

## SUPPLEMENTARY DATA

### Supplementary Material

#### *Evaluation of Millipore Proinsulin radioimmunoassay Kit by Northwest Lipid Research Laboratories (NWRL)*

1. Intra-Assay Variation: For the evaluation of intra-assay variation of the proinsulin assay, three serum samples received for analysis were selected to represent samples with Low, Medium, and High proinsulin levels. Each sample was analyzed 20 times in a single assay. The results of these analyses are summarized in Table S1.

**Supplementary Table S1. Intra-Assay Variation.**

Sample	Sample count	Mean proinsulin (pM)	Standard Deviation	Coefficient of Variation (%)
Low	20	4.7	0.2	5.0
Medium	20	10.8	0.7	6.0
High	20	61.1	0.9	1.4

2. Inter-Assay Variation: For the evaluation of inter-assay variation, the Low and High proinsulin controls provided by the kit manufacturer were utilized.

**Supplementary Table S2. Inter-Assay Variation.**

Sample	Sample count	Mean proinsulin (pM)	Standard Deviation	Coefficient of Variation (%)
Low	55	16.1	1.21	7.48
High	55	36.0	1.74	4.84

3. Assay Sensitivity: The sensitivity, or detection limit, of a radioimmunoassay is defined as the antigen concentration which can be distinguished from the zero concentration. For the evaluation of the assay sensitivity, assay buffer was run 20 times as an unknown sample in the proinsulin radioimmunoassay. The sensitivity was calculated as mean result for assay buffer +3SD. For the proinsulin assay, the calculated sensitivity was 0.51 pM.

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**Supplementary Table S3. Proinsulin Assay Sensitivity.**

Sample	Sample count	Mean proinsulin (pM)	Standard Deviation
Assay Buffer	20	0.03	0.16

4. Cross Reactivity: Purified human c-peptide and insulin preparations were used to determine cross-reactivity. Dilutions of c-peptide and insulin, prepared at concentrations matching those of the proinsulin standard curve, were analyzed in the RIA assay as unknown samples. For each assay ED50 of the proinsulin, c-peptide, and insulin curves were calculated. The percent cross-reactivity was calculated as: ED50 for proinsulin/ ED50 for c-peptide or insulin X 100. There was virtually no reaction of the proinsulin specific antibody used in the assay, and standard curves for insulin and c-peptide were flat. Based on the results obtained, insulin and c-peptide do not cross-react in the proinsulin assay. Thus, for both insulin and C-peptide, these were calculated as <0.1%.

Serial dilution of serum sample from an individual with T1D

Proinsulin was measured in serial dilutions of a banked serum sample from an individual with recent onset T1D and a proinsulin level of 42.61 pmol/L. Assay buffer was used to perform serial dilutions, and the total volume for each reaction was 200 µL. Results are shown in **Supplemental Figure S1**.

Evaluation of serum samples from individuals before and after pancreatectomy

Proinsulin values were assayed in serum obtained before and after pancreatectomy performed for resection of pancreatic adenocarcinoma.

**Supplementary Table S4. Proinsulin Value in Serum from Individuals Undergoing Pancreatectomy.**

Age (years)	Sex	Pre-surgery proinsulin (pM)	Post-surgery proinsulin (pM)	Days Post Procedure	Required Insulin Post-operatively
80	M	51.15	<3	4	Yes
58	F	5.73	<3	5	Yes
62	M	30.54	<3	3	Yes
52	M	35.45	<3	4	Yes
43	F	8.77	3.21	5	Yes
57	M	<3	<3	5	Yes

### *Proinsulin levels in Insulin Ab + samples*

To test whether elevated microinsulin autoantibody (miAA) could be an explanation for falsely elevated serum proinsulin values, proinsulin was measured using the Millipore human proinsulin RIA in fasting serum samples from 22 children with new-onset diabetes who were either miAA negative or positive (antibody testing performed by the Barbara Davis Autoantibody/HLA Service Center; normal miAA titer is <0.010). As shown in **Supplemental Figure S2**, no correlation between higher proinsulin values and higher absolute values of miAA titers was present.

### *Targeted mass spectrometry assays for C-peptide and Proinsulin in serum*

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To validate the detection of serum proinsulin by the Millipore RIA in samples from individuals with type 1 diabetes and undetectable stimulated serum C-peptide, we performed targeted mass spectrometry (MS) analyses based on selected reaction monitoring (SRM) to verify the presence or absence of serum C-peptide and proinsulin. The nature of insulin processing and proinsulin allows us to select two surrogate peptides (see **Supplemental Table S5** below and **Supplemental Figure S3**) for specific detection of C-peptide and proinsulin, respectively, using MS. The C-peptide sequence represents the result of mature insulin processing independent of trypsin digestion. The proinsulin surrogate sequence is derived from trypsin digestion of the proinsulin proteins where trypsin cleaves after R and K, resulting a peptide sequence with exactly one amino acid additional to the C-peptide. Synthetic heavy peptides (H) labeled with <sup>13</sup>C/<sup>15</sup>N on the C-terminal lysine (K) or glutamine (Q) with crude purity (~75% pure) were purchased from New England Peptide (Gardner, MA) as internal standards for quantification. Targeted MS assays for each peptide with at least three SRM transitions were developed and used to quantify the presence or absence of C-peptide and proinsulin. The specificity of endogenous analyte detection was assessed based on the relative intensity patterns between endogenous signals and heavy standards. Because crude heavy peptides were used as internal standards, targeted MS assays were used to verify the presence or absence of C-peptide and proinsulin as detected by the TOSOH and Millipore assays, rather than quantification of exact concentrations.

**Supplementary Table S5. Surrogate Peptides for Targeted Mass Spectrometry Assays.**

Protein	Surrogate peptide
C-peptide	EAEDLQVGQVELGGPGAGSLQPLALEGSL <b>Q</b>
Proinsulin	EAEDLQVGQVELGGPGAGSLQPLALEGSL <b>QK</b>

Aliquots of ~75 µL human sera from T1D patients and a nondiabetic control subject were used for the assays. An initial serum protein concentration was determined by the BCA protein assay (Pierce). Immunodepletion of fourteen high abundance serum proteins (albumin, IgG, α1-antitrypsin, IgA, IgM, transferrin, haptoglobin, α2-macroglobulin, fibrinogen, complement C3, α1-acid glycoprotein, apolipoproteins A-I, apolipoproteins A-II, and apolipoprotein B) was performed using a prepacked 12.7 mm × 39.5 mm IgY-14 affinity LC5 column (Sigma-Aldrich) on an Agilent 1100 series HPLC system (Agilent, Palo Alto, CA) as previously described (1). Protein was denatured, reduced, and digested by trypsin with a urea-based protocol.

25 µL peptide sample at 1 µg/µL was spiked with 100 fmol/µL heavy labeled peptide standards and fractionated using a high pH reverse phase cLC following the PRISM workflow as previously described (2). All fractions of the first serum sample were screened for the fraction number of surrogate peptides for C-peptide and Proinsulin using a nanoACQUITY UPLC system coupled online to a TSQ Vantage triple quadrupole mass spectrometer (Thermo Scientific, San Jose, CA). Final peptide fraction samples were analyzed with a BEH 1.7 µm C18 column (100 µm i.d. × 10 cm) with a ~35 min gradient. SRM data were analyzed using Skyline software (3). The specificity of endogenous analyte detection were assessed based on the relative intensity patterns between endogenous signals and heavy standards. A signal to noise (S/N) of surrogate endogenous peptides >10 is required for confident quantification.

Sera from 10 individuals with type 1 diabetes and undetectable stimulated C-peptide values, but detectable proinsulin values were tested. Consistent with our prior testing, targeted mass spectrometry measurements of each of the samples from individuals with type 1 diabetes demonstrated C-peptide was below the limit of detection (LOD), but proinsulin was clearly detectable (**Supplemental Table S6**). As a control, a serum sample from a nondiabetic individual was also tested, and in contrast to samples from individuals with type 1 diabetes, demonstrated both detectable C-peptide and proinsulin. Representative extracted ion chromatograms for the detection of C-peptide and Proinsulin in representative serum samples from a longstanding T1D subject and a nondiabetic control subject are shown in **Supplemental Figure S4**.

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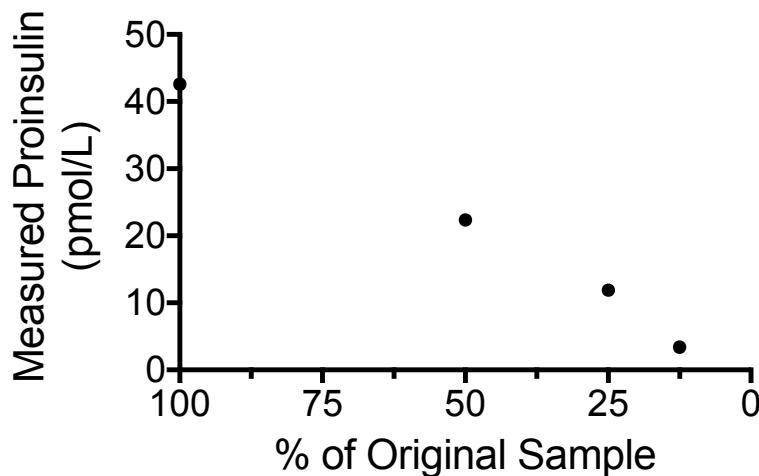
**Supplementary Table S6.** Measured peak areas of Light and Heavy C-peptide and proinsulin surrogate peptides (representing endogenous signal and internal standard, respectively) in sera.

Subject ID	C-peptide (TOSOH) (nmol/L)	Proinsulin (Millipore) (pmol/L)	EAEDLQVGQVELGGGP GAGSLQPLALEGSLQ (C-Peptide) <b>Endogenous Signal (L/H ratio)</b>	EAEDLQVGQVELGGGP GAGSLQPLALEGSLQK (Proinsulin) <b>Endogenous Signal (L/H ratio)</b>
T1D se137128202	<0.017	10.72	<LOD	0.00163
T1D se238594973	<0.017	17.31	<LOD	0.00152
T1D se327454919	<0.017	7.67	<LOD	0.00150
T1D se33975524	<0.017	7.19	<LOD	0.00149
T1D se453237507	<0.017	13.74	<LOD	0.00159
T1D se465483285	<0.017	18.26	<LOD	0.00152
T1D se467541771	<0.017	14.1	<LOD	0.00161
T1D se84755504	<0.017	45.32	<LOD	0.00170
T1D se88548588	<0.017	13.14	<LOD	0.00159
T1D se95377272	<0.017	11.99	<LOD	0.00163
Control Serum	n/a	n/a	0.00119	0.00150

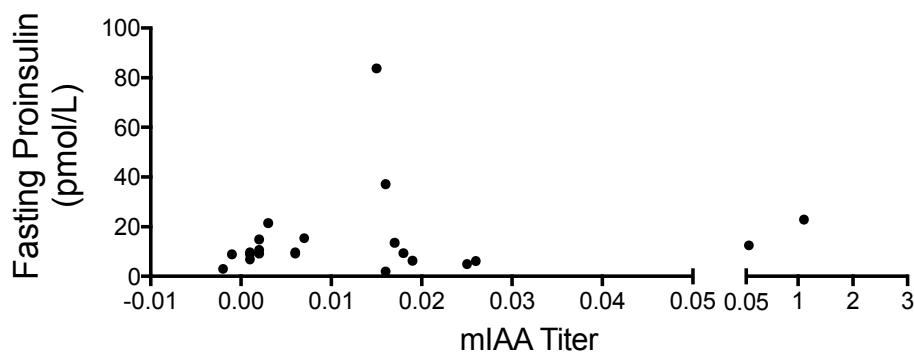
C-peptide and Proinsulin were measured as L/H ratio by targeted MS where 'L' is the endogenous light version of surrogate peptide and 'H' is the spike-in heavy-isotope labeled version of surrogate peptide as the internal standard. <LOD indicates that signals were below the level of detection based on either signal levels or background interference of signals.

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**Supplementary Figure S1. Proinsulin levels measured in serially diluted serum collected from an individual with recent onset diabetes.** Assay buffer was used to dilute serum collected from an individual with recent onset T1D and a proinsulin level of 42.61 pmol/L. The total volume for each reaction was 200  $\mu$ L. Results are graphed according to the % of original serum for each dilution (plotted on x-axis) compared to measured proinsulin values on y-axis.



**Supplementary Figure S2. No correlation exists between fasting proinsulin levels and microinsulin autoantibody titers in serum from individuals with new onset diabetes.** Fasting serum proinsulin values were obtained in 22 pediatric individuals with new onset type 1 diabetes who were either microinsulin autoantibody (miAA) negative ( $n=11$ ) or positive ( $n=11$ ; positive test defined as miAA titer  $>0.1$ ). miAA titer values are plotted on x-axis compared to fasting proinsulin values on y-axis.



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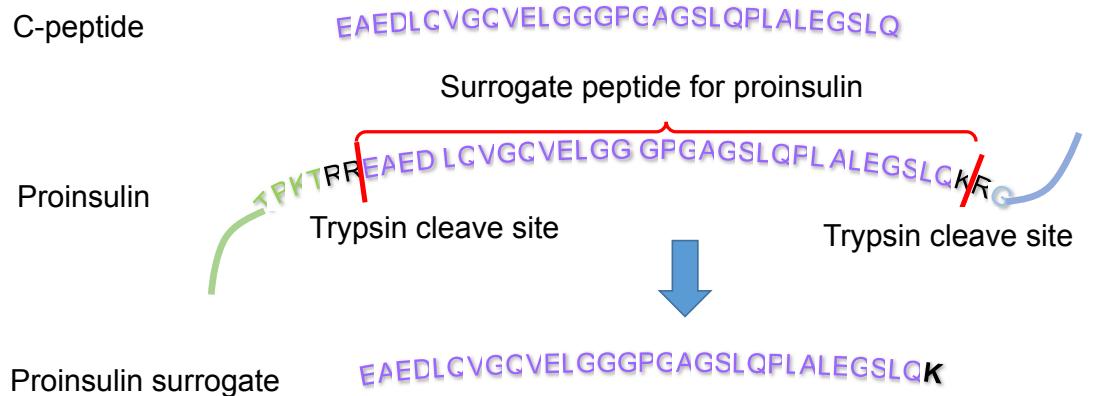
**Supplementary Figure S3. Illustration of surrogate peptides for C-peptide and proinsulin selected for targeted MS assays.** A. Sequence of insulin. B. Trypsin digestion results in a surrogate peptide of proinsulin with one additional Lysine to C-peptide while C-peptide remains intact.

**A.**

10	20	30	40	50
MALWMRLLPL	LALLALWGPD	PAAA	FVNQHLCGSHLVEALY	LVCGERGFFY
60	70	80	90	100
TPKT	REAEAD	LQVGQVELGG	GPGAGSLQPL	ALEGSLQKRG
IVEQCCTSIC				
110				
SLYQLENYCN				

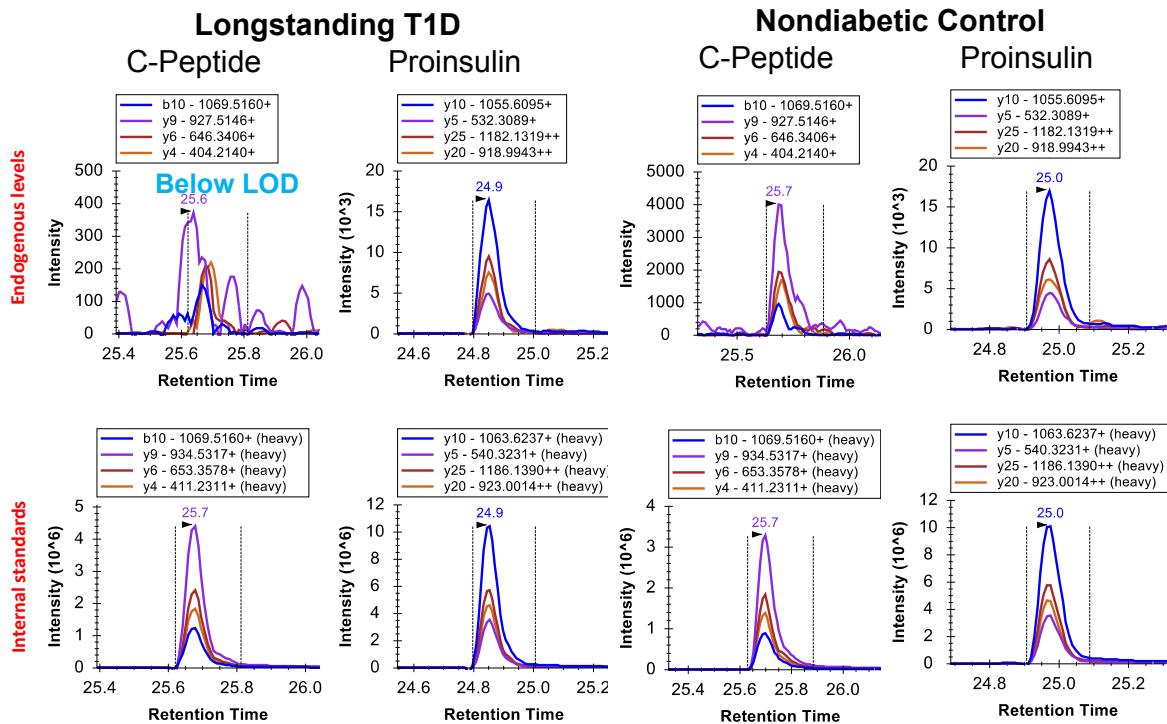
— A chain  
— C-peptide  
— B chain

**B.**



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**Supplementary Figure S4. Representative extracted ion chromatograms for the detection of C-peptide and Proinsulin in serum samples from a longstanding T1D subject and a nondiabetic control subject.** Each colored curve represents the signal of a specific fragment (or SRM transition) as indicated.



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### List of Investigators and Sites

A listing of the T1D Exchange Clinic Network sites participating in the Residual C-Peptide in Patients with Type 1 Diabetes Study with participating principal investigators (PI), co-investigators (I), coordinators (C), and number of participants recruited per site is included below:

**Philadelphia, PA Children's Hospital of Philadelphia** (n=5) Steven Willi (PI); Tammy Calvano (C)  
**Aurora, CO Barbara Davis Center for Childhood Diabetes** (n=49) Georgeanna Klingensmith (PI); Heidi Haro (C) **Syracuse, NY SUNY Upstate Medical University** (n=87) Ruth Weinstock (PI); Suzan Bzdick (C) **New York City, NY Naomi Berrie Diabetes Center, Columbia University P&S** (n=70) Robin Goland (PI); Ellen Greenberg (C) **Ann Arbor, MI University of Michigan** (n=11) Joyce Lee (PI); Ashley Eason (C) **Indianapolis, IN Riley Hospital for Children, Indiana University School of Medicine** (n=105) Linda DiMeglio (PI); Stephanie Woerner (C) **Portland, OR Harold Schnitzer Diabetes Health Center at Oregon Health and Science University** (n=89) Andrew Ahmann (PI); Rebecca Fitch (C) **Buffalo, NY Women and Children's Hospital of Buffalo Diabetes Center** (n=48) Kathleen Bethin (PI); Michelle Ecker (C) **Seattle, WA University of Washington, Diabetes Care Center** (n=11) Irl Hirsch (PI); Christina Peterson (C) **Idaho Falls, ID Rocky Mountain Diabetes & Osteoporosis Center, PA** (n=95) David Liljenquist (PI); Brandon Robison (C) **Minneapolis, MN International Diabetes Center/Park Nicollet Adult Endocrinology** (n=29) Richard Bergenstal (PI); Beth Olson (C) **New Haven, CT Yale Pediatric Diabetes Program** (n=18) Eda Cengiz (PI); Amy Steffen (C) **Los Angeles, CA University of Southern California - Community Diabetes Initiatives** (n=47) Anne Peters (PI); Perez Hinton (C) **St. Louis, MO Washington University** (n=32) Janet McGill (PI); Lori Buechler (C) **Iowa City, IA University of Iowa Children's Hospital** (n=5) Eva Tsakikian (PI); Joanne Cabbage (C) **Kansas City, MO Children's Mercy Hospital** (n=17) Mark Clements (PI); Lois Hester (C) **Detroit, MI Henry Ford Health System** (n=36) Davida Kruger (PI); Heather Remtema (C) **Gainesville, FL University of Florida** (n=20) Desmond Schatz (PI); Jamie Thomas (C) **Columbus, OH Central Ohio Pediatrics Endocrinology and Diabetes Services** (n=28) William Zipf (PI); Diane Seiple (C) **Tampa, FL University of South Florida Diabetes Center** (n=17) Henry Rodriguez (PI); Danielle Henson (C) **Nashville, TN Vanderbilt Eskind Diabetes Clinic** (n=17) Jill Simmons (PI); Faith Brendle (C) **Minneapolis, MN University of Minnesota** (n=9) Brandon Nathan (PI); Kara Schmid (C) **Ocean Springs, MS The Diabetes Center, PLLC** (n=40) Kathleen Arnold (PI); Sharon Sellers (C) **Worcester, MA University of Massachusetts Medical School** (n=13) David Harlan (PI); Lisa Hubacz (C) **Durham, NC University of North Carolina Diabetes Care Center** (n=35) John Buse (PI); Julie Tricome (C) **Philadelphia, PA University of Pennsylvania School of Medicine/Rodebaugh Diabetes Center** (n=12) Michael Rickels (PI); Cornelia Dalton-Bakes (C) **Findlay, OH Blanchard Valley Medical Associates** (n=6) Leroy Schroeder (PI); Amanda Roark (C) **Nashville, TN Vanderbilt Eskind Diabetes Clinic** (n=14) Amy Potter (PI); Faith Brendle (C)