.343                  | 19.230                 | 86.1         |
| C               | 9.752              | 19.752                  | 19.871                 | 100.8        |
| D               | 31.544             | 41.544                  | 43.049                 | 103.6        |

| RECOVERY SERUM | NEAT Conc (pmol/L) | PROJECTED Conc (pmol/L) | OBSERVED Conc (pmol/L) | Recovery (%) |
|----------------|--------------------|-------------------------|------------------------|--------------|
| A              | 4.943              | 14.943                  | 14.123                 | 94.5         |
| B              | 2.716              | 12.716                  | 12.759                 | 100.3        |
| C              | 1.246              | 11.246                  | 12.058                 | 107.2        |
| D              | 30.177             | 40.177                  | 41.635                 | 103.6        |

Table 15. Projected and observed proinsulin concentrations, with percentage recovery on addition of 10pmol/L proinsulin, in plasma and serum measured at 405nm.

| RECOVERY PLASMA | NEAT Conc (pmol/L) | PROJECTED Conc (pmol/L) | OBSERVED Conc (pmol/L) | Recovery (%) |
|-----------------|--------------------|-------------------------|------------------------|--------------|
| A               | 12.216             | 22.216                  | 20.364                 | 91.7         |
| B               | 12.343             | 22.343                  | 19.230                 | 86.1         |
| C               | 9.752              | 19.752                  | 19.871                 | 100.8        |
| D               | 31.544             | 41.544                  | 43.049                 | 103.6        |

| RECOVERY SERUM | NEAT Conc (pmol/L) | PROJECTED Conc (pmol/L) | OBSERVED Conc (pmol/L) | Recovery (%) |
|----------------|--------------------|-------------------------|------------------------|--------------|
| A              | 4.906              | 14.906                  | 13.953                 | 93.6         |
| B              | 2.618              | 12.618                  | 12.626                 | 100.1        |
| C              | 1.224              | 11.224                  | 11.872                 | 105.8        |
| D              | 30.051             | 40.051                  | 41.904                 | 104.6        |

Table 16. Projected and observed proinsulin concentrations, with percentage recovery on addition of 10pmol/L proinsulin, in plasma and serum measured at 450nm.

The recovery shown is generally excellent with the majority of samples demonstrating recovered values within +/-10% of the projected value.
Dilution Test

Test patients plasma and serum was diluted to 2 concentrations and the projected proinsulin concentration compared to the observed concentration. The diluent used was protein matrix solution (MultiAssay Diluent, Roche).

| DILUTION TEST PLASMA | DILUTION TEST SERUM |
|----------------------|----------------------|
| **Dilution Factor** | **Dilution Factor** | **Expected Conc (pmol/l)** | **Observed Conc (pmol/L)** | **Recovery (%)** | **Expected Conc (pmol/l)** | **Observed Conc (pmol/L)** | **Recovery (%)** |
| Plasma A             | Serum A              | 1 12.38 | 12.38 | 100.00 | 1 4.91 | 4.91 | 100.00 |
|                      |                      | 2 6.19 | 6.35 | 102.52 | 2 2.45 | 2.30 | 93.89* |
|                      |                      | 4 3.09 | 0.31 | 10.15  | 4 1.23 | 0.82 | 66.53* |
| Plasma B             | Serum B              | 1 12.53 | 12.53 | 100.00 | 1 2.62 | 2.62 | 100.00 |
|                      |                      | 2 6.26 | 5.85 | 93.31  | 2 1.31 | 0.94 | 71.81* |
|                      |                      | 4 3.13 | 0.86 | 27.55  | 4 0.65 | 0.43 | 65.70* |
| Plasma C             | Serum C              | 1 9.72 | 9.72 | 100.00 | 1 1.22 | 1.22 | 100.00* |
|                      |                      | 2 4.86 | 5.33 | 109.63 | 2 0.61 | 0.27 | 44.77* |
|                      |                      | 4 2.43 | 0.71 | 29.07* | 4 0.31 | Incalculable | No value* |
| Plasma D             | Serum D              | 1 31.61 | 31.61 | 100.00 | 1 30.05 | 30.05 | 100.00 |
|                      |                      | 2 15.81 | 20.87 | 132.07 | 2 15.03 | 15.91 | 105.90 |
|                      |                      | 4 7.90 | 6.68 | 84.53  | 4 7.51 | 6.98 | 92.91  |

Table 17. Projected and observed proinsulin concentrations with varying dilution factors, with percentage recovery shown, both in plasma and serum, read at 450nm.

| DILUTION TEST PLASMA | DILUTION TEST SERUM |
|----------------------|----------------------|
| **Dilution Factor** | **Dilution Factor** | **Projected Conc (pmol/I)** | **Observed Conc (pmol/L)** | **Recovery (%)** | **Projected Conc (pmol/I)** | **Observed Conc (pmol/L)** | **Recovery (%)** |
| Plasma A             | Serum A              | 1 12.22 | 12.22 | 100.00 | 1 5.60 | 5.60 | 100.00 |
|                      |                      | 2 6.11 | 6.73 | 110.20 | 2 2.80 | 2.76 | 98.46 |
|                      |                      | 4 3.05 | 0.55 | 18.01  | 4 1.40 | 1.22 | 87.17* |
| Plasma B             | Serum B              | 1 12.34 | 12.34 | 100.00 | 1 2.72 | 2.72 | 100.00 |
|                      |                      | 2 6.17 | 6.28 | 101.82 | 2 1.36 | 1.36 | 100.29* |
|                      |                      | 4 3.09 | 1.15 | 37.20  | 4 0.68 | 0.83 | 122.09* |
| Plasma C             | Serum C              | 1 9.75 | 9.75 | 100.00 | 1 1.25 | 1.25 | 100.00* |
|                      |                      | 2 4.88 | 5.77 | 118.23 | 2 0.62 | 0.70 | 111.88* |
|                      |                      | 4 2.44 | 1.94 | 42.78* | 4 0.31 | 0.11 | 34.03* |
| Plasma D             | Serum D              | 1 31.54 | 31.54 | 100.00 | 1 30.16 | 30.18 | 100.00 |
|                      |                      | 2 15.77 | 21.12 | 133.91 | 2 15.09 | 16.25 | 107.69 |
|                      |                      | 4 7.89 | 7.02 | 88.98  | 4 7.54 | 7.39 | 97.90  |

Table 18. Projected and observed proinsulin concentrations with varying dilution factors, with percentage recovery shown, both in plasma and serum, read at 450nm.

NB. Results marked with * have projected and/or initial concentrations outside the manufacturers designated assay range of 2.5-100pmol/L.

The dilution test show that in most observations a dilution factor of 2 will generally yield a comparable result. A dilution factor of 4 is, in most of the observed tests, not comparable. Considerations have to be made to the lower limit of detection and the amount of error that may be attributed to this. Also dilutions were made up by hand, which may also carry a portion of error.
Cross reactivity

As the proinsulin molecule is precursor to C-peptide and insulin, cross reactivity with these proteins have been tested to detect any competition or interference. Protein matrix solution was spiked with international standard proinsulin and international standard insulin or C-peptide for this test.

| Cross Reactivity with Insulin | Cross Reactivity with C peptide |
|--------------------------------|---------------------------------|
| **Insulin Conc (pmol/L)**     | **Cpeptide Conc (pmol/L)**     |
| Projected Proinsulin Conc (pmol/L) | Projected Proinsulin Conc (pmol/L) |
| Observed Proinsulin Conc (pmol/L) | Observed Proinsulin Conc (pmol/L) |
| Recovery (%)                  | Recovery (%)                   |
| 2500                          | 8250                           |
| 0.00                          | 0.00                           |
| 0.00                          | 0.00                           |
| 100.00                        | 100.00                         |
| 2500                          | 8250                           |
| 16.09                         | 16.09                          |
| 16.09                         | 16.96                          |
| 100.00                        | 105.45                         |
| 2500                          | 8250                           |
| 37.50                         | 37.50                          |
| 32.84                         | 31.93                          |
| 87.58                         | 85.15                          |

Table 19. Projected and Observed proinsulin concentrations and percentage recovery when spiked with either insulin or C-peptide, read at 405nm.

| Cross Reactivity with Insulin | Cross Reactivity with C peptide |
|--------------------------------|---------------------------------|
| **Insulin Conc (pmol/L)**     | **Cpeptide Conc (pmol/L)**     |
| Projected Proinsulin Conc (pmol/L) | Projected Proinsulin Conc (pmol/L) |
| Observed Proinsulin Conc (pmol/L) | Observed Proinsulin Conc (pmol/L) |
| Recovery (%)                  | Recovery (%)                   |
| 2500                          | 8250                           |
| 0.00                          | 0.00                           |
| 0.00                          | 0.00                           |
| 100.00                        | 100.00                         |
| 2500                          | 8250                           |
| 16.07                         | 16.07                          |
| 16.28                         | 16.65                          |
| 101.32                        | 103.62                         |
| 2500                          | 8250                           |
| 37.50                         | 37.50                          |
| 32.84                         | 32.11                          |
| 87.58                         | 85.62                          |

Table 20. Projected and Observed proinsulin concentrations and percentage recovery when spiked with either insulin or C-peptide, read at 450nm.

As can be seen by these data the presence of insulin or C-peptide in samples is unlikely to interfere with determination of proinsulin in this assay, with recovery generally as expected.
Freeze/Thaw Test

Pooled serum was spiked with proinsulin international standard and frozen then defrosted 1 to 5 times pre analysis to test the effect of freeze/thaw on measured proinsulin concentration. Baseline measurement of 1 freeze thaw cycle was decided on as this is the most likely scenario of preanalytic sample storage in a clinical laboratory.

### Freeze/thaw Plasma & Serum

| No. F/T cycles | Plasma Proinsulin Conc (pmol/L) | Recovery Plasma (%) | Serum Proinsulin Conc (pmol/L) | Recovery Serum (%) |
|----------------|---------------------------------|---------------------|---------------------------------|-------------------|
| 1              | 34.56                           | 100.00              | 6.57                            | 100.00            |
| 2              | 33.49                           | 96.90               | 6.89                            | 104.79            |
| 3              | 37.40                           | 108.22              | 6.21                            | 94.42             |
| 4              | 36.69                           | 106.15              | 5.95                            | 90.46             |
| 5              | 38.14                           | 110.35              | 7.34                            | 111.62            |

Table 2. Proinsulin concentration measured after stated number of freeze/thaw cycles and percentage difference from the baseline point calculated, measured at 405nm.

![Graph](image)

**Figure 1.** Graph of recovery of proinsulin from baseline after repeated freeze/thaw cycles, measured at 405nm.

### Freeze/thaw Plasma & Serum

| Number of Freeze Thaw cycles | Plasma Proinsulin Conc (pmol/L) | Recovery Plasma (%) | Serum Proinsulin Conc (pmol/L) | Recovery Serum (%) |
|------------------------------|---------------------------------|---------------------|---------------------------------|-------------------|
| 1                            | 34.62                           | 0.00                | 6.73                            | 0.00              |
| 2                            | 33.38                           | -3.57               | 7.08                            | 5.17              |
| 3                            | 37.12                           | 7.21                | 6.41                            | -4.70             |
| 4                            | 36.90                           | 6.58                | 6.02                            | -10.62            |
| 5                            | 38.02                           | 9.81                | 6.55                            | -2.64             |

Table 22. Proinsulin concentration measured after stated number of freeze/thaw cycles and percentage difference from the baseline point calculated, measured at 450nm.
Figure 2. Graph of recovery of proinsulin from baseline after repeated freeze/thaw cycles, measured at 450nm.

The relationship between the freeze thaw cycle and change in the proinsulin concentration measured does not appear to be linear, so drawing conclusions about the effect of repeated freeze/thaw on samples for proinsulin analysis is difficult. In both serum and plasma on two freeze thaw cycles proinsulin concentrations are around or below 5% difference which would be considered acceptable, and account for expected analytical variation. At greater levels of free/thaw cycles the percentage change generally increases to that greater than acceptable, with this difference probably attributable to repeated freeze thaw of the samples.

In looking at these results the previously observed intra assay precision has to also be taken into account. When read at 405nm with a proinsulin concentration of approximately 5pmol/L the coefficient of variation is 7.5%, so the level of error in measurement of proinsulin concentration is already high, so how much of the difference in these values is attributable only to the effect of freeze thaw is difficult to determine.
Linearity

To test the linearity of the assay the calibration points were plotted against the given concentration of proinsulin and Pearson’s R correlation coefficient calculated. As standards are assayed in duplicate and the data was taken from 2 plates and average of the 4 values of optical density are used.

**Figure 3.** Graph of average optical density at given proinsulin concentration, read at 405nm, with linear trend line and correlation coefficient given.

**Figure 4.** Graph of average optical density at given proinsulin concentration, read at 450nm, with linear trend line and correlation coefficient given.
Figure 5. Graph of average optical density at given proinsulin concentration, read at both 405nm and 450nm, with linear trend line and correlation coefficients given.

As can be seen from this data the linearity of the calibrators is very good, with the correlation coefficient at 450nm excellent, $R^2=0.9999$. When the data from the reads at the two wavelengths are plotted against each other it can be seen that when read at 450nm the range of concentrations is much reduced but as the spread over the optical density is greater; this gives rise to the greater sensitivity at the lower end of the measurement scale.
Interference from Haemolysis

To test for possible interference from haemolysis in samples, a sample spiked with proinsulin international standard had additional solution added at varying levels of haemolysis. The level of haemolysis for each spiked sample was determined by analysing on the Roche (Hitachi) P Module before analysis of proinsulin concentration. Where the haemolysis level equated to zero this was considered the baseline and the percentage difference from this proinsulin concentration calculated.

**EFFECT OF HAEMOLYSIS**

| HAEMOLYSIS (AU/L) | CONC (pmol/L) | Recovery from HAEM0 (%) |
|------------------|---------------|-------------------------|
| HAEM0            | 0             | 28.28                   | 100.00                  |
| HAEM1            | 50            | 27.94                   | 98.82                   |
| HAEM2            | 100           | 27.36                   | 96.75                   |
| HAEM3            | 149           | 30.97                   | 109.52                  |
| HAEM4            | 200           | 29.03                   | 102.66                  |

Table 23. Proinsulin concentration and percentage difference from haemolysis = 0, read at 405nm.

**Figure 6.** Graph of recovery from haemolysis = 0, with increasing levels of haemolysis read at 405nm.

**EFFECT OF HAEMOLYSIS**

| HAEMOLYSIS (AU/L) | CONC (pmol/L) | Recovery from HAEM0 (%) |
|------------------|---------------|-------------------------|
| HAEM0            | 0             | 28.60                   | 100.00                  |
| HAEM1            | 50            | 28.50                   | 99.65                   |
| HAEM2            | 100           | 27.81                   | 97.22                   |
| HAEM3            | 149           | 31.49                   | 110.09                  |
| HAEM4            | 200           | 29.54                   | 103.26                  |

Table 24. Proinsulin concentration and percentage difference from haemolysis = 0, read at 450nm.
Figure 7. Graph of recovery from haemolysis =0, with increasing levels of haemolysis read at 405nm.

There appears to be no clear relationship between increasing haemolysis and change in measured proinsulin concentration. The point measuring 149 AU/L haemolysis, at 10.1% different from the value measured at haemolysis=0, is still within acceptable levels of analytical variability. This data supports no interference from haemolysis up to a level of 200 AU/L.
Comparison of data obtained at 405nm and 450nm

As the recommendation is to run the assay with 2 reads at 405nm and 450nm a comparison of the obtained proinsulin concentrations has been made to assess the performance of each wavelength read.

Figure 8. Graph comparing obtained concentrations between readings at 405nm and 450nm, N=102. R² value given.

Across the manufacturers range of 2.5pmol/L to 40pmol/L comparable between the two wavelengths there was excellent agreement in the concentration of proinsulin obtained between the reads at the two wavelengths with an R² value of 0.9994.

As the read at 450nm is recommended due to the greater sensitivity at the lower end of the measuring range this comparison was repeated with only values up to 10pmol/L.

Figure 9. Graph comparing obtained concentrations between readings at 405nm and 450nm, range 2.5pmol/L to 10pmol/L, N=42. R² value given.
As can be seen the agreement at the lower end of the measuring range is not as good as generally over the whole range but this is expected with the increased sensitivity at 450nm. Also as has been previously shown with Intraassay and limit of quantification experiments, the precision particularly at 405nm, is not acceptable below 8pmol/L so the likelihood of disagreement on obtained concentration at the two wavelengths is greater.

Figure 10. Bland-Altman Difference plot with 450nm as the test method. N=99.

From the above difference plot it can be seen that at 450nm the concentrations obtained tend to be greater than those at 405nm, with several outliers from this. This difference is consistent across the given range of 2.5pmol/L to 40 pmol/L. Relatively though this effect is probably not that great, at the lower end of the measurement scale the precision has been shown to be poorer and at the higher scale a difference of up to 0.6pmol/L is proportionally low.

Conclusions

From the experiments and data analysis performed some conclusions can be drawn in assessing the performance characteristics of the TECO Medical Intact Proinsulin ELISA kit.

- The measuring range of the assay at each wavelength can confidently be defined as;
  - 5 – 40pmol/L measuring range at 450nm
  - 8 – 100pmol/L measuring range at 405nm
  The intraassay precision within these ranges has been shown as good, the limit of detection is acceptable, as is the upper limit of quantification.
- Recovery of both serum and plasma on addition of a known proinsulin concentration is good.
- Dilution of samples with MultiAssay Diluent up to x2 is acceptable.
- There is no interference with insulin up to 2500 pmol/L or C-peptide up to 8250 pmol/l.
- Freeze/Thaw up to 2 cycles is acceptable, and possibly acceptable up to 5 cycles, with no real significant differences observed between serum and plasma samples.
- The assay calibration is linear.
- Samples are unaffected by Haemolysis up to 200AU/L.
- There are no real differences between measurement at 450nm and 405nm although the greater sensitivity at the lower range has shown value in analysing data at both wavelengths to extend the lower reporting range.