

ONLINE ONLY APPENDIX

Validity and Reproducibility of Measurement of Islet Autoreactivity by T-cell Assays in Subjects with Early Type 1 diabetes

Kevan C Herold MD, Barbara Brooks-Worrell PhD, Jerry Palmer MD, H Michael Dosch MD, Mark Peakman MD, Peter Gottlieb MD, Helena Reijonen PhD, Sefina Arif PhD, Lisa Spain PhD, Clinton Thompson, M.S., John M. Lachin ScD, and The Type 1 Diabetes TrialNet Research Group*

* The TrialNet Study Group is presented at www.diabetestrialnet.org.

From: The Biostatistics Center, The George Washington University (J.M. Lachin, C Thompson), Rockville, MD; Yale University (K. Herold), University of Washington (J Palmer and B Brooks-Worrell), University of Toronto (HM Dosch), Dept of Immunobiology and NIHR Biomedical Research Centre at Guy's and St Thomas' NHS Foundation Trust and Kings College, London, UK (S Arif and M Peakman), University of Colorado (P. Gottlieb), Benaroya Research Institute (H Reijonen), and NIDDK

Participating Investigators

Type 1 Diabetes TrialNet T-cell Validation Study I:

Clinical Investigators:

Dorothy Becker MD, Children's Hospital of Pittsburgh, Pittsburgh, PA
Penelope Bingley MD, University of Bristol, Bristol, UK
Emanuele Bosi MD, San Raffaele Hospital, Milan, IT
Bruce Buckingham MD, Stanford University, San Francisco, CA
H. Peter Chase MD, Barbara Davis Center for Childhood Diabetes, Denver, CO
Peter Colman MD, Walter and Eliza Hall Institute, Melbourne, AU
Mark Daniels MD, Children's Hospital of Orange County, CA
Stephen Gitelman MD, University of California San Francisco, San Francisco, CA
Robin Goland MD, Columbia University, NYC, NY
Peter Gottlieb MD, Barbara Davis Center for Childhood Diabetes, Denver, CO
Leonard Harrison MBBS, MD, DSc; Walter and Eliza Hall Institute/Royal Melbourne Hospital, Melbourne, AU
Kevan Herold MD, Yale University School of Medicine, New Haven, CT
Francine Kaufman MD, Children's Hospital Los Angeles, Los Angeles, CA
Jennifer Marks MD, University of Miami, Miami, FL
Toni Moran MD, University of Minnesota, Minneapolis, MN
Tihamer Orban MD, Joslin Diabetes Center, Boston MA
Philip Raskin MD, University of Texas, Houston, TX
Henry Rodriguez MD, Indiana University, Indianapolis, IN
Desmond Schatz MD, University of Florida, Gainesville, FL
Robert Sherwin MD, Yale University
Diane Wherrett, MD; Hospital for Sick Children; Toronto, Canada
Darrell Wilson MD, Stanford University, San Francisco, CA

T-Cell Laboratories:

Cellular Immunoblot: Barbara Brooks-Worrell PhD, Jerry Palmer MD, Puget Sound Health Care System, University of Washington, Seattle, WA;

T-cell Proliferation: Michael Dosch MD, University of Toronto, Toronto, Canada.

Tetramer: Helena Reijonen MD, Will Leighty, Gerald Nepom MD,, Benaroya Research Institute, Seattle WA.

Cytokine ELISpot, USA: Peter Gottlieb MD,, Barbara Davis Diabetes Center, University of Colorado, Denver Colorado.

Cytokine ELISpot, UK: Mark Peakman MD,, University of Bristol, Bristol, UK.

Central Laboratories:

The Beta Cell Function Laboratory: Jerry Palmer MD,, University of Washington, Seattle, WA;

The Central ICA Laboratory: William Winter MD, University of Florida, Gainesville, FL

The Central Autoantibody Laboratory: George Eisenbarth MD, PhD, Barbara Davis Diabetes Center, University of Colorado, Denver, CO.

Coordinating Center:

John M. Lachin ScD, Heidi Krause-Steinrauf MS, Mary Foulkes PhD, Paula Friedenberg-McGee MS, Clinton Thompson MS, The George Washington University Biostatistics Center, Rockville, MD.

Chairman's Office:

Jay Skyler MD: University of Miami, Miami, FL;

Carla Greenbaum MD: Benaroya Research Institute, Seattle, WA

TrialNet Study Group: www.Diabetestrialnet.org

This Online Only Appendix presents supplemental material not contained in the published manuscript.

Detailed Laboratory Methods:

The detailed methods for each laboratory assay follow.

Cellular Immunoblotting. Cellular immunoblotting was performed as previously described(1; 2) . Human pancreata were obtained from the NIH Islet Consortium. Islet cells were subjected to preparative 10% SDS-PAGE. Following electrophoresis, the gels were electroblotted onto nitrocellulose (BioRad, Richmond, CA) at 30 mA overnight. The blots were sliced into 18 sections by apparent size, and nitrocellulose particles were prepared from the individual sections, and the nitrocellulose particles used to stimulate PBMCs in vitro. Control wells contained nitrocellulose particles without antigen. **Table A1** describes the characteristics of the individual blots.

A stimulation index (SI) for each molecular weight section was calculated as the ratio of the mean CPM among the experimental wells relative to the CPM of the control wells. Positive proliferation for a given blot was considered to be an SI > 2.0 which corresponds to greater than the mean + 3 SD of control values (2). Antigen doses and specificity of PBMC responses of type 1 diabetes patients to the islet protein preparations and known islet autoantigens using cellular immunoblotting have been previously described (2). PBMC responses to tetanus toxoid were used as a control antigen along with the PBMC responses to mitogens, which were included to test for viability of the cultures. PBMC responses of Type 1 diabetes patients, autoantibody positive type 2 diabetes patients, autoantibody negative Type 2 diabetes patients, and controls to tetanus toxoid have been shown to be similar. Based upon results in over 60 controls and 60 patients with Type 1 diabetes, cellular immunoblotting is considered positive when > 3 blot sections have an SI > 2.0 (2).

T-cell Proliferation: Peripheral blood mononuclear cells, from blood samples shipped overnight by courier, were prepared by Ficoll-Hypaque centrifugation as described (3-5). The average cell yield was near 1×10^6 /ml of heparinized blood. Washed and viable cells were seeded (1×10^5 /well) into BD flat bottom microplates containing test or control antigens in 200 μ L in serum- and protein-free Ex-Cell Hybri-Max medium (Sigma, St. Louis, MO). Test antigens were used at 1-4 concentrations (0.1-10 μ g), depending on cell yields. Ten units of recombinant human IL-2 were added to all test wells as described previously (4). Depending on the cell yield as well as the blood volume transmitted, not all test antigens could be used in all tests. Cultures were incubated (37°C, 5% CO₂) for five days, receiving a tritiated thymidine pulse (1 μ Ci) for the last 18 hours. Cultures were then harvested and counted by liquid scintillation. To compare results from different donors, results were transformed into stimulation indices (SI, cpm test antigen/cpm antigen free control cultures). An SI of 1.5 or larger was considered a positive response. To distinguish different test antigens, these were separated into different groupings (3). **Table A2** describes the antigens used for the T cell proliferation assay.

An autoreactive T cell score was generated from all positive autoantigen responses divided by the total number of autoantigens tested. Test antigens were also scored for an environmental score, i.e. the number of positive responses to environmental antigens tested. In addition, an overall T cell score was generated, summarizing all positive responses. These

T cell scores were used in the analysis. An overall T cell score of 3 or larger was considered evidence for the presence of autoimmunity in a given sample.

UK-ELISPOT: Peripheral blood mononuclear cells (PBMCs) producing interferon- γ (IFN- γ) in response to stimulation with synthetic peptides representing naturally processed and presented epitopes of IA-2, GAD65 and proinsulin were detected by cytokine ELISPOT as described (6). Only samples from HLA-DR3 or 4+ individuals were used. **Table A3** describes the synthetic peptides used.

All single peptides were tested at 10 μ M final concentration; the pool was tested at 3 μ M and 10 μ M (final concentration of individual peptide components). Control analytes were peptide diluent alone (negative control wells; DMSO) or a pentavalent preparation of pertussis, diphtheria, *Haemophilus influenza* B, polio and tetanus toxoid vaccines (control positive wells; Pediacel, Sanofi Pasteur MSD, Maidenhead, UK). A stimulation index (SI, derived as number of spots in test analyte wells/number in negative control wells) of ≥ 3.0 was designated as a positive response to an analyte. Samples showing a response to one or more analytes were classified as positive indicating disease. Blood samples were excluded from analysis on the basis of poor quality if the viability according to dye exclusion was <95%; the yield of PBMCs was <0.5x10⁶/ml blood; or there was no response (SI<3.0) to Pediacel.

US-ELISPOT: Samples were shipped from the No American Clinical Centers via overnight courier. PBMCs producing interferon- γ (IFN- γ) in response to stimulation with synthetic peptides representing naturally processed and presented epitopes of IA-2, GAD65 and proinsulin were detected by cytokine ELISPOT as previously described by the UK ELISPOT assay (6).

Tetramer: The construction of the expression vectors for generation of soluble DR0401 (DRA*0101/DRB1*0401), DR0404 (DRA1*0101/DRB1*0404) or DR0301 (DRA1*0101/DRB1*0301) molecules has been described previously (7). Briefly, a site-specific biotinylation sequence was added to the 3' end of the DRB1*0401 or DRB1*0404 leucine zipper cassette, and the chimeric cDNA was subcloned into a Cu-inducible *Drosophila* expression vector. DR-A and DR-B expression vectors were co-transfected into Schneider S-2 cells, the class II monomers then purified, concentrated and biotinylated. The desired peptide was loaded for 48-72 hours, and tetramers were formed by incubating class II molecules with PE-labeled streptavidin.

Peripheral blood mononuclear cells (PBMC) were separated from the heparinized blood by gradient centrifugation (Lymphoprep, Nycomed, Oslo, Norway) and expanded *in vitro* in the presence of a specific peptide. **Table A4** describes the peptides that were used:

Briefly, CD4+ cell population was separated from the PBMC using “no-touch” CD4+ T cell isolation technique (Miltenyi Biotec, Bergisch Gladbach, Germany). Antigen presenting cells were adhered from the CD4 negative cell population on the 48-well tissue culture plate for at least 1 hr (3x10⁶/well). After removing the non-adherent cells and gentle washing, CD4+ cells were added into the wells (2x10⁶/well). Cells were cultured with RPMI 1640 containing 10% v/v pooled human serum in the presence of pooled or single peptides (selected by the HLA type) or a positive control peptide (a flu peptide chosen by the HLA type) at a concentration of 10 μ g/ml for 14 days. When peptide pool was used the concentration of each peptide in the mixture was 10 μ g/ml. On day 7 and, if needed, on day

10, IL-2 was added into the culture. On day 14 the cells were stained using 10 μ g/ml of PE-labeled HLA-DR0401/04 or 0301 tetramers containing a specific or a negative control peptide for 2 hours at 37°C, and then stained on ice for 20 min with CD3-FITC and CD4-PerCP antibodies. Cells were washed with PBS containing 1% Fetal Bovine Serum and the flow cytometry analysis was performed by using FACS-Calibur flow cytometer (BD Biosciences, San Jose, CA). The data analysis was performed by using the FloJo program (Tree Star Inc, Ashland, OR). The cut-off for negative tetramer staining using the irrelevant tetramer was set at 0.25%. The frequency of tetramer positive cells using the relevant tetramer was determined and the ratio of tetramer positive cells above the background was assigned. The following criteria was used to determine the positivity of the sample:

Positive: Ratio 4x or higher above the background (1.0% or more)

Indeterminate = Low positive: Ratio 2 to 4x above the background (0.5 to 0.99%)

Negative: Ratio less than 2x above the background (less than 0.5%)

Additional Statistical Methods:

Quantitative reproducibility of the individual analytes was assessed using the reliability coefficient (8).

Additional Results:

Influence of Elapsed Interval Between Tests. To assess the influence of the elapsed duration between the two successive tests of subjects on the reproducibility of each assay, the subjects were stratified according to the elapsed duration into three categories: 2-7 days, 8-14 days and >14 days. Within each laboratory the kappa agreement statistic was computed for each of these categories, and the differences among categories tested using a general test for homogeneity.

Table A5 shows the resulting kappa statistics within each laboratory. There was no heterogeneity in kappa among the duration categories for autoantibodies. However, there was nominally significant heterogeneity among the duration categories for all but the UK ELISpot laboratory, with a consistent trend towards decreasing agreement as the elapsed duration increases only among the TCP and tetramer laboratories, not the US ELISpot or cellular immunoblot laboratories. Thus it is possible that the reproducibility among some, if not all, of the laboratories was influenced by the duration between the two successive collections.

Influence of Indeterminate Assay Results. Each laboratory could classify a specimen as positive for T-cell activity, or negative, or indeterminate when neither a clear positive or negative result was observed. Since the objective was to detect diabetes versus not, such indeterminate results were included as a negative classification. As shown in Table 2 of the main paper, such results were observed for the CI, Tetramer and UK-ELISPOT assays. The data from these three laboratories were re-analyzed with the indeterminate results treated as missing or non-evaluable and the principal results for each are shown in **Table A6**.

Table A6.A presents the discriminant validity of each assay excluding the indeterminate results that should be compared to Table 2 of the main paper. Likewise Table A3.B presents the reproducibility of each that should be compared to Table 4 of the main paper. Excluding the indeterminate results has minor effects on the properties of the cellular CI and UK ELIPOT laboratories owing to the small number of such specimens. The

sensitivity and odds ratio for the tetramer laboratory increased slightly (from 46% and 2.1 to 61% and 2.37), as did the kappa and the Entropy R^2 (from 0.41 and 0.13 to 0.68 and 0.39). However, there were far fewer subjects with evaluable and non-indeterminate specimens with the tetramer than the other assays.

Qualitative Analyte Results. Table A7 presents the summary of the discriminant validity (sensitivity, specificity, etc.) for each individual analyte from each individual laboratory, and the quantitative reproducibility of that analyte from the duplicate collections, represented by the reliability coefficient (ρ) that represents the proportion of all variation that is attributable to the variation in the hypothetical true values in the population. In the present context with repeat collections 2 to 28 days apart, the remainder is the proportion of total variation due to random within subject variation and to laboratory variation. Thus a value $\rho = 0.8$ implies that 20% of the variation among observed values is attributable to random sources of variation.

The tetramer assay did not measure all peptides in all samples. The DR3 peptides were only measured in subjects with HLA DR3, and likewise the DR4 antigens were measured in DR4 subjects. Also, depending on volume, the pool peptides were only assayed in a subset. Thus the sample sizes vary among the different peptides.

No one analyte from any laboratory showed jointly a sensitivity, specificity and reliability of at least 80%. The maximum quantitative reliability (74%) applied to a Tetramer pool peptide that was assayed in a small number of the DR4 subjects.

Separately for each laboratory, GEE logistic regression models assessed the ability of the analyte qualitatively (positive versus negative) for each assay to distinguish those with diabetes versus controls. For each laboratory, analyses were conducted for each analyte individually in univariate analyses, and of all analytes jointly in an aggregate multivariate analysis. Table A8 presents the univariate and multivariate (jointly-adjusted) odds ratio for the analytes of each laboratory.

For the CI laboratory, a number of blot sections were statistically significant with odds ratios ranging up to 23.7 for blot 16. However, owing to the intercorrelations among the blot sections, only blot 16 remained statistically significant at $p < 0.01$ with an adjusted odds ratio of 12.8. While blot 8 had a univariate odds ratio of 7.89 ($p < 0.01$), its adjusted odds ratio increased to 17.5, however, the confidence interval is wider and the p-value higher.

For the TCP laboratory, all but one of the analytes had a statistically significant univariate odds ratio, but none exceed 4.0. In the multivariate model, a number of analytes were redundant when included in the model and it was required that they be removed in order to fit a multivariate model. In this model only one analyte was significant at the 0.01 level and its positivity was associated with a reduced odds of having diabetes when adjusted for other analyte effects, an indication that there is redundancy in the information provided by the set of analytes in the model.

For the Tetramer laboratory, no analyte was significant univariately. It was not possible to fit any multivariate models because the DR3 and DR4 peptides were measured in different subjects, the pool peptides in only a subset of these, and the sample size was inadequate to fit separate logistic models using either the two DR3 peptides among DR3 subjects, or using the two DR4 peptides among DR4 subjects.

For the US and UK ELISPOT laboratories, no analyte was significant.

Table A1: The molecular weight ranges for each blot section in the Cellular Immunoblot (CI)

Blot Number	MW range
1	177+ kDa
2	151-177 kDa
3	131-151 kDa
4	109-131 kDa
5	93-109 kDa
6	79-93 kDa
7	66-79 kDa
8	56-66 kDa
9	48-56 kDa
10	41-48 kDa
11	34-41 kDa
12	29-34 kDa
13	24-29 kDa
14	21-24 kDa
15	17-21 kDa
16	15-17 kDa
17	13-15 kDa
18	5-13 kDa

Table A2: Antigens used for the T cell proliferation assay (TCP) dosch edits

Analyte name	Antigen
MIPBLG	β -lactoglobulin
MIPBSA	Bovine serum albumin
MIPAB	BSAp149 (ABBOS epitope in BSA)
MIPCAES	Casein
ISLAT69	Tep69 (ICA69 epitope)
ISLAGAD	GAD
ISLAGAD55	GAD555
ISLAPI	Proinsulin
GLIAS100	S100 β
GLIAGF	GFAP
CNSAMBP	Myelin basic protein
CNSAEX2	EX-2

Table A3: Peptides used for the UK ELISPOT assay

Analyte name	Peptide
P1	proinsulin C19-A3
R2	IA-2 ₈₅₃₋₈₇₂
R3	IA-2 ₇₉₃₋₈₁₇
R5	IA-2 ₇₀₉₋₇₃₆
3.1	GAD65 ₃₃₅₋₃₅₂
4.11	GAD65 ₅₅₄₋₅₇₅
IA2/P1 pool 3 μ M	Pool of R3, R5, and P1
IA2/P1 pool 10 μ M	Pool of R3, R5, and P1

Table A4: Peptides used to prepare Class II tetramers

Analyte name	Peptide
MDR4GAD555	DR4-GAD65 555-567 tetramer, stimulation with the DR4 binding peptide pool
MDR4GAD274	DR4-GAD274-286 tetramer, stimulation with the DR4 binding peptide pool
DR4GAD555	DR4-GAD65 555-567 tetramer, stimulation with the GAD65 555-567 peptide
DR4GAD274	DR4-GAD65 274-286 tetramer, stimulation with GAD65 274-286 peptide
MDR3PRO	DR3-ProIns B24-C36 tetramer, stimulation with the DR3 binding peptide pool
MDR3GAD247	DR3-ProIns B24-C36 tetramer, stimulation with the DR3 binding peptide pool
DR3PRO	DR3-ProIns B24-C36 tetramer, stimulation with the ProIns B24-C36 peptide
DR3GAD247	DR3-GAD65 247-266 tetramer, stimulation with the GAD65 247-266 peptide

Table A5: Time-stratified Kappa coefficients for each Lab, by Diabetes status. The value in parenthesis is the standard error associated with the kappa statistic and the p-value tests for kappa homogeneity across the time-strata. Kappa is missing when all results are constant for the two collections within an interval (e.g. only 'Positive' interpretations rendered on the first visit).

Laboratory	2 – 7 days		8 – 14 days		> 14 days		Homogeneity p <
	N	Kappa (SE)	N	Kappa (SE)	N	Kappa (SE)	
US ELISpot	7	-0.40 (0.19)	14	0.43 (0.23)	8	0.38 (0.30)	0.01
Cellular Immunoblot	13	0.58 (0.25)	19	0.68 (0.17)	13	0.53 (0.23)	<0.0001
T cell proliferation	26	0.69 (0.14)	22	0.44 (0.19)	15	0.32 (0.24)	<0.0001
Tetramer	16	0.58 (0.21)	18	0.44 (0.20)	16	0.09 (0.26)	0.002
UK ELISpot	19	0.16 (0.22)	16	0.25 (0.21)	14	-0.14 (0.25)	0.48
Autoantibodies	44	0.65 (0.13)	44	0.82 (0.09)	42	0.61 (0.12)	0.33

Table A6. Principal results from a reanalysis with indeterminate specimen results treated as missing, for those three laboratories with such results.

A. Numbers of specimens assayed and measures* of the ability to discriminate between subjects with and without T1D for each T-cell assay.

	# (%) Specimens†	Sensitivity	Specificity	Odds Ratio‡	p value
Cellular Immunoblot	116 (65%)	81%	87%	29.4	<0.0001
UK ELISpot	101 (93%)	66%	67%	3.91	0.0016
Tetramer	85 (46%)	61%	61%	2.37	0.093

*Sensitivity and specificity estimated from a GEE model using all collections in all subjects

† The number and percent of all specimens received that were considered evaluable and not indeterminate among 179 collections for CI; and 183 DR3 and/or DR4 in North America for Tetramer and 109 in the UK Elispot.

‡ The ratio of the odds that a specimen is from a subject with T1D for a positive assay versus that for a negative assay

B. The numbers of subjects with evaluable assays from the two successive visits, the simple proportion of agreement between visits, the Kappa index of agreement and its 95% confidence limit and the Entropy R^2 .

	n*	% Agreement	Kappa	95% CI	Entropy R^2
Cellular Immunoblot	42	86%	0.70	0.49, 0.92	0.414
UK ELISpot	43	56%	0.118	-0.19, 0.41	0.009
Tetramer	25	88%	0.68	0.40, 0.96	0.39

* The number of subjects for whom the laboratory considered the specimen from the two visits to be evaluable and not indeterminate.

Table A7. Separately for each diagnostic analyte within each laboratory, the numbers of evaluable specimens, the discriminant validity (sensitivity, specificity, positive and negative predictive values) and the quantitative reproducibility (reliability coefficient).

A. Cellular Immunoblot

Analyte	# Specimens Control / T1D	Sens	Spec	PPV	NPV	Reliability (95% CI)
BLOT1	74 / 45	29%	85%	56%	67%	0.07 (0, 0.36)
BLOT2	74 / 45	15%	93%	58%	64%	0.26 (0, 0.53)
BLOT3	74 / 45	38%	91%	72%	71%	0.42 (0.18, 0.66)
BLOT4	74 / 45	48%	86%	70%	74%	0.26 (0, 0.53)
BLOT5	74 / 45	46%	88%	71%	74%	0.53 (0.32, 0.74)
BLOT6	74 / 45	27%	89%	62%	67%	0.14 (0, 0.42)
BLOT7	74 / 45	15%	93%	58%	64%	0.30 (0.04, 0.57)
BLOT8	74 / 45	25%	96%	80%	68%	0.28 (0.01, 0.55)
BLOT9	74 / 45	35%	86%	63%	70%	0.43 (0.19, 0.67)
BLOT10	74 / 45	23%	86%	52%	65%	0.13 (0, 0.42)
BLOT11	74 / 45	38%	91%	72%	71%	0.46 (0.23, 0.69)
BLOT12	74 / 45	19%	92%	60%	65%	0.25 (0, 0.52)
BLOT13	74 / 45	29%	84%	54%	67%	0.41 (0.16, 0.65)
BLOT14	74 / 45	33%	89%	67%	69%	0.24 (0, 0.51)
BLOT15	74 / 45	42%	89%	65%	72%	0.25 (0, 0.53)
BLOT16	74 / 45	50%	85%	89%	77%	0.60 (0.41, 0.78)
BLOT17	74 / 45	46%	93%	81%	75%	0.55 (0.34, 0.75)
BLOT18	74 / 45	6%	93%	38%	62%	0.36 (0.10, 0.61)

B. TCP

Analyte	# Specimens Control / T1D	Sens	Spec	PPV	NPV	Reliability (95% CI)
CNSAEX2	93 / 58	47%	74%	53%	69%	0.23 (0, 0.46)
CNSAMBP	93 / 58	48%	75%	55%	70%	0.35 (0.13, 0.57)
GLIAGF	93 / 58	50%	68%	49%	68%	0.26 (0.03, 0.49)
GLIAS100	93 / 58	57%	66%	51%	71%	0.41 (0.21, 0.62)
ISLAGAD	93 / 58	62%	69%	55%	74%	0.42 (0.22, 0.62)
ISLAGAD55	93 / 58	60%	67%	53%	73%	0.39 (0.18, 0.60)
ISLAPI	93 / 58	64%	62%	51%	73%	0.41 (0.20, 0.61)
ISLAT69	93 / 58	60%	69%	55%	74%	0.30 (0.07, 0.52)
MIPAB	93 / 58	60%	69%	55%	74%	0.33 (0.11, 0.55)
MIPBLG	93 / 58	60%	66%	52%	73%	0.32 (0.10, 0.55)
MIPBSA	93 / 58	60%	66%	52%	73%	0.32 (0.10, 0.54)
MIPCAES	93 / 58	55%	69%	52%	71%	0.28 (0.05, 0.50)

C. Tetramer

Analyte	# Specimens Control / T1D	Sens	Spec	PPV	NPV	Reliability (95% CI)
MDR4GAD555	14 / 8	50%	71%	50%	71%	0.74 (0.33, 1)
MDR4GAD274	14 / 8	0%	93%	0%	62%	0.46 (0, 1)
DR4GAD555	34 / 32	16%	85%	50%	52%	0.09 (0, 0.48)
DR4GAD274	31 / 31	32%	100%	100%	60%	0.19 (0, 0.59)
MDR3PRO	16 / 6	83%	13%	26%	67%	*
MDR3GAD247	12 / 6	33%	67%	33%	67%	*
DR3PRO	27 / 28	89%	7%	50%	40%	0.67 (0.44, 0.90)
DR3GAD247	23 / 26	12%	61%	25%	38%	0.29 (0, 0.72)

* Too few subjects measured to allow computation of the reliability coefficient.

D. UK-ELISPOT

Analyte	# Specimens Control / T1D	Sens	Spec	PPV	NPV	Reliability (95% CI)
P1	58 / 51	22%	84%	55%	55%	0 (0, 0.14)
R2	58 / 51	16%	90%	57%	55%	0 (0, 0.25)
R3	58 / 51	27%	84%	61%	57%	0.21 (0, 0.51)
R5	58 / 51	20%	91%	67%	56%	0.03 (0, 0.35)
3.1	58 / 51	20%	91%	67%	57%	0 (0, 0.30)
4.11	58 / 51	20%	88%	59%	56%	0 (0, 0.29)
IA2/P1 pool 3 μ M	58 / 51	14%	90%	54%	54%	0 (0, 0.30)
IA2/P1 pool 10 μ M	58 / 51	22%	88%	73%	58%	0.02 (0, 0.33)

E. US-ELISPOT

Analyte	# Specimens Control / T1D	Sens	Spec	PPV	NPV	Reliability (95% CI)
G3IFNG	48 / 39	15%	85%	60%	55%	0.25 (0, 0.61)
P1IFNG	48 / 39	10%	90%	57%	56%	0 (0, 0.34)
R2IFNG	48 / 39	5%	90%	29%	54%	0.44 (0.14, 0.73)
R3IFNG	48 / 39	18%	77%	54%	54%	0.27 (0, 0.61)
R5IFNG	48 / 39	5%	81%	25%	54%	0.26 (0, 0.62)

Table A8: Odds ratio for having diabetes versus not as a function of positivity for each analyte individually (univariate), and jointly (multivariate), separately for each laboratory. The sample sizes for each analyte are presented in Table A7.

A. CI

Analyte	Univariate Models			Multivariate Model		
	Odds Ratio	95% CI	P	Odds Ratio	95% CI	P
BLOT1	2.36	(1.00, 5.55)	<0.05	0.37	(0.09, 1.62)	0.19
BLOT2	2.36	(0.74, 7.49)	0.15	1.29	(0.32, 5.15)	0.72
BLOT3	5.74	(2.21, 14.94)	<0.01	4.21	(1.28, 13.87)	<0.05
BLOT4	5.89	(2.5, 13.86)	<0.01	1.11	(0.34, 3.66)	0.86
BLOT5	6.11	(2.42, 15.41)	<0.01	3.15	(0.70, 14.24)	0.14
BLOT6	3.06	(1.24, 7.59)	<0.05	2.18	(0.28, 16.95)	0.46
BLOT7	2.36	(0.63, 8.75)	0.20	2.30	(0.61, 8.70)	0.22
BLOT8	7.89	(2.11, 29.56)	<0.01	17.46	(1.68, 181.24)	<0.05
BLOT9	3.51	(1.30, 9.49)	<0.05	1.14	(0.31, 4.24)	0.85
BLOT10	1.90	(0.81, 4.47)	0.14	0.16	(0.03, 0.99)	<0.05
BLOT11	5.74	(2.33, 14.14)	<0.01	1.23	(0.37, 4.06)	0.74
BLOT12	2.62	(0.80, 8.58)	0.11	0.76	(0.11, 5.22)	0.78
BLOT13	2.13	(0.85, 5.33)	0.11	1.56	(0.45, 5.44)	0.49
BLOT14	4.13	(1.67, 10.2)	<0.01	0.75	(0.16, 3.56)	0.71
BLOT15	4.09	(1.62, 10.32)	<0.01	0.82	(0.13, 5.14)	0.83
BLOT16	23.67	(4.79, 116.93)	<0.01	12.83	(0.93, 177.59)	<0.1
BLOT17	11.68	(3.51, 38.80)	<0.01	3.03	(0.50, 18.45)	0.23
BLOT18	0.92	(0.19, 4.52)	0.92	0.08	(0.01, 0.82)	<.05

B. TCP

Analyte	Univariate Models			Multivariate Model		
	Odds Ratio	95% CI	P	Odds Ratio	95% CI	P
CNSAEX2	2.50	(1.17, 5.36)	<0.05	1.12	(0.31, 3.98)	0.87
CNSAMBP	2.84	(1.26, 6.43)	<0.05	2.90	(0.64, 13.26)	0.17
GLIAGF	2.10	(0.98, 4.49)	<0.1	0.13	(0.03, 0.53)	<0.01
GLIAS100	2.52	(1.14, 5.55)	<0.05	0.26	(0.02, 2.97)	0.28
ISLAGAD	3.61	(1.63, 8.00)	<0.01	*	*	*
ISLAGAD55	3.04	(1.37, 6.75)	<0.01	10.73	(0.21, 551.04)	0.24
ISLAPI	2.92	(1.32, 6.57)	<0.01	1.85	(0.43, 8.02)	0.41
ISLAT69	3.36	(1.50, 7.53)	<0.01	*	*	*
MIPAB	3.36	(1.50, 7.53)	<0.01	*	*	*
MIPBLG	2.90	(1.32, 6.39)	<0.01	1.74	(0.31, 9.76)	0.53
MIPBSA	2.90	(1.32, 9.41)	<0.01	*	*	*
MIPCAES	2.72	(1.23, 6.00)	<0.05	0.85	(0.08, 9.27)	0.89

* Analyte deleted from the multivariate model owing to inability to fit the model otherwise.

C. Tetramer

Analyte	Univariate Models			Multivariate Model		
	Odds Ratio	95% CI	P	Odds Ratio	95% CI	P
MDR4GAD555	2.50	(0.35, 18.0)	0.37			
MDR4GAD274	*	*	*			
DR4GAD555	1.07	(0.29, 4.02)	0.92			
DR4GAD274	*	*	*			
MDR3PRO	0.71	(0.05, 9.85)	0.81			
MDR3GAD247	1.00	(0.12, 8.23)	1.00			
DR3PRO	0.50	(0.89, 2.85)	0.44			
DR3GAD247	0.22	(0.03, 1.38)	0.11			

Multivariate models could not be fit that allowed for the correlation among repeat collections either using the DR3 peptides alone, or using the DR4 peptides alone, even using just the non-pool peptides.

* Sample size is too small to provide model estimates (MDR4GAD274), or the model is degenerate owing to constant values (all negative) among control subjects (DR4GAD274).

D. UK-ELISPOT

Analyte	Univariate Models			Multivariate Model		
	Odds Ratio	95% CI	P	Odds Ratio	95% CI	P
P1	1.50	(0.58, 3.87)	0.405	1.39	(0.39, 4.99)	0.615
R2	1.61	(0.55, 4.72)	0.384	0.98	(0.26, 3.73)	0.979
R3	2.06	(0.74, 5.71)	0.165	1.14	(0.30, 4.33)	0.850
R5	2.59	(0.84, 8.00)	0.010	1.59	(0.39, 6.41)	0.516
3.1	2.59	(0.84, 7.97)	0.098	2.22	(0.63, 7.83)	0.215
4.11	1.78	(0.53, 5.98)	0.353	1.25	(0.33, 4.71)	0.746
IA2/P1 pool 3 μ M	1.38	(0.39, 4.93)	0.621	0.84	(0.22, 3.25)	0.806
IA2/P1 pool 10 μ M	3.71	(0.88, 15.69)	0.074	2.76	(0.56, 13.64)	0.213

E. US-ELISPOT

Analyte	Univariate Models			Multivariate Model		
	Odds Ratio	95% CI	P	Odds Ratio	95% CI	P
G3IFNG	2.00	(0.49, 8.18)	0.335	2.45	(0.46, 12.95)	0.291
P1IFNG	1.714	(0.39, 7.60)	0.478	2.19	(0.37, 12.99)	0.390
R2IFNG	0.465	(0.09, 2.53)	0.376	0.52	(0.08, 3.54)	0.506
R3IFNG	1.531	(0.42, 5.23)	0.515	1.36	(0.38, 4.92)	0.636
R5IFNG	0.378	(0.07, 1.93)	0.242	0.23	(0.04, 1.30)	0.096

References

1. Brooks-Worrell B, Gersuk VH, Greenbaum C, Palmer JP: Intermolecular antigen spreading occurs during the preclinical period of human type 1 diabetes. *J Immunol* 166:5265-5270, 2001
2. Brooks-Worrell BM, Starkebaum GA, Greenbaum C, Palmer JP: Peripheral blood mononuclear cells of insulin-dependent diabetic patients respond to multiple islet cell proteins. *J Immunol* 157:5668-5674, 1996
3. Winer S, Astsaturov I, Cheung R, Gunaratnam L, Kubiak V, Cortez MA, Moscarello M, O'Connor PW, McKerlie C, Becker DJ, Dosch HM: Type I diabetes and multiple sclerosis patients target islet plus central nervous system autoantigens; nonimmunized nonobese diabetic mice can develop autoimmune encephalitis. *J Immunol* 166:2831-2841, 2001
4. Dosch H, Cheung RK, Karges W, Pietropaolo M, Becker DJ: Persistent T cell anergy in human type 1 diabetes. *J Immunol* 163:6933-6940., 1999
5. Miyazaki I, Cheung RK, Gaedigk R, Hui MF, Van der Meulen J, Rajotte RV, Dosch HM: T cell activation and anergy to islet cell antigen in type I diabetes. *J Immunol* 154:1461-1469., 1995
6. Arif S, Tree TI, Astill TP, Tremble JM, Bishop AJ, Dayan CM, Roep BO, Peakman M: Autoreactive T cell responses show proinflammatory polarization in diabetes but a regulatory phenotype in health. *J Clin Invest* 113:451-463, 2004
7. Novak EJ, Liu AW, Nepom GT, Kwok WW: MHC class II tetramers identify peptide-specific human CD4(+) T cells proliferating in response to influenza A antigen. *J Clin Invest* 104:R63-67, 1999
8. Lachin JM: The role of measurement reliability in clinical trials. *Clinical Trials* 1:553-566, 2004