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PRINCIPAL INVESTIGATOR: Massimo Trucco, M.D.

CONTRACTING ORGANIZATION: Children's Hospital of Pittsburgh
Pittsburgh, Pennsylvania 15213

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6. AUTHOR(S) Massimo Trucco, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Children's Hospital of Pittsburgh Pittsburgh, Pennsylvania 15213 E-Mail: mnt@pitt.edu	8. PERFORMING ORGANIZATION REPORT NUMBER
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13. ABSTRACT (Maximum 200 Words)

Type 1 diabetes is considered an autoimmune disease characterized by the presence of lymphocytes in the islets of Langerhans. The majority of infiltrating cells are actually T lymphocytes that are considered responsible for the destruction of the insulin producing β -cells present in the islets. When the number of dead cells reach 85-90% of the originally existing β -cells, the disease presents, frequently with an abrupt and clinically serious onset. Individuals are considered at high risk to develop the disease, based on their genetic susceptibility (as determined by the presence of susceptibility alleles at various HLA loci) and on the presence in the serum of autoantibodies directed against islet specific autoantigens (the most indicative being GAD65, IA-2, and insulin). In individuals genetically at risk for the disease an environmental component is generally considered to be the triggering event. Viruses, and in particular enteroviruses, are among the most influential environmental triggers of the autoimmune reaction that brings about the disease onset. The aim of this program is to determine whom among the Army personnel is at high risk to develop the disease in order to prevent the unexpected onset of the disease that may be associated with tragic consequences.

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INTRODUCTION

Type 1, or insulin-dependent diabetes mellitus (IDDM), is an autoimmune disease characterized by the presence of infiltrating lymphocytes in the islets of Langerhans. This pathologic scenario is normally defined as "insulinitis". The majority of infiltrating cells are actually T lymphocytes that are considered responsible for the destruction of the insulin producing β -cells present in the islets. When the number of dead cells reach 85-90% of the originally existing β -cells, the disease presents, frequently with an abrupt and clinically serious onset.

Diabetes currently affects an estimated 16 million Americans, and about 800,000 new cases are diagnosed each year. New, promising human trials, aimed at preventing the dramatic onset of the disease, are sponsored by the NIH under the organization umbrella known as TrialNet.

Individuals considered at high risk to develop the disease, based on their genetic susceptibility (as determined by the presence of susceptibility alleles at various HLA loci) and on the presence in the serum of autoantibodies directed against islet specific autoantigens (the most indicative being GAD65, IA-2, and insulin) are included in these trials.

Since being at-risk for the disease is not alone sufficient for one to eventually become diabetic, an environmental component is generally considered to be the triggering event. Viruses, and in particular enteroviruses, are considered among the most influential environmental triggers of the autoimmune reaction that brings about the disease onset.

To determine whom among the Army personnel is at high risk to develop the disease in order to prevent the unexpected onset of the disease that may be associated with tragic consequences if the individual is performing a particularly difficult task (e.g., the pilot of a bomber plane), we proposed three major aims for our study:

- 1) Defining a prophylactic approach against possibly diabetogenic forms of enteroviruses.**
- 2) Setting up an efficient, reliable, and inexpensive molecular approach for typing very large numbers of individuals for the genetic markers associated with susceptibility or resistance to type 1 diabetes.**
- 3) Making available a high-throughput, sensitive screening protocol for diabetes clinical onset prediction (i.e., autoantibody titer testing) in genetically at-risk individuals.**

To reach these aims, members of the Division of Immunogenetics at the Children's Hospital of Pittsburgh (CHP), will be helped by experts in fluorochromes and robotic devices at Carnegie-Mellon University in Pittsburgh (CMU), and specialists in microarray-based ELISA technology from the University of Tübingen, Germany.

1. Characterization of α and β chains of T cell receptor (TCR) from diabetogenic T cell clones.

(William Rudert, M.D., Ph.D., Division of Immunogenetics, CHP)

Our studies of the Coxsackievirus B (CVB), as the possible trigger of a T-cell mediated disease process that brings about overt diabetes, have been focused on obtaining the nucleic acid sequences of the TCR(s) of those individual T-cells which are involved in the attack against the β -cells of the pancreas. The α - and β -chains of the TCR contain variable regions which may differ in each T-cell, determining the specific interactions of each individual T-cell with the antigenic peptide presented by the antigen presenting cell (APC) in its HLA class II molecule.

In order to develop a model system using disease specific TCRs, it is necessary to obtain DNA sequences of both TCR chains present in the same cell. Our current strategy to achieve this goal has already yielded promising preliminary data when applied to peripheral blood samples from healthy individuals (Figure 1).

V α 3	uN	J α	
CATD	V	NRDDKIIFGKGTRLHILP	J α 30
CATD	V	NRDDKIIFGKGTRLHILP	J α 30
CATD	V	NRDDKIIFGKGTRLHILP	J α 30
CATD	V	NRDDKIIFGKGTRLHILP	J α 30
CAT	G	NRDDKIIFGKGTRLHILP	J α 30
CATD	ALE	PLVFGKGTRLSVIA	J α 29
CATD	ALE	PLVFGKGTRLSVIA	J α 29
CA	P	PLVFGKGTRLSVIA	J α 29
CAT	S	NSGNTPLVFGKGTRLSVIA	J α 29
CATD	ED	SGNTPLVFGKGTRLSVIA	J α 29
CATD	ED	SGNTPLVFGKGTRLSVIA	J α 29
CATD	E	SGNTPLVFGKGTRLSVIA	J α 29
CATD	AD*	SGNTPLVFGKGTRLSVIA	J α 29
CATD	A	NRDDKIIFGKGTRLHILP	J α 30
CATD	A	NRDDKIIFGKGTRLHILP	J α 30
CA		SDGQKLLFARGTMLKVDL	J α 16
CATD		GQNFVFGPGTRLSVLP	J α 26
CATD		SGAGSYQLTFGKGTKLSVIP	J α 28

Figure 1. Amino acid sequences encoded by the V α variable region. The sequences were obtained from the expansion of two individual cells (first nine and second group of nine sequences), which expressed the V α 3 mRNA. Only the 3' end of the V α 3 variable region is shown. The abundance of PCR clones containing two different J α s suggests that two V α s were being expressed in each cell. The variation including and surrounding the N region may indicate that some editing was occurring during the expansion of the cells in culture. In the second sample group, the presence but lower abundance of the sequences containing J α 16, J α 26, and J α 28 requires further investigation. The * indicates that a frame shift was required to obtain the J α amino acid sequence.

To begin, we sort single T cells which express TCRs containing the V β 7 chain into 96-well plates. An excess of V β 7 positive T cells was found among the T cells present in the islets of the Langerhans at the onset of diabetes (Conrad et al., *Nature* 371:351, 1994; Luppi et al., *Diabetologia*43:1484, 2000). To facilitate the division and growth of the individual cells, the each well also contains 25 Jurkat cells. The cells are co-cultured for two weeks. Jurkat is a T cell line that does not express TCR V β 7. Because the TCRs of the Jurkat cells contain V α 2 and V β 8 chains, this approach will not be useful for obtaining the sequences of TCRs which utilize those chains. However, the parallel use of a second T-cell line with different known TCR chains will allow the analysis of patient cells that contain the V α 2 and V β 8 chains as well.

The next technical problem was to be able to obtain the maximum amount of the cellular mRNA as possible for the analysis of both the V α and V β chains present in the cells derived from the single original cell (i.e., each T cell clone). This was achieved by using the ExpressDirect™ mRNA Capture and RT System (Pierce, Rockford, IL). The mRNA is captured and purified from the total RNA by binding it to oligo dT segments which have been anchored into a PCR tube. cDNA is synthesized by priming with the oligo dT and becomes covalently anchored to the tube. This approach has two specific advantages for our method. First, all the cDNA prepared from the total amount of captured mRNA is carried into the following PCR reaction, and second, after removal of the PCR reaction, the anchored cDNA may be used for additional PCR reactions. Thus, we can first perform V α amplifications and then a V β amplification using the same cDNA derived from the single original cell clone.

The first V α amplification is performed using a mix of 28 specific forward V α primers (excluding the Jurkat specific V α 2 primer) and the constant region reverse primer for 13 cycles. This first round PCR product is then divided into 28 tubes containing separate V α primers for a second round of 30 cycles. It is expected that only 1 (or 2) of the primers will produce a positive band from the cDNA of any single well. The cDNAs obtained from several wells are tested in parallel to collectively provide negative controls or internal controls for artifacts which are seen with every sample. Positive bands with the correct expected sizes are then cloned and sequenced.

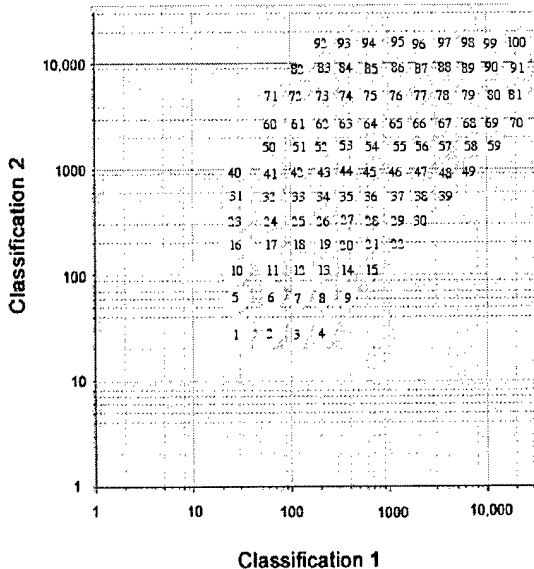
After the first round of V α PCR is performed, the PCR tube with the anchored cDNA is washed twice with TE buffer, and a nested PCR specific for V β 7 is performed. These PCR products are then cloned and sequenced to obtain the V β sequences.

The results obtained in this way have already proven useful in obtaining specific T-cell sequences from the PBL of patients which exhibit a high V β 7 abundance. Further attempts will be made to refine the method to be able to directly obtain the results from single cells in individuals at the onset of the disease.

2. Molecular HLA typing based on fluorescent microspheres in suspension.

(Angela Alexander, Ph.D., Division of Immunogenetics, CHP)

The principle utilized in the DNA analysis portion of this project involves the use of fluorescent microspheres. By combining two different fluorescent dyes in various concentrations during bead production, there are theoretically up to 100 discreetly distinguishable



fluorescent emission spectra possible (Figure 2). Additionally, the introduction of a third fluorescent dye can increase the number of unique spectra to 1000. (Our collaborators at CMU are working towards this specific goal). Individual reactants (i.e., oligonucleotides) anchored to a particular set of microspheres can then be identified based on their unique fluorescence. The Luminex¹⁰⁰ machine analyzes these microspheres in a flow stream where they are individually classified based on their specific fluorescence. The presence of a reporter molecule (e.g., PE) can also be detected and quantified. The combination of these data (presence/ absence of reporter on each particular beadset) allows the accumulation of data from multiple tests from a single sample (Figure 3).

Figure 2

This technology offers several distinct advantages:

1. Capability of simultaneously monitoring up to 100 different analytes within a single sample.
2. The specificity of this system allows the detection of polymorphisms as small as a single nucleotide change.
3. It is easily adaptable to almost any gene of interest.

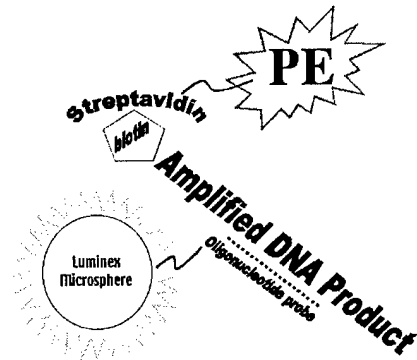
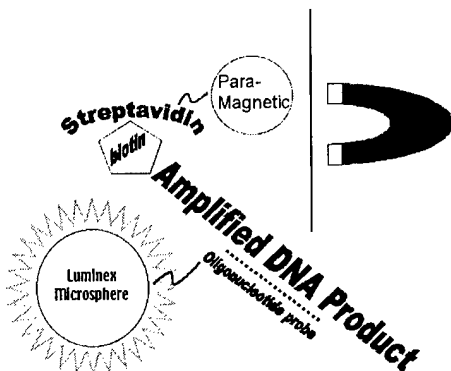


Figure 4

Figure 3



Commercial kits for the analyses of DNA polymorphic sequences using this approach are currently available. However, to make a more generally usable test, rather than the additional step of a reporter molecule, we set up to use the labeled PCR-amplified DNA to physically capture the positive reacting beads and simply detect their presence or absence in each sample (Figure 4).

Using this technology, HLA typing to identify the presence of alleles known to be associated to the development of diseases such as type 1 diabetes, can be performed in a matter of hours instead of days as required for the current standard technology. In order to achieve this, the specificity and reliability of the procedure had first be determined and optimized.

Several steps are important in the bead production and use. First the oligonucleotide probes specific for the HLA alleles must be attached to the microspheres (beads). Once this coupling reaction is optimized, the structure of the oligos themselves (length, branched or straight, number and size of linker components, etc.) as well as hybridization conditions should be varied until the maximum signal is obtained. Interestingly, the structure of the probe that gave the strongest fluorescent signal (branched oligo dendrimer) with a reporter molecule did NOT perform equally well in the magnetic bead capture assay (Figure 5).

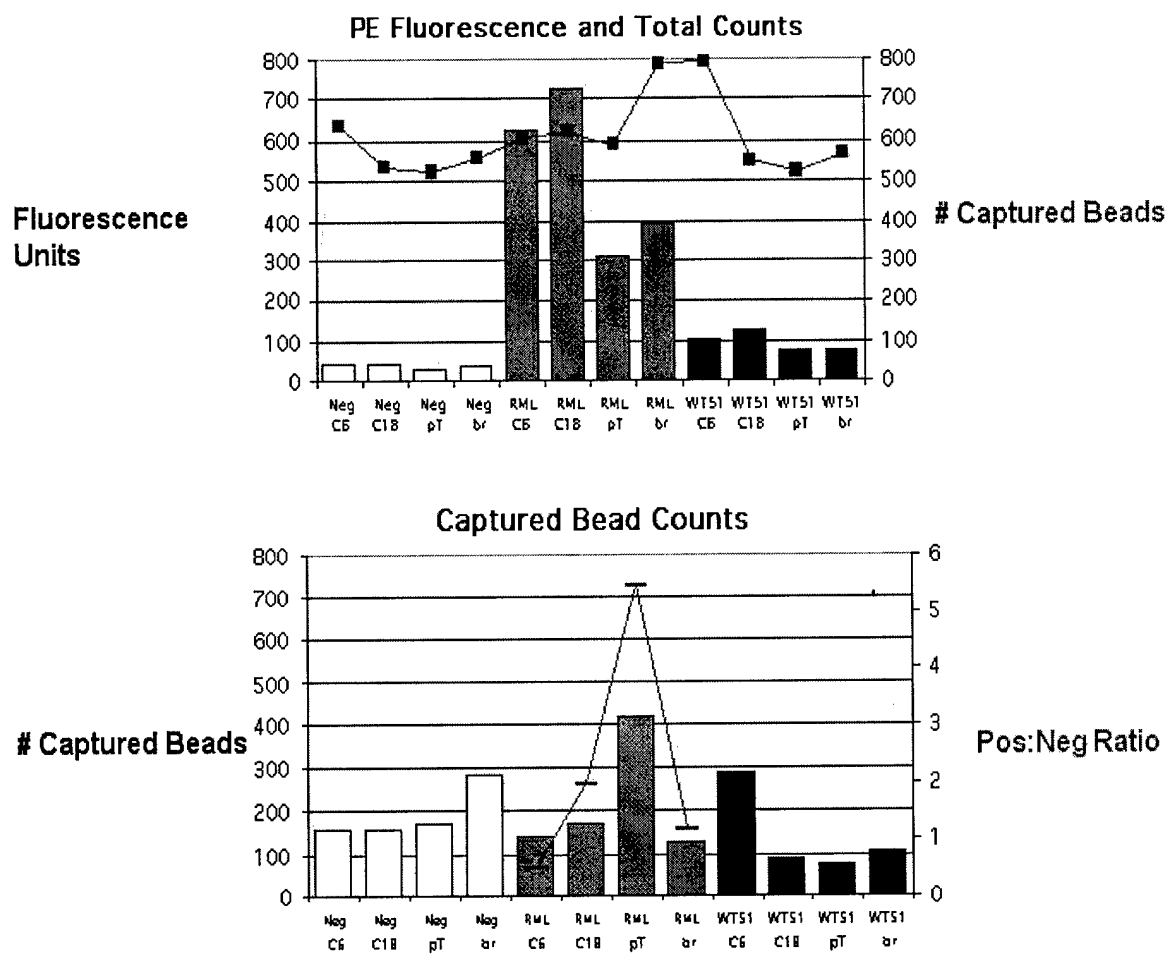


Figure 5. Neg= No DNA, RML= positive cell line DNA, WT51= negative cell line DNA, C6= 6 carbon linker, C18= 18 carbon linker, pT= 12 thymidine residues as linker, br= pT plus branch to incorporate 2 oligo probes on one link to bead.

Our final oligo selection is based on the ability of these beads to bind to Streptavidin (SA)-magnetic beads after hybridization with biotinylated PCR product. The oligonucleotide structure consists of a double probe sequence in head to tail arrangement, a spacer made up of 15 thymidine residues and finally an amino group to enable coupling of the sequence to the microspheres.

Currently the entire panel of oligo probes needed to perform the HLA DQB1 typing is being synthesized and prepared for analysis. Once the entire panel is established, actual typing and fine-tuning of the conditions can occur.

A further increase in specificity (i.e., reduction of background) was achieved by utilizing information learned from the Pyrosequencing experiments (see next section). Here we found that during the PCR reaction, the DQB1 primers also amplify a pseudogene present near the DQB1 locus, i.e., DQB2. Designing a new primer set resulted in a vast reduction of this contaminating amplification and a significantly lowered background fluorescence in the assay (Figure 6).

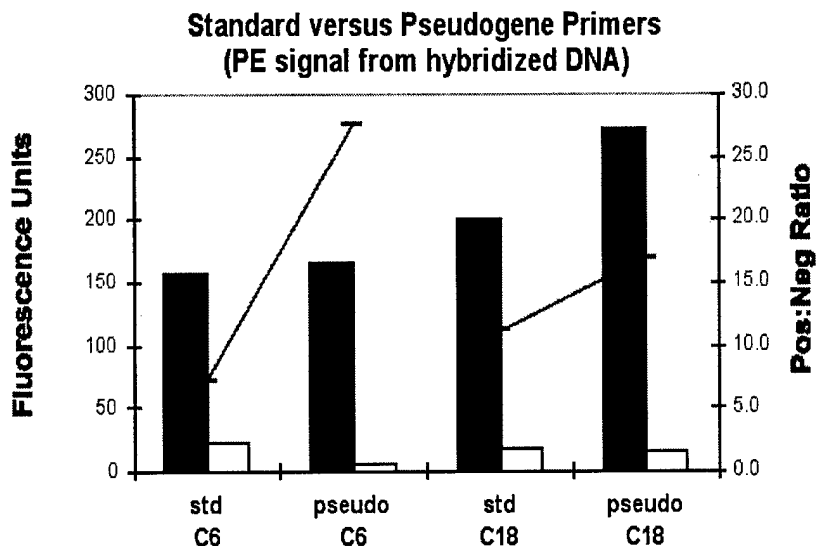


Figure 6.

Additionally, the use of multi-well plates coated with streptavidin is being investigated as an alternative means of capturing and isolating the positively hybridized fluorescent microspheres, thus eliminating the need of magnetic bead separation.

3. Pyrosequencing diabetogenic HLA alleles

(Steve Ringquist, Ph.D., Division of Immunogenetics, CHP)

In parallel with the microsphere approach, we also tested the adaptability of the nucleotide pyrosequencing approach to molecular HLA typing. The advantage of the sequencing versus the hybridization approach is obvious: final nucleotide sequences are defined directly and not inferred based solely on stringent hybridization results to oligonucleotide probes.

Technical Background

The pyrosequencing method, summarized in Figure 7, is a four enzyme process combining the activities of DNA polymerase, ATP sulfurylase, luciferase, and apyrase. Briefly, the method can be described in 5 steps: (1) hybridization of sequencing primer to a single stranded DNA template; (2) incorporation of a complementary nucleotide and release of pyrophosphate; (3) use of ATP sulfurylase activity to convert pyrophosphate and exogenous adenosine 5' phosphosulfate into ATP, followed by conversion into light via the activity of luciferase; (4) degradation of unincorporated dNTP by means of apyrase; and (5) reiteration of reaction steps 1 through 4 using the next dNTP to be tested for incorporation into the nascent nucleotide chain.

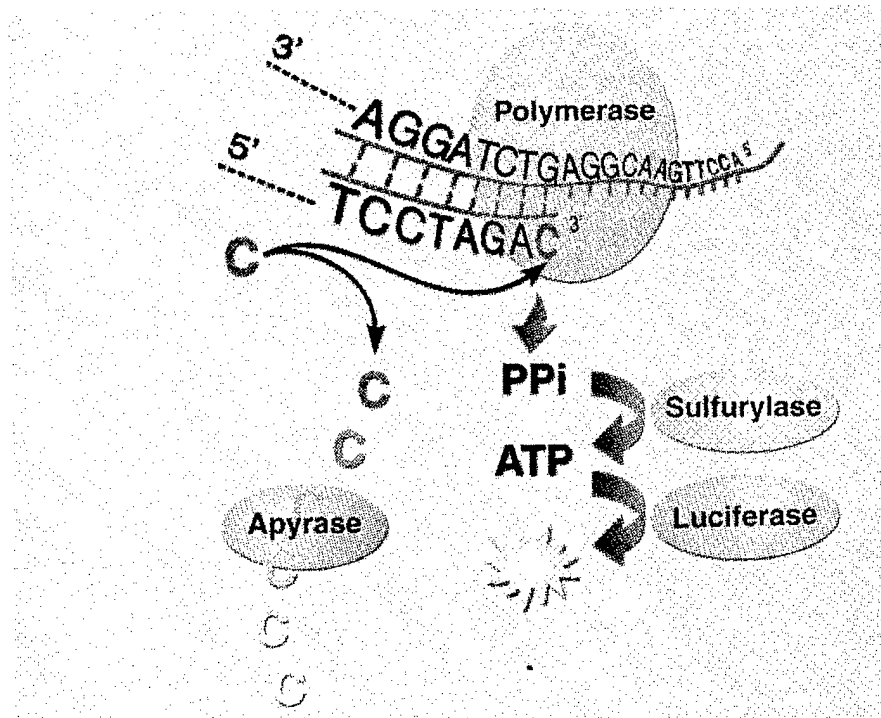


Figure 7

Signal intensities from the pyrograms in Figure 9 were quantified by measuring the peak heights. Additional analysis of the data was performed by comparing the measured peak heights obtained from the mixture of oligonucleotides SR11 and SR14 with the calculated peak heights expected by addition of the signal intensities obtained by individually sequencing the oligonucleotides.

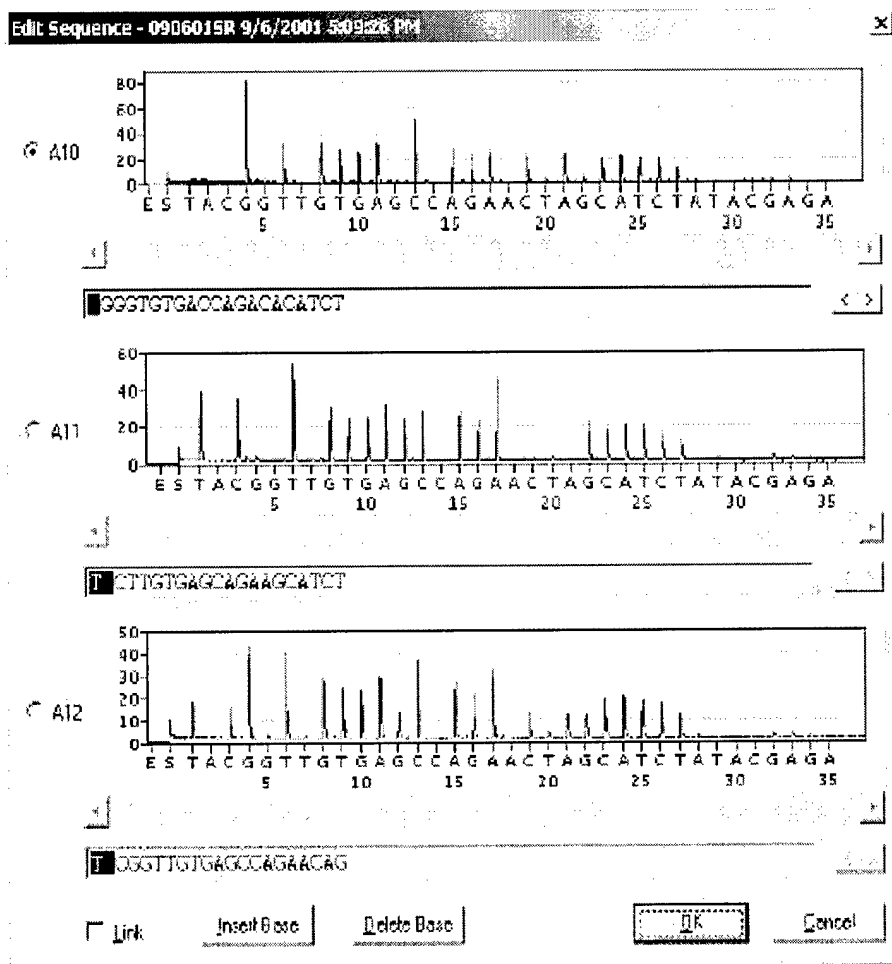


Figure 9

As illustrated in Figures 10 and 11, comparison of the measured and calculated signal intensities resulted in a linear relationship with a slope of about 1.06 and a correlation coefficient of approximately 0.99, indicating an additive, predictable signal.

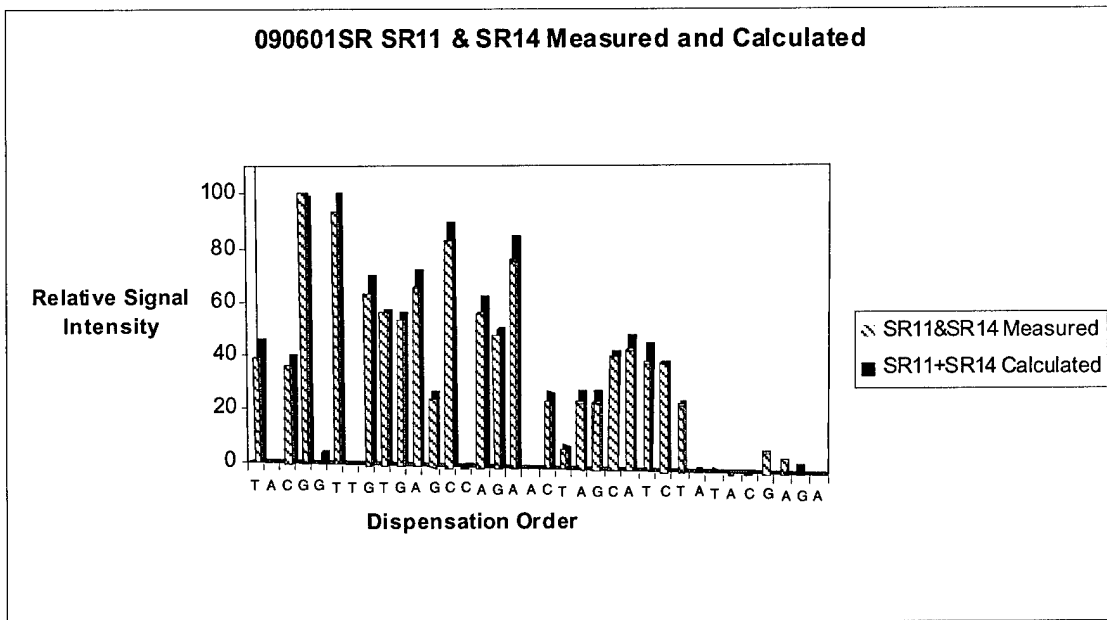


Figure 10

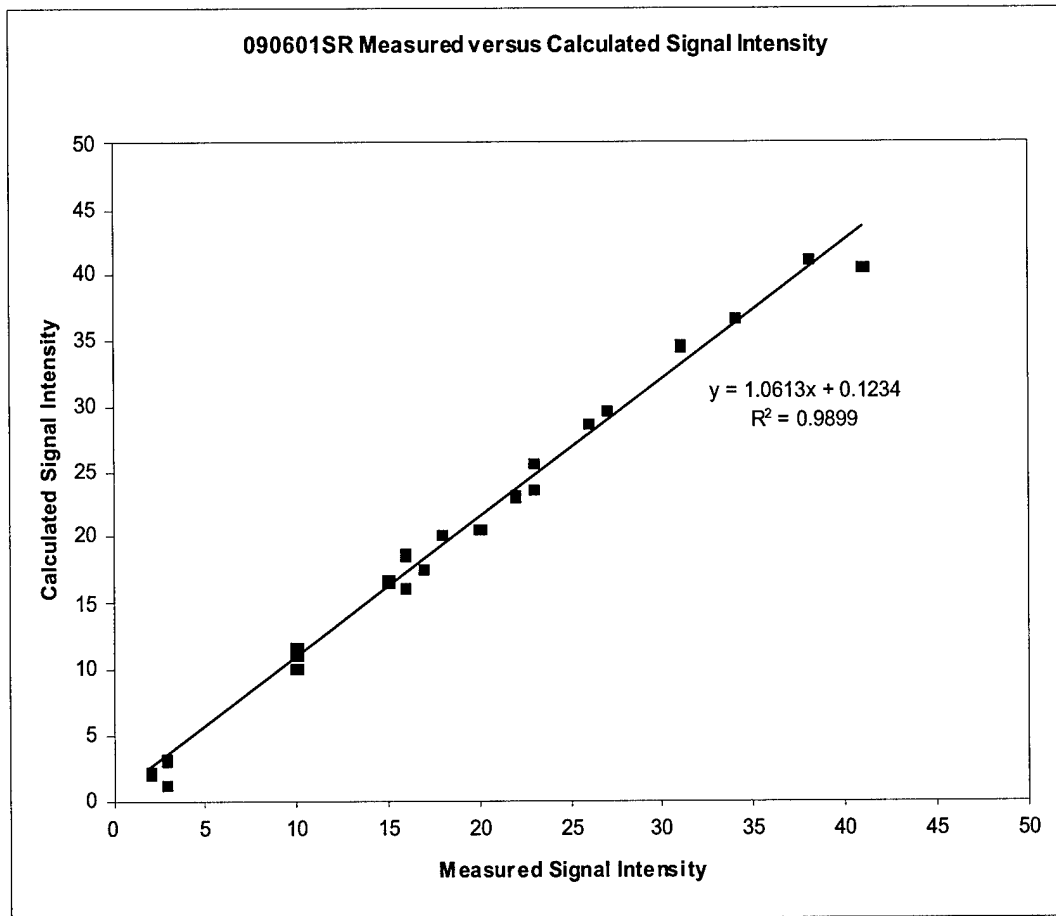


Figure 11

Pyrosequence Based Typing

DNA containing the HLA DQB1 gene was prepared by PCR amplification using the most polymorphic exon 2 specific primers DQBampA and DQBampB. Genomic DNA, obtained from the HLA containing cell lines listed in Table 1, resulted in a single PCR product of the expected molecular weight when analyzed by agarose gel electrophoresis (data not shown).

Table 1. Summary of HLA Containing Cell Lines and DQB1 Alleles

Cell Line	DQB1 Allele
BM16	0301
CB6B	0603
COX	0201
DKB	03032
EHM	0501
EK	0503
FURO	05031, 0601
HO301	0609
LKT3	0401
PGF	0602
SPOO10	0502
WT47	0604
WT49	0201
WT51	0302

All cell lines, except FURO, are homozygotes for DQB1.

The resulting pyrogram, however, indicated the presence of at least 2 sequences when DNA from cell lines BM16 or EHM were examined (Figure 12). Quantification of the peak heights followed by subtraction of the signal expected from the known DQB1 sequence resulted in the unmasking of a second sequence, associated with a correlation coefficient of approximately 0.92 (Figures 13 and 14). Analysis of the contaminating sequence was performed by a BLAST search against the publicly available human database, indicating a 100% match to a 27 nucleotide region of the HLA pseudogene DQB2. Moreover, a BLAST search using the PCR primers also resulted in a 100% match to regions of the pseudogene such that the expected PCR product would have a molecular weight identical to that obtained from amplification of the DQB1 gene.

Re-engineered PCR primers were synthesized following the recommendations of Pera et al., *Tissue Antigens* 55:275, 2000, with the intention of minimizing amplification of the pseudogene. PCR amplification from genomic DNA yielded a product of the expected molecular weight when analyzed by gel electrophoresis (data not shown). Pyrosequencing (Figure 15) resulted in a cleaner pyrogram for the DQB1 sequence, the result of a roughly 80% reduction in signal associated with the contaminating pseudogene.

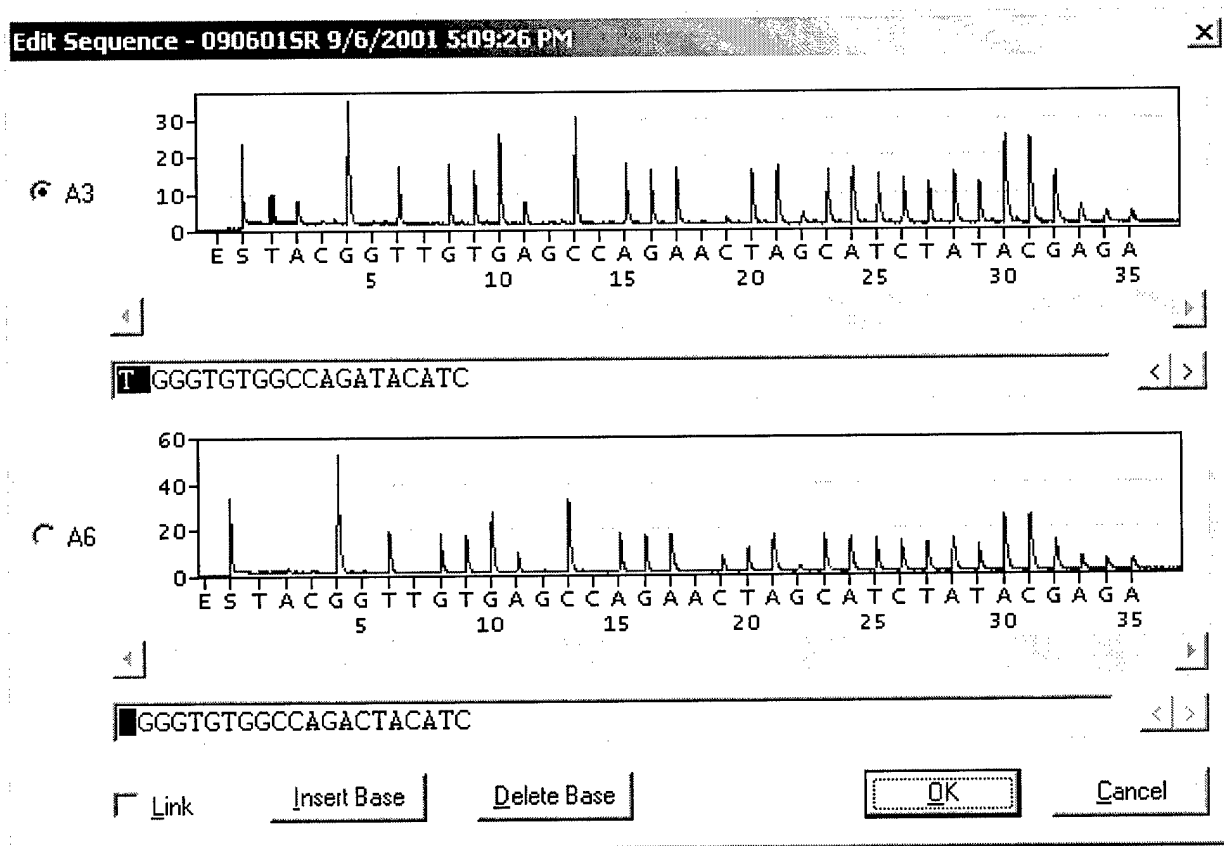


Figure 12

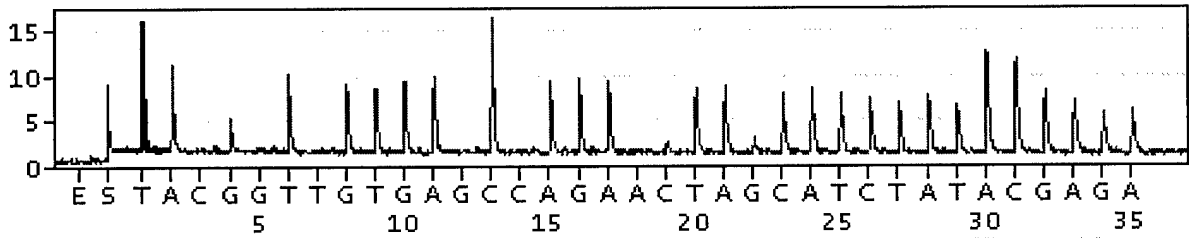
Pyrosequence analysis of amplified DNA from cell lines CB6B and EHM, representing alleles DQB1*0603 and *0501, was performed individually and in combination (Figure 16). Data representing a 62 nucleotide read length were obtained, exhibiting the expected peak intensities. Sequencing of the mixture of CB6B and EHM also resulted in the expected signal intensities (Figures 17-18), resulting in a slope of 1.16 and a correlation coefficient of 0.84 when the measured and calculated signal intensities were compared.

Future Experiments

The next series of experiments will seek to first optimize the experimental conditions and then gain experience with typing samples of human origin. Experiments designed to optimize the pyrosequencing protocol will maximize the sequence read length (perhaps to as many as a 100 nucleotides) and choose the best set of sequencing primers for efficient coverage of the polymorphic residues. In the second experimental series, pyrosequence based typing of human blood samples will be performed using previously sequence specific oligonucleotide probe (SSOP) typed blood samples along with samples from the HLA containing cell lines. Samples will be chosen, initially, based on their expected frequency in our healthy population and from our diabetic patient pool. Additional samples will then be studied based on their expected complexity for haplotype identification.



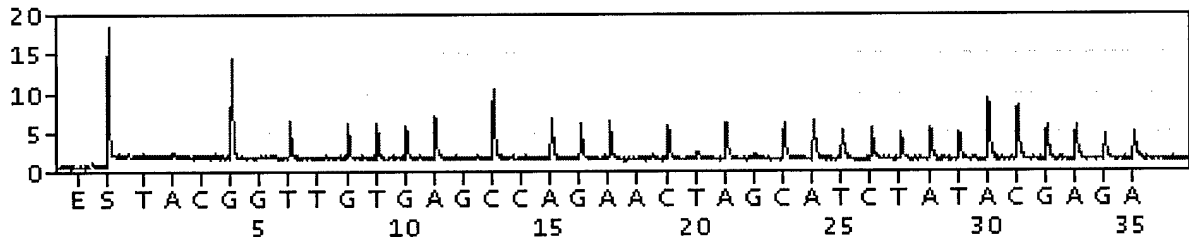
A3



TT ATGTGACCAGATACATCT



A7



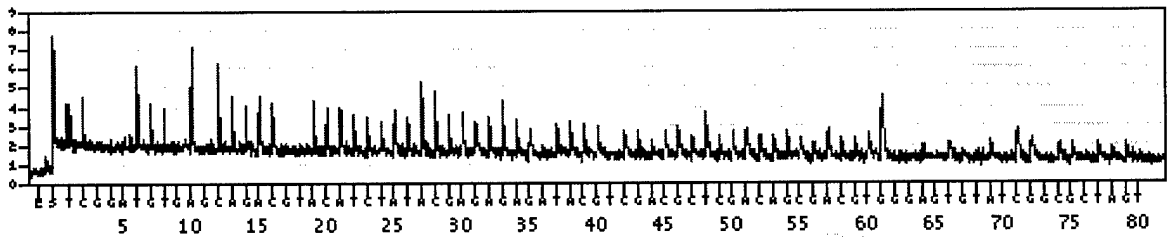
GGGTGTGACCAGACACATCT



Link

Figure 15

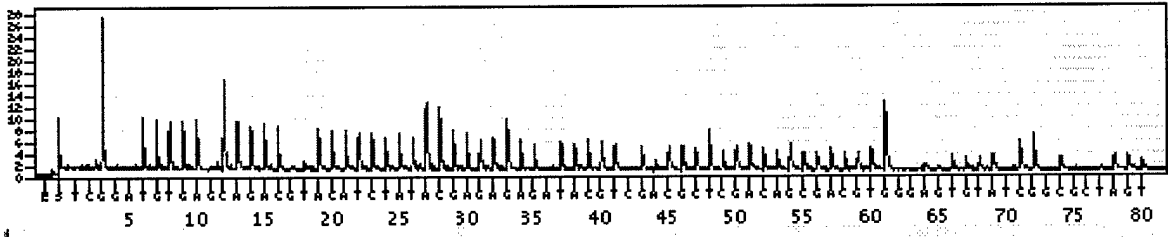
A3



CTTGTAACCAGACACATCT

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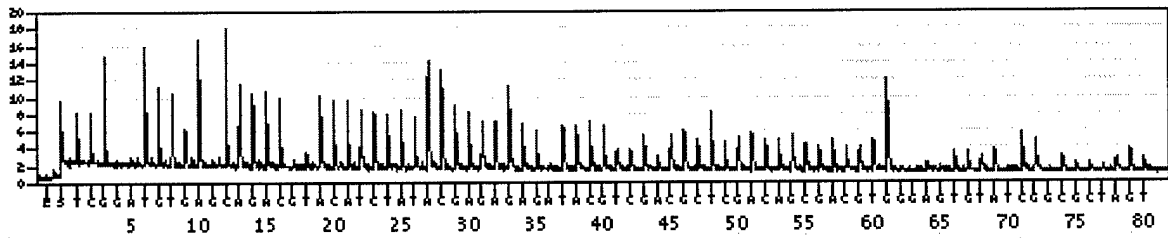
A7



GGGGTGTGACCAGACATC

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B4



CGGTTGTGAACCCAGACAC

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Link

Insert Base

Delete Base

OK

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Figure 16

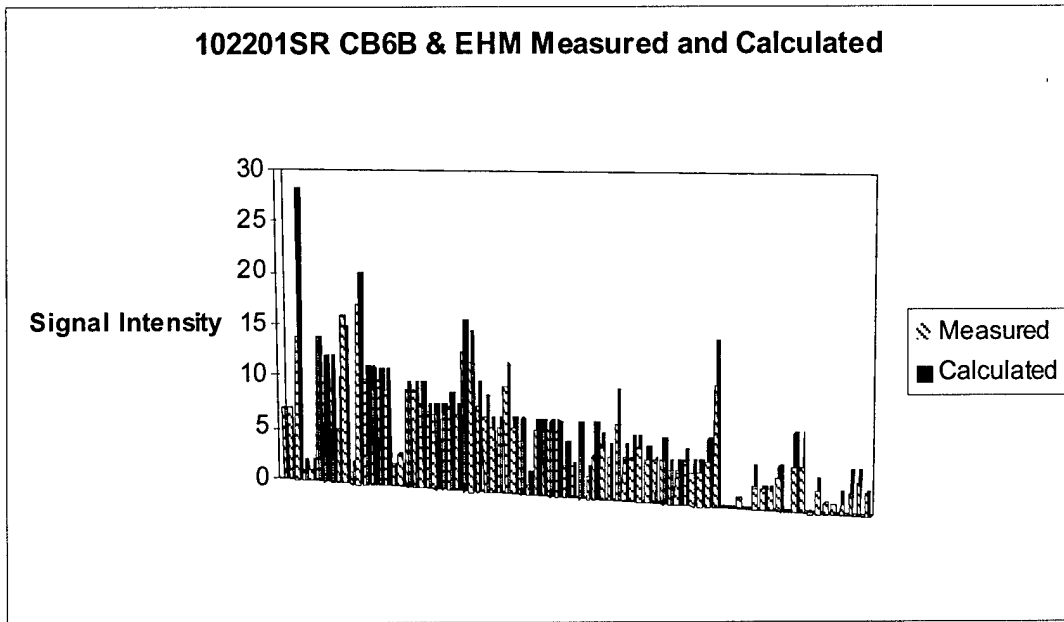


Figure 17

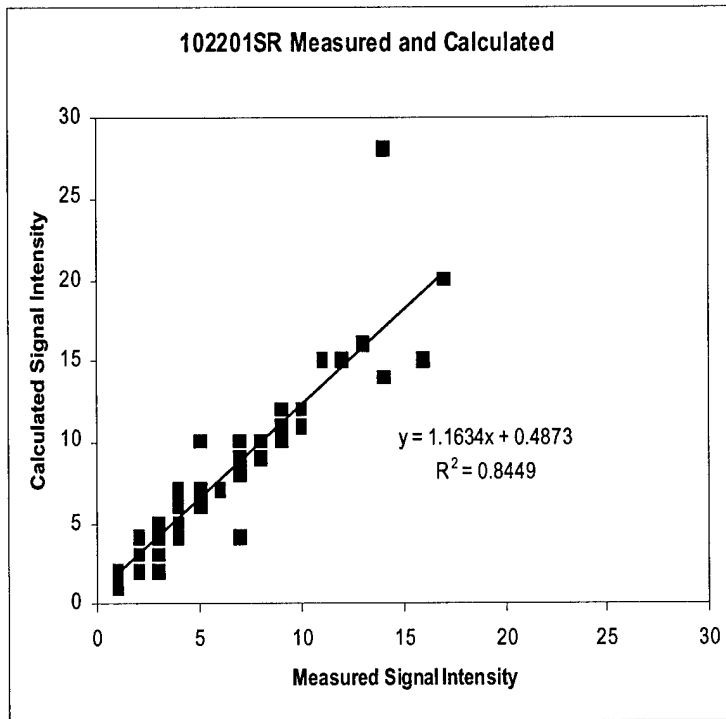


Figure 18

4. Multiplex-Immuno Assays for IDDM Diagnostics

(Petra C. Traub, Ph.D. and Thomas O. Joos, Ph.D., University of Tübingen, Germany)

As Type 1 diabetes is an autoimmune disease characterized by destruction of the insulin-producing β -cells in the islets of Langerhans, early prediction before the clinical onset of IDDM could be achieved by the detection of IDDM specific autoantibodies, e.g. antibodies against GAD 65, IA-2 and insulin, in the sera of individuals.

Currently the standard technique to determine the presence of autoantibodies in the sera of individuals genetically at-risk for type 1 diabetes is the ELISA (Enzyme linked immunosorbent assay). It consists of the use of plates containing 96 wells on the bottom of which are coupled the different antigens to be checked. Serum of a single patient is added to each well and any autoantibodies specific for the antigen present in that well are bound to the antigen. After some washes (to eliminate antibodies not specifically bound) a secondary, anti-human antibody (coupled with an enzymatic, radioactive or fluorescent "detector") is added. After a new wash step, to eliminate the unbound secondary Ab, the plate is analyzed. The ELISA utilizes 7 μ l of patient serum to be tested. This is a limit for this assay, particularly if the serum is from small amounts of archived samples.

In order to quantify in parallel individual autoantibodies of patients suffering from IDDM autoimmune disease, a planar microarray based immunoassay was created that allows the simultaneous analysis of known autoantigens like IA-2, GAD 65 and insulin from minimal amounts of patient sera. Protein microarrays with recombinant antigens (IA-2, GAD 65) and insulin together with control proteins were immobilized on aldehyde activated glass slide supports (CEL Associates, Inc., Pearland, TX, USA) by means of contact printing using a GMS417 ring and pin system (Affymetrix, Santa Clara, CA, USA). Microarrays were incubated over night in humidified chamber to allow covalent binding of the autoantigens to the solid support. To block remaining aldehyde groups and to prevent non-specific binding the microarrays were incubated with blocking buffer (1.5 % bovine serum albumin, 5 % low fat dry milk, 0.1 % Tween 20 in PBS) for 60 min. Microarrays were incubated with 0.1 – 0.4 μ l patient sera diluted in 20 μ l of incubation buffer (1.5% bovine serum albumin, 2.5 % low fat dry milk, 0,1 % Tween 20 in PBS) for 3 hours at room temperature, followed by four washes with PBS, 0.1 % Tween 20. Cy5 conjugated secondary goat anti-human antibody (7.5 μ g/ml in incubation buffer) was added onto the microarray to allow binding to the captured autoantibodies. Subsequently the arrays were washed four times with PBS, 0.1% Tween 20, dried with a stream of nitrogen and scanned with a GMS 418 biochip Reader (Affymetrix, Santa Clara, CA, USA). Digitized fluorescence signals were quantified using the ImaGene 4.1 software (BioDiscovery, Marina Del Rey, CA, USA).

In the first period of the project the assay described above was optimized for the following components:

- Efficient binding of the antigens to the surface.
- Optimize blocking procedure to increase signal/noise ratio.

- Assay performance, e.g. incubation and washing times, as well as concentrations of serum and detection antibodies.
- Stability of the antigens.

So far we have screened 81 sera, previously characterized by ELISA or RIA. The established planar microarray assay results correlated well by detecting 37 out of 39 GAD65 positive and 24 out of 32 IA-2 positive sera (see Figure 19 and 20).

In addition we have started to include peptides into the microarray, which are known to react with IDDM specific autoantibodies. Sixty-three RIA-characterized sera (25 IA-2 positive, 21 GAD65 positive, and 17 double positive) were screened with peptides immobilized on the planar microarray. From the 14 peptides tested, 8 produced positive signals with known sera positive for either IA-2 or GAD65 autoantibodies. However, six of these peptides also produced positive signals with sera negative for these autoantibodies (Table 2).

Table 2: Screening with IDDM-relevant Peptides.

Peptide	Sequence	Source	Positive Signals
P1	PEVKEK	GAD	0
P2	FIEWLKVKILPEVKEK	GAD	6 D*
P3	KILPEVKEKHEFLSRL	GAD	4 nD*** + 3D
P4	WLKVKILLPEVKEKHEF	GAD	3nD + 4D
P5	AFIKATGKKEDE	ICA	0
P6	VIVMLTPLVEDGVKQC	IA-2	1 nD
P7	KEVPALTAVETGATC	Polio virus	all
P8	AVTPLRIFIVPPAAEY	Epstein Barr virus	3 D
P9	GPPAAGPPAAGPPAA	Epstein Barr virus	8 nD + 2 D
P10	KSWFTPP	random	0
P11	HFHRPAG	random	0
P12	HWHRPAG	random	0
P13	CPRDLNGKSGC	CH1p	3 nD + 4 D
P14	KEVPALTAVETGAT	Polio virus	all

* D: diabetic sera (i.e. IA-2 and/or GAD 65 positive).

** D: non diabetic sera (i.e. no IA-2 or GAD 65 antibodies)

Microarrays for IDDM Diagnostics: Sera-Test

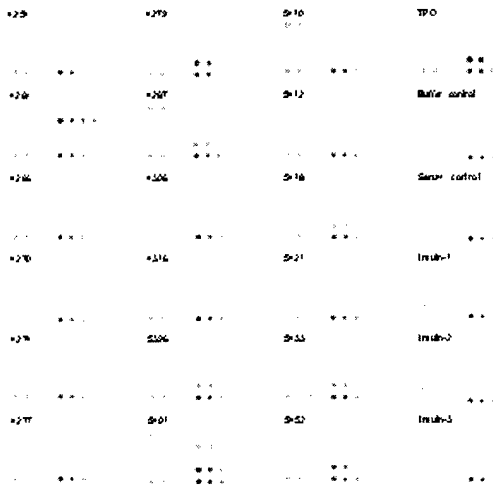


Figure 19

Table: Comparison of a conventional ELISA and a microarray based system .

Antigen	ELISA: detected positives	Microarray: detected positives
GAD	14	14/14 detected
IA-2	11	8/11 detected 1 additional positive
Insulin	3	3/3 detected 14 additional

Figure 20

Pittsburgh Serum Samples

Spotting Scheme

IA-2		Av-Cy5
BSA		21-H
GAD		hulgG
TPO		A-hulgG
Insulin		peptide

Table: Comparison of a RIA and a microarray based system

Antigen	RIA: detected positives	Microarray: detected positives
GAD	25	23/25 detected
IA-2	21	16/21 detected

Control:
PU-5401



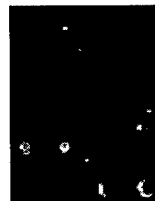
DMLT-NP:



35750103:



35000104:



DMLT-TM:



Another approach for highly parallelized and miniaturized assay systems is provided by the bead based multianalyte profiling technology of Luminex. After detailed introduction to the technology in Pittsburgh and performance of first experiments at CHP, the expectations and limitations of this technology will be evaluated at the University of Tübingen and at CHP.

Next, the microarray immuno assay will be adapted to the bead based Luminex technology. The data obtained with the two different solid phase based immunoassays, (microarray and bead based) will be compared and validated with the fluid phase based RIA assay routinely performed at the CHP.

Miniaturized and highly parallelized immunoassays like these will improve efficiency by greatly increasing the number of assays that can be performed with a single serum sample and will reduce cost by dramatically decreasing reagent consumption. Multiplexed immunoassays will significantly facilitate and accelerate the screening of a large number of patient sera for the presence of different types of IDDM specific autoantibodies.

5. Suspension microarrays for detection of immunological predictors of IDDM

(Giuseppe Montefalcone, Ph.D., Division of Immunogenetics, CHP)

As an alternative to the ELISA, the system that we are developing is based on fluorescent beads coupled with the antigen and analyzed by a flow cytometer. (See section 2). The system is based on a mixed set of microspheres, each set labeled internally with a specific ratio of orange and red dyes, and each coupled with a different antigen (Figure 21).

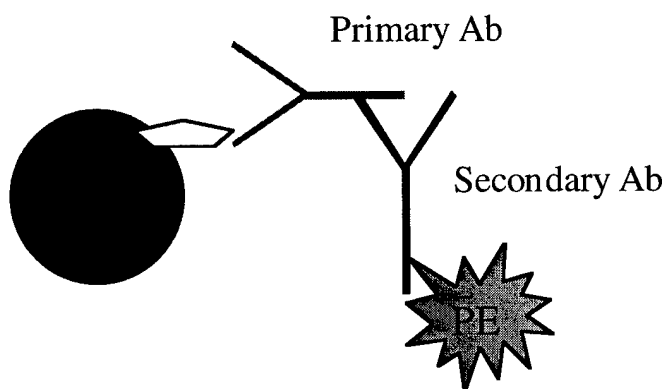


Figure 21

Eventually it will become possible to test a patient in a more complete way for the presence of a vast number of antibodies by using a minimal amount (~5-10 μ l) of serum.

Currently we are focusing on detection of insulin autoantibodies. We have coupled beads with insulin and then tested the technique using different types of antibody (Ab), polyclonal or monoclonal, produced by different sources (mouse or rabbit), choosing the one producing the best signal to analyze the system further and to establish what to expect as an ideal positive control in the experiment with patient sera.

To prove the specificity of the binding between primary antibody and insulin we used isotype controls (antibodies recognizable by the secondary, but not binding to insulin). As expected, beads coupled with insulin and incubated with isotype control and PE-labeled secondary Ab resulted in signals equivalent to autofluorescence. When anti-insulin Ab were used, the signal produced was strongly positive (roughly 6500-fold signal over background and background is about the same signal as the bead autofluorescence) (Table 3).

Table 3: Values obtained against the Isotype Control

Beads coupled with:	Primary Ab	Secondary Ab	Mean fluorescent signal
Insulin	anti-insulin	Yes	6515
BSA	anti-insulin	Yes	9
Insulin	isotype control	Yes	8
BSA	isotype control	Yes	2

In order to validate the specificity of these results, we made a competition experiment showing that increasing amounts of free insulin in the suspension media induces a reduction of the positive signal to the same level as the negative controls (Figure 22).

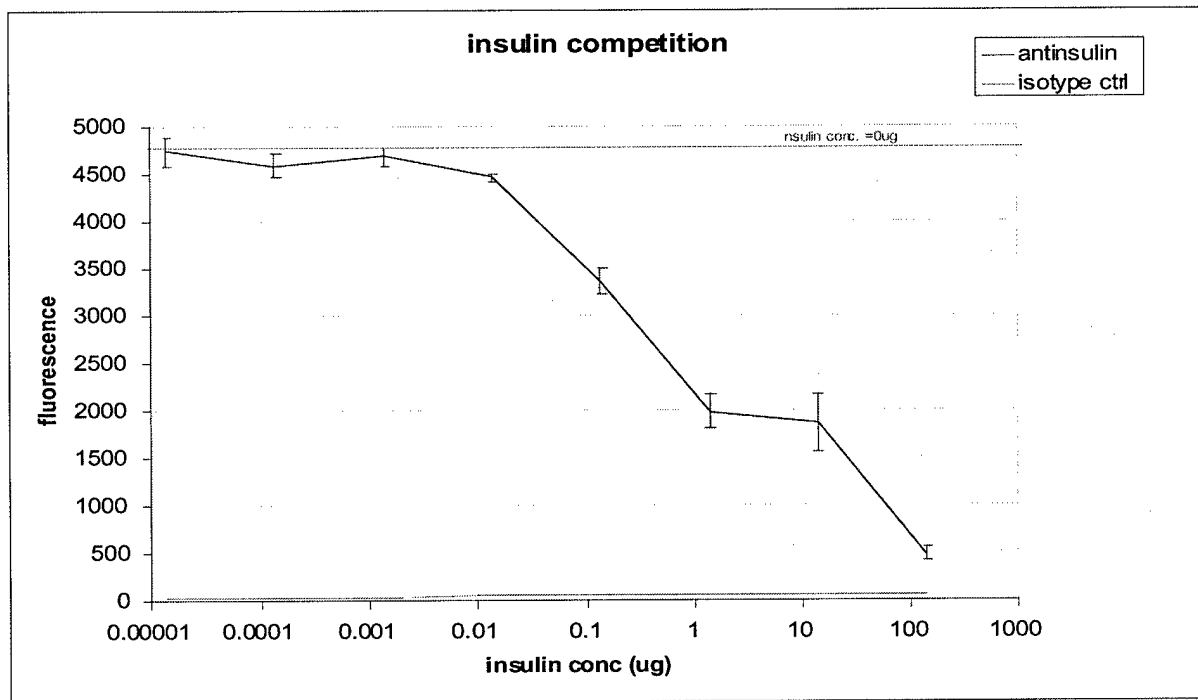


Figure 22

Further proof that there is a direct proportional relationship between antigen and primary Ab has been obtained by decreasing the amount of primary Ab added to the same bead preparation and verifying that the signal measured decreased proportionally (Figure 23).

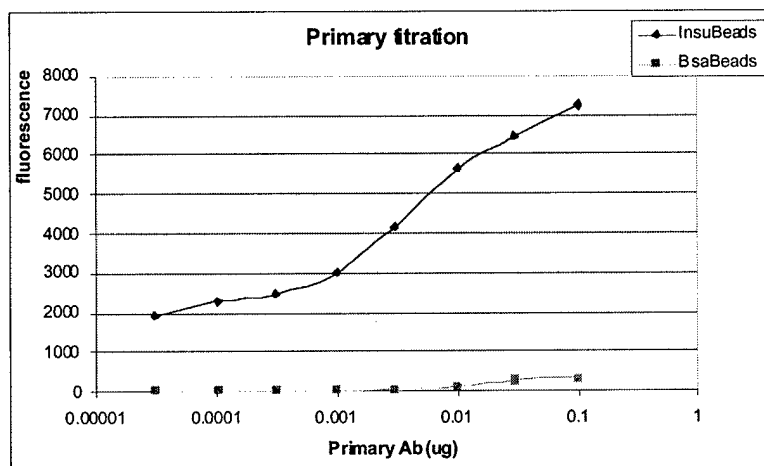


Figure 23

Each of these experiments has been done in triplicate, resulting in a high level of precision, and compared with a series of negative controls. A further source of potential bias is the influence of human serum on the level of the signal. We then performed our test by adding increasing amounts of human serum to the samples and verifying that its presence lowers the signal proportionally to the quantity added, but without losing specificity (Figure 24).

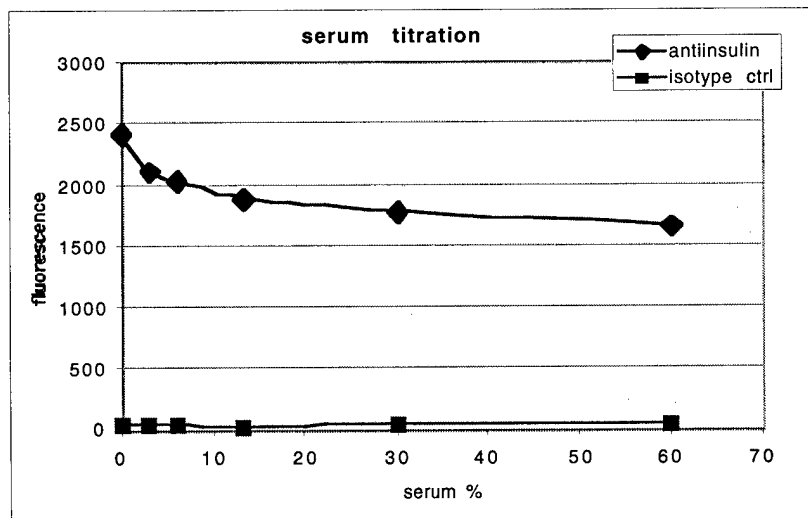


Figure 24

Our next step will be to establish the experimental parameters for human antibodies from patient sera. After the study with insulin is completed, we will proceed coupling the other IDDM-related antigens on beads to explore their effects. Subsequently, we will evaluate the multiples of antigens using the property of the flow cytometer to discriminate between different groups of fluorescent beads.

KEY RESEARCH ACCOMPLISHMENTS

After the first 12 months of operation, we have been able to:

- Clone the α chain mRNA from TCR-V β 7 positive T cells. The cloned cDNA will be transfected into the TCR-negative mutant of the T cell line Jurkat, which will then be used to test *in vitro* its reactivity against viral protein epitopes, and *in vivo* its possible diabetogenicity.
- Pyrosequence the genes encoding HLA-DQB1 alleles in homozygous and heterozygous cell lines, solving the problem of coamplification of the pseudogene DQB2, and increasing the size of each sequenced segment to ~60 nucleotides.
- Obtain readable results using fluorescent microspheres carrying insulin to test specific mouse monoclonal antibodies in high dilutions. Sera from patients will be tested next and the results compared with those obtained using conventional RIA.

REPORTABLE OUTCOMES / CONCLUDING REMARKS

Encouraged by the presented preliminary results obtained working in a synergistic effort with our colleagues at CMU and in Germany, we are confident that three initial reports (i.e., HLA molecular typing by pyrosequencing; Autoantibody screening by ELISA in suspension; and Characterization of α - and β chains of the T cell receptor molecules each expressed by single T cells) will be generated in the coming months which will constitute the basis on which the expansion and completion of this study will stand.