A Multiplexed Assay Panel for Detecting Autoantibodies Implicated in Human Type 1 Diabetes and Celiac Disease

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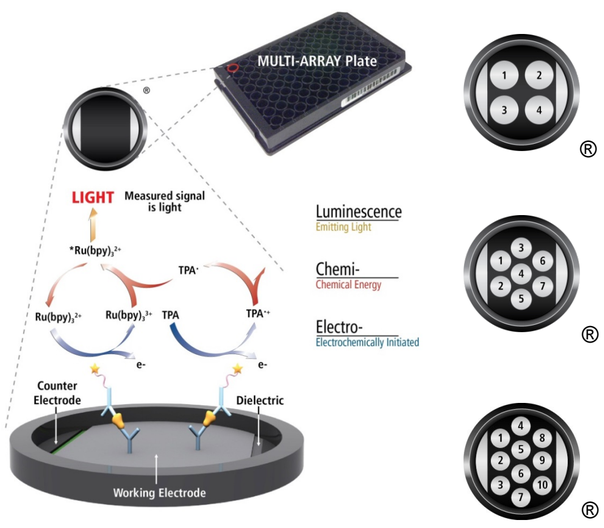
1 Introduction

Simultaneous detection of autoantibodies implicated in type 1 diabetes (T1D) is critical for early diagnosis and prediction of the disease. We report the development of a research-use only multiplexed assay panel that can simultaneously detect autoantibodies against insulin, Islet antigen 2 (IA2), glutamic acid decarboxylase 65 (GAD65), zinc transporter 8 (ZnT8), and transglutaminase (TGM2). MSD's U-PLEX® platform was used for the development and validation of the multiplexed immunogenicity assays for the detection of the five autoantibodies. Development of the individual assays took protein concentrations, dynamic range, specificity, matrix tolerance, and assay robustness into consideration. Three production kit lots have been generated from independent component lots. A sample aliquot as small as 15–30 µL can be tested simultaneously for all analytes in the panel.

2 Methods

MSD's electrochemiluminescence detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® and MULTI-SPOT® microplates.

The assays in this panel uses a bridging format on MSD's U-PLEX platform: autoantibodies are bridged by biotin conjugated protein and SULFO-TAG conjugated proteins.



Electrochemiluminescence Technology

- Minimal non-specific background and strong responses to analyte yield high signal-to-background ratios.
- The stimulation mechanism (electricity) is decoupled from the response (light signal), minimizing matrix interference.
- Only labels bound near the electrode surface are excited, enabling non-washed assays.
- Labels are stable, non-radioactive, and directly conjugated to biological molecules.
- Emission at ~620 nm eliminates problems with color quenching.
- Multiple rounds of label excitation and emission enhance light levels and improve sensitivity.
- Carbon electrode surface has 10X greater binding capacity than polystyrene wells.
- Surface coatings can be customized.

Assay Protocol

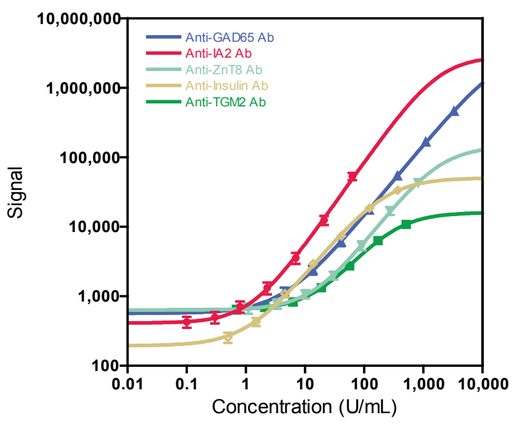
1. Prepare a multiplex U-PLEX linker-coupled biotin protein solution.
2. Prepare a multiplex SULFO-TAG conjugated protein solution.
3. Combine the multiplex biotin protein solution and SULFO-TAG protein solution with sample in a polypropylene plate and incubate 1 hour.
4. Transfer solution to the U-PLEX plate, incubate 1 hour, and wash.
5. Add MSD GOLD™ Read Buffer and analyze plate.

Development and Validation

The assays were developed and analytically validated following design control procedures. Accuracy, precision, and specificity were evaluated using independently built kit lots to test calibrators, controls, and serum samples over multiple days and runs.

3 Calibration Curves and Assay Range

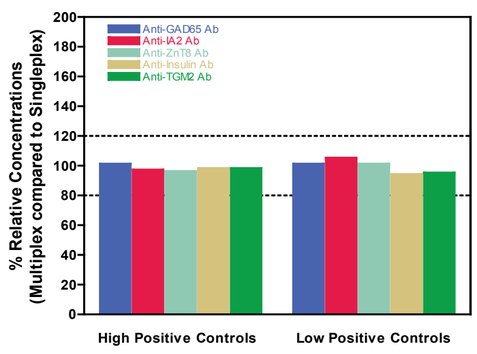
Calibration curves shown are the average from 50 independent runs. The blended calibrator is diluted serially (3-fold) to generate a 7-point standard curve. Anti-GAD65 and anti-IA2 calibrators were standardized against the NIBSC's Islet Cell Antibodies WHO reference.



	Anti-GAD65 Ab	Anti-IA2 Ab	Anti-ZnT8 Ab	Anti-Insulin Ab	Anti-TGM2 Ab
LOB (U/mL)	1.2	0.4	4	0.5	4.7
LLOQ (U/mL)	8.7	2.7	10.8	1.6	20.6
Cut-Point (U/mL)	13.5	1.6	6.3	0.7	6.9

Table includes lower limit of quantitation (LLOQ, n=6 runs) values and limits of blank (LOB, n=50 runs). Samples from 98 individuals (42 males; 56 females; age 20-30 years) with no known diagnosis of T1D were tested, and 98th percentile of sample distribution was used to set the cut-point for each assay.

4 Specificity



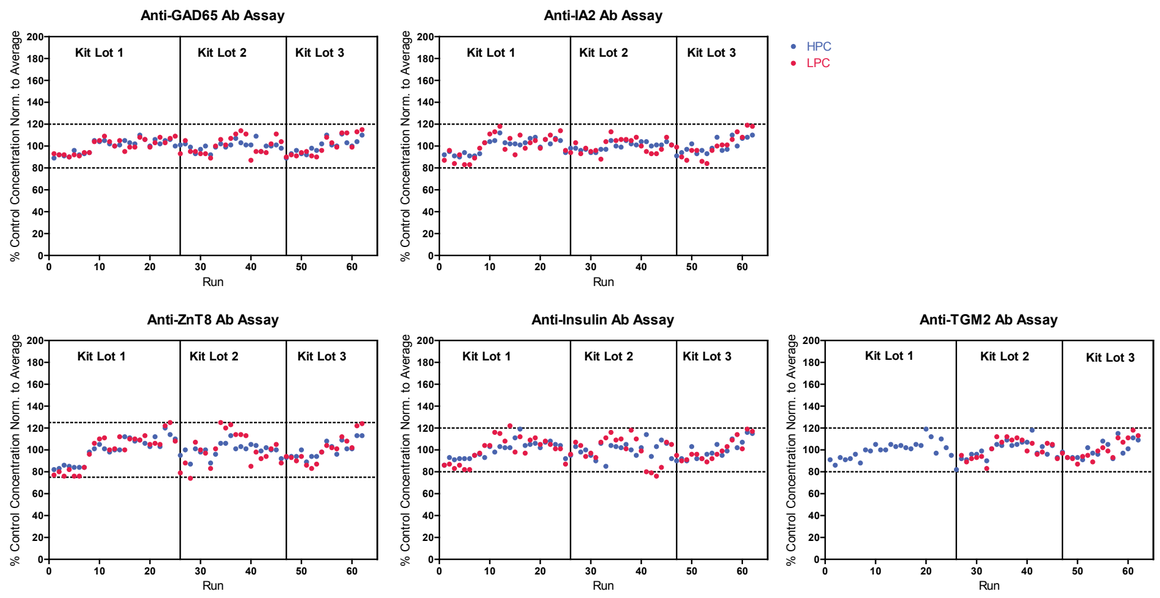
Control samples were tested in singleplex as well as in 5-plex assay format. The difference between the two formats was less than 10% for all positive samples. Negative samples were measured as negative on both formats.

5 Precision and Accuracy

High positive control (HPC), low positive control (LPC), and negative controls were used to evaluate the reproducibility of the assays. The controls were measured using a minimum of two replicates tested over multiple days for a total of 13 runs.

- CVs within a run were typically <5%.
- CVs between runs were typically <10%.

Controls for each assay were tested in 65 runs over three kit lots.



Control concentrations from each run were typically within 80-120% of the average for all the assays. Negative controls were also tested and showed as negative in all of the runs.

6 Sample Testing

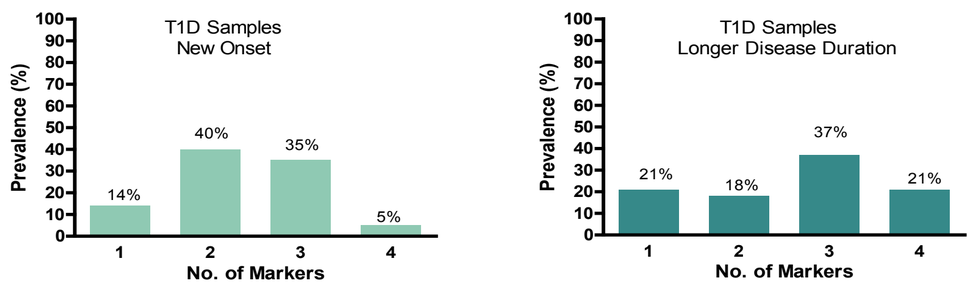
Human serum samples from 43 individuals with **new onset T1D** were tested on the multiplex panel. These samples were obtained as part of the 2018 Islet Autoantibody Standardization Program (IASP).

	Control Samples	T1D Samples	Sensitivity (%)	Specificity (%)	AUC	Accuracy
Anti-GAD65 Ab	90	50	78	94	0.91	89
Anti-IA2 Ab	90	50	67	94	0.9	84
Anti-ZnT8 Ab	90	50	72	90	0.76	83
Anti-Insulin Ab	90	50	26	99	0.8	72

The overall mean sensitivity of all the methods submitted to the 2018 IASP were: GAD65 66%; IA2 59%; ZnT8 57%; insulin 34%. The overall mean specificity of all the methods were: GAD65 98%; IA2 97%; ZnT8 96%; insulin 93%. (IASP 2018 Cumulative Performance Summary)

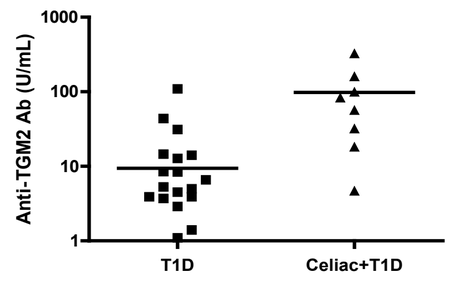
Human serum samples from another group of 38 individuals with **longer disease duration** (0.75–30 years) were also tested.

	Control Samples	T1D Samples	Sensitivity (%)	Specificity (%)	AUC	Accuracy
Anti-GAD65 Ab	30	38	58	93	0.89	74
Anti-IA2 Ab	30	38	63	88	0.81	75
Anti-ZnT8 Ab	30	38	63	93	0.81	75
Anti-Insulin Ab	30	38	68	93	0.87	79



More than one autoantibody was detected in most of the T1D samples.

Compared to individuals with longer disease duration, individuals with new onset T1D showed lower prevalence of anti-insulin antibody, coinciding with depletion of β cells during this disease stage. Similarly, the higher prevalence seen in individuals with longer disease duration was possibly due to insulin treatment.



Eight samples from individuals with both celiac disease and T1D were tested using the panel; all tested positive with anti-TGM2 antibody. In general, the levels of anti-TGM2 antibodies were higher in the celiac+T1D group.

7 Conclusion

A multiplex immunoassay panel developed on MSD's MULTI-ARRAY platform has been analytically validated for measurement of key T1D autoantibodies in human serum. All assays were individually optimized to achieve the best assay performance, as well as for ease of use. The simple 3-hour protocol requires just 15–30 µL of sample to measure all five analytes. These assays have excellent sensitivity and reproducibility to facilitate any T1D research or drug development program.

Acknowledgements

T1D samples with longer disease duration and celiac samples were from Dr. Mark Atkinson, Univ. of Florida.

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