Measurement of Differentially Methylated *INS* DNA Species in Human Serum Samples as a Biomarker of Islet β Cell Death

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Summary

Islet β cell death precedes development of type 1 diabetes, and detecting this process may allow for early therapeutic intervention. Here, we provide a detailed description of how to measure differentially methylated *INS* DNA species in human serum as a biomarker of β cell death.

The death of islet β cells is thought to underlie the pathogenesis of virtually all forms of diabetes and to precede the development of frank hyperglycemia, especially in type 1 diabetes. The development of sensitive and reliable biomarkers of β cell death may allow for early therapeutic intervention to prevent or delay the development of diabetes. Recently, several groups including our own have reported that cell-free, differentially methylated DNA encoding preproinsulin (*INS*) in the circulation is correlated to β cell death in pre-type 1 diabetes and new-onset type 1 diabetes. Here, we present a step-by-step protocol using digital PCR for the measurement of cell-free *INS* DNA that is differentially methylated at cytosine at position -69 bp (relative to the transcriptional start site). We demonstrate that the assay can distinguish between methylated and unmethylated cytosine at position -69 bp, is linear across several orders of magnitude, provides absolute quantitation of DNA copy numbers, and can be applied to samples of human serum from individuals with new-onset type 1 diabetes and disease-free controls. The protocol described here can be adapted to any DNA species for which detection of differentially methylated cytosines is desired, whether from circulation or from isolated cells and tissues, and can provide absolute quantitation of DNA fragments.

Keywords

Genetics; Issue 118; Biomarker; Islet; Diabetes; Insulin; Digital PCR; Epigenetics

A complete version of this article that includes the video component is available at http://dx.doi.org/10.3791/54838.

Disclosures

The authors have nothing to disclose.

Introduction

Type 1 diabetes (T1D) is an autoimmune disease that is characterized by the destruction of insulin-producing islet β cells by autoreactive T cells[1]. The diagnosis of T1D is typically made upon measurement of hyperglycemia (blood glucose > 200 mg/dl) in a lean, young individual, who might present with ketoacidosis as evidence of insulin deficiency. At the time of diagnosis of T1D, there is evidence for substantial loss of β cell function and mass (from 50 – 90%)[2]. In clinical studies, several immune modulatory drugs that were instituted at the time of diagnosis resulted in the stabilization of β cell function (and presumably mass), but none have resulted in clinical remission of disease, a finding that has raised the call for the development of biomarkers for earlier detection of the disease and for the longitudinal tracking of effectiveness of combination therapies[3,4]. Efforts by international consortia, such as the Human Islet Research Network Consortium at the National Institutes of Heath[5], have emphasized the need to develop biomarkers that focus on β cell stress and death in T1D.

In line with these efforts, our group and others have recently developed biomarker assays that measure circulating, epigenetically modified DNA fragments that arise primarily from dying β cells[6–9]. In all of the published assays to date, the focus has been on the quantitation of the human gene encoding preproinsulin (*INS*), which demonstrates greater degrees of unmethylated CpG sites in the coding and promoter regions compared to other cell types. The liberation of unmethylated *INS* DNA fragments was hypothesized as arising primarily from dying (necrotic, apoptotic) β cells. Our recent studies showed that in youth, elevations in both unmethylated and methylated *INS* DNA at position -69 bp (relative to the transcriptional start site) were observed in new-onset T1D, and together served as specific biomarkers for this population[6]. These biomarker assays involve the isolation of cell-free DNA from serum or plasma using commercial spin kits, followed by a bisulfite conversion of the isolated DNA (to convert non-methylated cytosines to uracils, leaving methylated cytosines intact).

In this report, we describe the technical aspects of serum sample collection, isolation of cell-free DNA from serum, bisulfite conversion, and performance of droplet digital PCR (henceforth, digital PCR) for differentially methylated *INS* DNA.

Protocol

Ethics Statement: Protocols were approved by the Indiana University Institutional Review Board. Parents of subjects provided written informed consent, and children older than 7 years provided assent for their participation.

1. Serum Processing

NOTE: The assay as described has been rigorously tested using human serum isolated as follows.

1. Collect blood in one red top (no-additive; uncoated) blood collection tube.
2. Let sit at room temperature for 30 min to allow the clot to form.

3. Centrifuge tube at 2,000 × g for 10 min at room temperature. Transfer the supernatant (serum) to a new tube designated for serum storage (polypropylene screw top tubes, size dependent on samples).

4. Store samples at −80 °C until ready for DNA isolation.

2. Serum DNA Extraction

NOTE: DNA is extracted with a DNA extraction kit using 50 µl of serum (recommended), following manufacturer's protocol with some modifications.

1. Prepare 200 µl lysis buffer + 1 µl poly(A) (5 µg) per sample in an microcentrifuge tube. Vortex for 15 sec. Set aside until Step 2.3.

2. Take serum samples from −80 °C freezer and thaw at room temperature. Bring serum sample volume up to 200 µl total using phosphate-buffered saline (PBS, with calcium chloride and magnesium chloride).

3. Lyse the sample by adding 20 µl protease (1.4 Anson units/ml) to sample, followed by 200 µl lysis buffer/polyA mix (from Step 2.2) and vortex for 8 sec. Incubate sample at 56 °C for 10 min, then briefly centrifuge for 7 sec at top speed in a microcentrifuge (>10,000 × g).

4. Precipitate DNA by adding 230 µl 100% ethanol to sample and vortex for 8 sec. Briefly centrifuge for 7 sec at top speed (>10,000 × g).

5. Apply the DNA to the spin column by adding 600 µl sample mixture to mini spin column. Centrifuge at 6,000 × g for 1 min. Discard the flow-through and place column in a clean tube.

6. Wash the DNA by adding 500 µl wash buffer 1 to the column. Centrifuge at 6,000 × g for 1 min. Discard the flow-through and place column in a clean tube. Add 500 µl wash buffer 2 to column and centrifuge at top speed (>10,000 × g) for 3 min. Place column in a new 1.5 ml microcentrifuge tube and centrifuge at top speed again for 1 min.

7. Elute the DNA by placing column in a new 1.5 ml microcentrifuge tube and adding 60 µl elution buffer directly on the filter. It is important to switch tips between samples to prevent cross contamination. Incubate at room temperature for 5 min, then centrifuge at 6,000 × g for 1 min. Add another 60 µl of elution buffer and centrifuge at 6,000 × g for 1 min.

8. Store DNA at −20 °C for later use, or proceed immediately to bisulfite conversion.

3. Bisulfite Conversion

NOTE: Bisulfite conversion is performed using a bisulfite conversion kit, following the manufacturer's protocol with some modifications.

1. To convert unmethylated cytosines to uracils, add 130 µl of bisulfite conversion reagent to 20 µl of DNA from step 2.7 in a PCR tube (single 0.2 ml PCR tube or

8-strip PCR tubes). Save the remaining 40 µl of DNA for future use, or use in replicate reactions. Mix 20 times by pipetting up and down and centrifuge briefly to make sure no drops are on sides or lid. Incubate in a water bath or thermal cycler as follows: 98 °C for 8 min, 54 °C for 60 min, 4 °C for 5 min.

1. Proceed to step 3.2 or store at 4 °C for up to 20 hr.
2. Add 600 µl of binding buffer to spin column and place the column in the provided collection tube. Apply the bisulfited DNA to column and mix by pipetting up and down 10 times. Centrifuge at top speed (>10,000 × g) for 30 sec. Discard flow-through.
3. Wash DNA by adding 100 µl of wash buffer to column and centrifuge at top speed (>10,000 × g) for 30 sec.
4. Perform desulphonation to remove sulphonate group to finalize the conversion of unmethylated cytosines to uracil by adding 200 µl of desulphonation buffer to the column. Let stand at room temperature for 20 min, then centrifuge at top speed (>10,000 × g) for 30 sec.
5. Wash DNA by adding 200 µl of wash buffer to the column and centrifuge at full speed for 30 sec. Discard the flow-through and repeat this wash step.
6. Place the column into a 1.5 ml microcentrifuge tube. Elute DNA by adding 10 µl elution buffer directly to column filter. Incubate for 1 min. Centrifuge for 30 s at full speed to elute DNA. Quantify recovered DNA by spectrophotometry at A260.

   NOTE: The DNA detected by spectrophotometry represents primarily the carrier polyA DNA that was added at step 2.3 above. Typically, recovery of polyA is ≥85% of the input (>4.25 µg).
7. Store DNA at −20 °C until ready to proceed with digital PCR.

4. Multiplex Digital PCR

1. Make a master mix with enough solution for each sample and control. Include at least one sample containing water (negative control), one sample of a plasmid containing unmethylated *INS* (1 pg, positive control), one sample of plasmid containing methylated *INS* (1 pg, positive control), and one sample containing a 1:1 mixture of plasmids.

1. To prepare the PCR master mix, add 12.5 µl per reaction of PCR buffer (*e.g.,* 125 µl per 10 samples), 1.25 µl per reaction primer/probe mix (*e.g.,* 12.5 µl per 10 samples), 8.25 µl per reaction water (*e.g.*, 82.5 µl per 10 samples), 0.5 µl per reaction EcoR1 enzyme (*e.g.,* 5 µl per 10 samples).

   NOTE: Primers and probes used here are as follows: Forward Primer: 5′-GGAAATTGTAGTTTTAGTTTTTAGTTATTTGT-3′; Reverse Primer: 5′-AAAACCCATCTCCCCTACCTATCA-3′; FAM probe: FAM-5′-ACCCCTACCACCTAAC-3′-MGB; VIC probe: VIC-5′-ACCCCTACCGCCTAAC-3′-MGB.

2. Set up multiplex PCR reaction in a 96-well plate Add 19.5 µl of master mix to each well. Add 2.5 µl of bisulfite converted DNA sample into each appropriate well (save the remaining 7.5 µl of bisulfite converted DNA or use in replicate reactions). Mix by pipetting up and down several times.

   NOTE: All 8 wells in a row must contain sample or buffer control.

3. Seal with a foil seal using a plate sealer and centrifuge in a plate spinner until there is no liquid on the sides of the wells.

4. Set up the automated droplet generator on the touchscreen.

   NOTE: All 8 wells in the cartridge must contain sample or buffer control.

   1 Set the number of rows being used by touching the row(s) in which samples are loaded into on the 96-well plate from step 4.2 and highlight the row in blue to indicate an active row.

   2 Load consumables from back to front to avoid contamination. To start, add cartridges along the back row of the instrument. Load tips into center row of the instrument.

   NOTE: The cartridges can only fit into the holders in the correct orientation.

   3 Place 96-well plate into the instrument. Remove a cold block from −20 °C freezer and place a new skirted 96-well plate into it. Place it inside the instrument next to the sample loaded 96-well plate.

   4 Add oil to dispenser in the front of the instrument. Select type of oil on touchscreen.

   5 Touch the blue start button to start the run. Confirm plate setup and touch start run button to begin.

   5 Once finished, remove the 96-well plate that contains the newly formed droplets and seal with a foil seal using a plate sealer.

   6 Perform PCR_{10} on a thermal cycler using the following program: 95 °C for 10:00 min; 40 cycles at: 94 °C for 00:30 min, 57.5 °C for 01:00 min; 98 °C for 10:00 min; 12 °C for 10 min or up to 24 hr.

   7 Place the plate on the droplet reader and set up the reader.

1. Click on the setup tab. Open a new template by clicking template > new. Enter a file name.

2. Use the well editor to set the parameters for each sample. Give each sample a unique name, set experiment to Rare Event Detection (R-RED), and set master mix to the mix used in step 4.1.

3. Give Target 1 a name (*i.e.,* unmethylated) and set Target 1 as unknown (U). Give Target 2 a name (*i.e.*, methylated) and set Target 2 as reference (R).

4. Click Run tab to start the run. In the run options window, select the detection chemistry (FAM/VIC).

5. Data Analysis

1. Open results in analyze tab and analyze using 2-D plot based on positive controls (see Figure 1).
2. Export CSV file generated by droplet reader to a spreadsheet program for data analysis.
3. To generate “copies per µl” of serum, use the formula: (concentration*250)/(volume of serum used for DNA isolation in µl).[6]

   NOTE: The data is typically converted to $\log_{10}$ when acquired from human serum to ensure normal distribution. Alternatively, data can be plotted on a $\log_{10}$ scale and analyzed by non-parametric statistics.

Representative Results

To interpret data appropriately, we use plasmid controls for both the unmethylated and methylated target *INS* DNA in each digital PCR run. These controls ensure that signals corresponding to methylated and unmethylated DNA are clearly distinguishable. Figure 1 shows the 2-D scatter plots corresponding to droplets for plasmid controls containing bisulfite-converted unmethylated *INS* DNA (Figure 1A), methylated *INS* DNA (Figure 1B), and a 1:1 mixture of the two plasmids (Figure 1C). Plasmid containing unmethylated *INS* is indicated by the droplets positive for the 6-Carboxyfluorescein (FAM) signal, whereas plasmid containing methylated *INS* is indicated by the droplets positive for the VIC signal. In the 1:1 plasmid mixture, a population of double-positive droplets is seen, corresponding to droplets containing both species of DNA. To distinguish positive droplets from negative droplets, data are gated using these 2-D plots. Note that in Figure 1A, there is a slight shift of the FAM-positive droplets into the VIC channel, indicating that the probe for the methylated *INS* DNA exhibits some cross-reactivity for the unmethylated *INS* DNA. Similarly, in Figure 1B, there is a very slight shift of the VIC-positive droplets into the FAM channel, indicating cross-reactivity of the probe for the unmethylated DNA with methylated DNA. A major advantage of digital PCR is it can still discriminate specific signals even when probes are not 100% specific. For appropriate Poisson statistical calculations by the software, each PCR reaction should partition into at least 10,000 total droplets, display a cluster of negative droplets, and show a clear amplitude difference between negative and positive droplets (for reliable gating). To demonstrate linearity of our primers, we performed mixtures of the two plasmids across several orders of magnitude. As shown in Figure 2, varying concentrations of one plasmid can be linearly detected in the presence of a constant amount of the second plasmid. For new labs performing this procedure, we recommend constructing a similar mixture experiment to ensure that the assay is operating in a linear detection fashion.

Next, we obtained serum of three subjects with new-onset T1D (within 2 days of diagnosis) and three control individuals without T1D (see Table 1 for clinical characteristics). Protocols were approved by the Indiana University Institutional Review Board. Parents of subjects provided written informed consent. Figure 3 shows the quantitation of unmethylated and methylated *INS* DNA copy numbers/µl serum, converted to $\log_{10}$ from these controls and

T1D subjects. The data show that individuals with T1D exhibit elevated levels of both methylated and unmethylated *INS* DNA compared to controls, similar to data reported previously[6].

Discussion

Methylation of cytosines by DNA methyltransferases allows for the epigenetic control of transcription at many genes. The *INS* gene in humans is almost exclusively expressed in islet β cells, and there appears to be a correlation between the frequency of methylation of cytosines in the *INS* gene to silencing of its transcription[11]. As such, most cell types show substantially higher frequencies of methylation of the *INS* gene at various cytosines compared to β cells[11–13]. It has been proposed that the prevalence of β cell death could be monitored by analysis of the serum levels of cell-free unmethylated *INS* DNA, which would arise largely from dying β cells that liberate their DNA[12].

The bisulfite reaction converts unmethylated cytosines into uracils, leaving methylated cytosines unchanged[14]. Sequencing or PCR analysis of bisulfite-converted DNA therefore allows determination of whether the original cytosine was methylated or not. Methylation-specific PCR using dye-based technology exploits differences in PCR efficiency owing to 3′ base-pair mismatches between primers and template, and thereby allows distinction of unmethylated vs. methylated cytosines following bisulfite conversion of DNA[15]. Accordingly, dye-based PCR was used to quantitate the relative ratio of unmethylated to methylated *INS* in early studies of T1D subjects[12,16]. However, dye-based PCR technology has several drawbacks, including limited specificity, lack of absolute quantitation of DNA fragment concentrations, and the inability to multiplex (*i.e.*, detect both methylated and unmethylated DNA species in the same reaction). Other groups have performed direct sequencing of cell-free DNA fragments, a technique that allows monitoring of several differentially methylated CpG sites with much greater specificity[9]. However, the sequencing technique requires large sample volumes, is not easily amenable to use of banked samples, and is expensive.

As a result of these limitations, our group and others have employed digital PCR, a technique that provides absolute quantitation of DNA fragments, requires only microliter quantities of serum from fresh or banked samples, is amenable to multiplexing, shows greater sensitivity than traditional quantitative PCR, and is less expensive than direct sequencing. Digital PCR technology involves the use of conventional primer/probe assays and a microfluidics-based partitioning of a water-in-oil PCR reaction into ~ 20,000 droplets. Following thermal cycling of the partitioned reaction, the droplets are subsequently analyzed by a flow cytometer to identify droplets with positive and negative signals. Poisson statistics is used to calculate absolute quantities (copy numbers) of each DNA species. The conceptual details of digital PCR have been described elsewhere[17].

This protocol describes the measurement of β cell death in humans by measuring differentially methylated *INS* DNA by digital PCR. Our data presented here and elsewhere[6] show that primer/probe combinations that detect differential methylation of cytosine at position -69 (relative to the transcriptional start site of *INS*) exhibit sufficient specificity for

either the unmethylated cytosine (FAM probe) or the methylated cytosine (VIC probe) (see Figure 1A and B). Mixtures of plasmids containing both methylated and unmethylated *INS* fragments at position -69 bp (*i.e.*, bisulfite-converted DNA having either uracil or cytosine at position -69 bp) exhibit droplets containing one or the other plasmid, or both plasmids (double-positive signals in Figure 1C). The Poisson statistical calculation utilizes the droplet numbers that are positive for a given probe (whether single-positive or double-positive) to derive the copy numbers of each methylated or unmethylated DNA fragment.

When utilizing human serum for the measurement of differentially methylated cytosines at position -69 bp in cell-free DNA (Figure 3), our data demonstrate two key features: (1) levels of methylated *INS* are 5–10-fold higher in these subjects than unmethylated *INS* (regardless of diagnosis), and (2) absolute levels of both unmethylated and methylated *INS* are higher in subjects with new-onset T1D compared to controls. Whereas the higher levels of methylated *INS* compared to unmethylated *INS* likely reflect the greater burden of cell-free DNA emanating from non-β cells, the higher levels of both species of *INS* in new-onset T1D subjects likely reflects both an increase in prevalent β cell death (unmethylated *INS*), and possibly death/turnover of other cell types that are associated with the disease state (*e.g.,* innate and adaptive immune cells). Further speculation on the elevations of both species of *INS* in new-onset T1D subjects was discussed previously[6]. Regardless of the sources of both species of *INS* in serum from these individuals, it is notable that measurement of ratios of unmethylated to methylated *INS* (as reported by other investigators using dye-based PCR) would not have picked up the significant differences in absolute levels described here and in our recent publication[6], thus emphasizing the power of digital PCR for this type of biomarker analysis.

Some precautions and limitations should be noted. We feel it is important that blood is processed within 4 hr of collection and either used immediately for DNA isolation or stored at −80 °C for future use. In unpublished studies, we have observed some loss of DNA recovery after 4 hr of collection, perhaps owing to DNA degradation, as seen in studies of long-term storage of samples[18]. Because of the increased sensitivity of digital PCR, special care should be taken to avoid contamination of samples that would otherwise not be noticeable in traditional qPCR Finally, a key limitation of the technique is the analysis of samples in which droplet generation does not exceed ~10,000 droplets. Such samples do not provide reliable Poisson statistics, and as such may be considered uninterpretable. Multiple causes of such low droplet generation might include technical errors in sample handling, technical issues with the droplet generator, or issues related to quality of reagents used.

In summary, this report describes the detailed protocol for measurement of differentially methylated, cell-free *INS* DNA at position -69 bp by digital PCR. The protocol described here can be adapted to any DNA species for which detection of differentially methylated cytosines is desired, whether from circulation or from isolated cells and tissues, and can provide absolute quantitation of DNA fragments.

Materials

Name	Company	Catalog Number	Comments
Red Top Vacutainer	Beckon Dickinson	366441	no additive, uncoated interior, 10 ml
Cryovial Tube	Simport	T310-3A	polypropylene, screw cap tube, any size
QIAamp DNA Blood Mini Kit	Qiagen	51106	
Poly(A)	Sigma	P9403	disloved in TE buffer (10 mM Tris-Cl pH 8.0 + 1 mM EDTA) to 5 μg/μl
Absolute Ethanol (200 Proof)	Fisher Scientific	BP2818-500	
DPBS (with $CaCl_2$ and $MgCl_2$)	Sigma	D8662	
0.2 ml PCR 8-strip Tubes	MidSci	AVST	
8-strip Caps, Dome	MidSci	AVSTC-N	
EZ DNA Methylation-Lightning Kit	Zymo	D5031	
ddPCR Supermix for Probes (No dUTP)	Biorad	1863024	
Buffer Control for Probes	Biorad	1863052	
Human Unmethylated/Methylated Primer/Probe mix	Life Technologies	AH21BH1	
EcoR1	New England Biolabs	R0101L	
twin.tec PCR Plate 96, semi-skirted	Eppendorf	951020346	
Pierceable Foil Heat Seal	Biorad	1814040	
PX1 PCR Plate Sealer	Biorad	1814000	
QX200 AutoDG Droplet Digital PCR System	Biorad	1864101	
Automated Droplet Generation Oil for Probes	Biorad	186-4110	
DG32 Cartridge for Automated Droplet Generator	Biorad	186-4108	
Pipet Tips for Automated Droplet Generator	Biorad	186-4120	
Pipet Tip Bins for Automated Droplet Generator	Biorad	186-4125	
C1000 Touch Thermal Cycler	Biorad	1851197	
QX200 Droplet Reader	Biorad	186-4003	
ddPCR Droplet Reader Oil	Biorad	186-3004	

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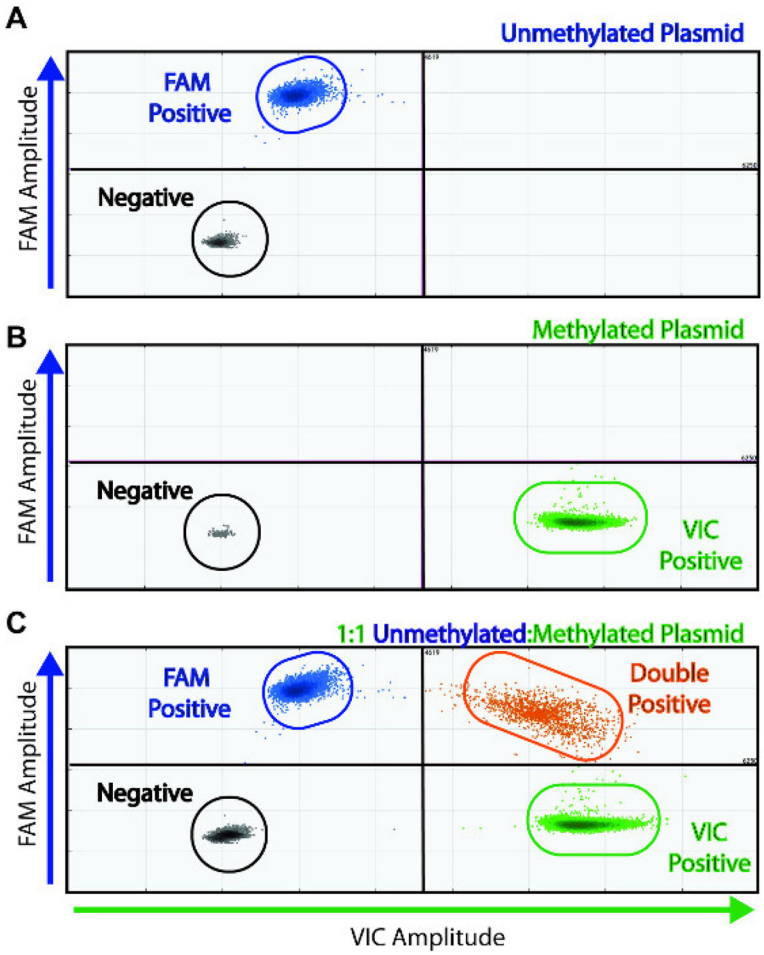

Figure 1. Representative 2-D Plots of Plasmid Controls

Plasmid standards were generated by cloning fragments of bisulfite-converted *INS* DNA harboring unmethylated or methylated cytosine at position -69 bp relative to the *INS* transcriptional start site. Shown are 2-D plots using plasmid containing unmethylated **(*A*)** and methylated **(*B*)** *INS* DNA and for a 1:1 mixture of the two plasmids **(*C*)**. Arrows identify the unmethylated, methylated, and unmethylated + methylated (double-positive) *INS* DNA-containing droplets. FAM = Target 1; VIC = Target 2. Please click here to view a larger version of this figure.

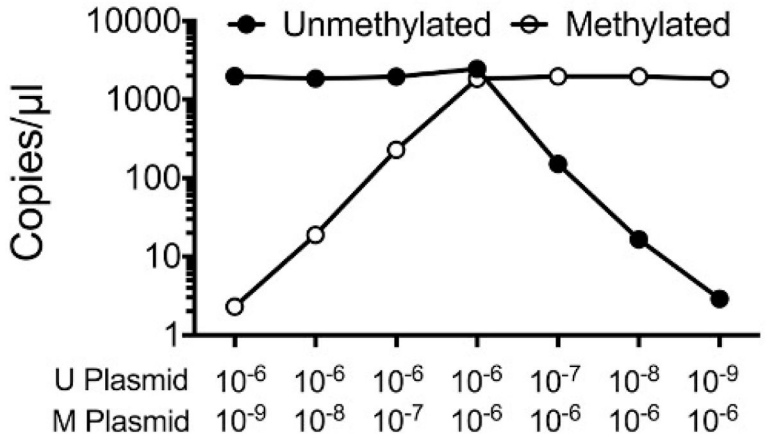

Figure 2. Quantitation of DNA Targets using Control Plasmid Mixtures

Plasmid standards containing cloned fragments of bisulfite-converted *INS* DNA harboring unmethylated or methylated cytosine at position -69 bp relative to the *INS* transcriptional start site were mixed at the various combinations shown, then subjected to multiplex digital PCR. The figure shows the quantitation of target DNA fragments, presented as copies/µl; r^2 = 0.989 for unmethylated *INS* DNA and r^2 = 0.998 for methylated *INS* DNA. Please click here to view a larger version of this figure.

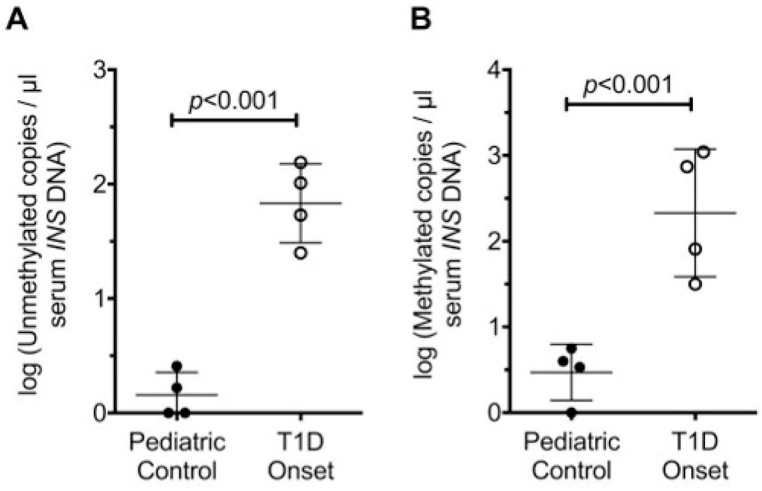

Figure 3. Circulating Unmethylated and Methylated *INS* DNA Levels in Controls and Subjects with T1D

Serum samples were collected from 4 youth with new-onset T1D and from 4 disease-free, unrelated controls, then processed for measurement of differentially methylated *INS* DNA by digital PCR. Shown are results of digital PCR assays for unmethylated **(*A*)** and methylated **(*B*)** *INS* DNA. Data is displayed as individual points and mean ± SEM. Statistics were analyzed by a two-tailed parametric Students *t* test. Please click here to view a larger version of this figure.

Table 1

Demographics of Controls and Subjects with T1D.

	Controls	T1D at onset
Age (years)	10.3 ± 1.3	9.2 ± 1.0
Female/Male	1:3	1:3
BMI Z-Score	0.30 ± 0.9	0.74 ± 0.9
HbA_{1C} (%)		11.1 ± 0.6
C-Peptide (pmol/L)		285.5 ± 37.0