Automation of a multiplex agglutination-PCR (ADAP) type 1 diabetes (T1D) assay for the rapid analysis of islet autoantibodies

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ABSTRACT

Screening for islet autoantibody markers to identify individuals who are at high risk for developing type 1 diabetes (T1D), often years in advance of clinical symptoms, is both a challenge and a necessity. Identifying high-risk individuals not only reduces hospitalization and rates of life-threatening diabetes ketoacidosis (DKA), but also directs enrollment into prevention trials that require patients who are in the early stages of disease. Here we describe an automated high-throughput multiplex islet autoantibody assay that integrates antibody detection by agglutination-PCR (ADAP) chemistry on the Hamilton Microlab STAR liquid handling platform. The automated system features on-deck thermal cycling and plate sealing to minimize the level of human intervention. The automated multiplex ADAP T1D assay performed similarly to that of manual methods using two distinct cohorts of clinical specimens obtained from the Lucile Packard Children’s Hospital at Stanford University and the 2018 Islet Autoantibody Standardization Program (IASP). Notably, the automated assay requires only 4 μL of serum sample for the simultaneous analysis of GAD, IA-2 and insulin autoantibodies.

Up to 96 samples may be processed in as little as 3 hours, and the only user intervention required is to transfer a final sealed 96-well plate containing PCR amplicons onto a quantitative PCR (RT-qPCR) instrument for quantification. The automated system is particularly well suited for large-scale analysis of islet autoantibodies in a reproducible, timely, and cost-effective manner.

Introduction

Type 1 diabetes (T1D) is a chronic disease characterized by progressive loss of pancreatic function and life-long reliance on administration of insulin [1]. The humoral immune response in T1D patients leads to the production of autoantibodies against glutamic acid decarboxylase (GAD), islet antigen 2 (IA2), and the insulin and zinc Transporter 8 (ZnT8) within pancreatic β-cells [1]. The presence of two or more of these canonical autoantibodies indicates a likelihood approaching 100% that an individual will develop type 1 diabetes [2]. Detection of anti-islet autoantibodies also differentiate autoimmune-based diabetes, such as T1D and latent autoimmune diabetes of the adult (LADA), from other forms of diabetes, and this may be used to inform optimal treatment [1]. Islet autoantibodies appear many years in advance of symptom onset, and early diagnosis of asymptomatic pre-diabetic patients reduces both disease complication and fatality [2].

Traditional methods for the detection of islet autoantibodies rely on the radioactive and solution-phase radio-binding assay (RBA), because it is sensitive and it preserves antigen conformation, ensuring good clinical performance [3]. However, reagents for this assay are both hazardous and expensive, and this has limited the adoption of RBA testing on a large scale. Additionally, RBA assays are not multiplexed, which necessitates the use of large sample volumes and repeat testing for multiple islet autoantibodies. Various improved methods have been developed to overcome the limitations of RBA [4–9], but extensive validation of a fully automated sample-to-answer islet autoantibody testing solution has not been published. Moreover, automation remains critical to the success of widespread adoption of T1D risk screening campaigns.

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We previously reported an improved multiplex antibody detection by agglutination-PCR (ADAP) T1D assay for the detection of islet autoantibodies whereby DNA-barcoded islet autoantigens are used as reporters to create distinct PCR amplicons for multiplex quantification (Fig. 1) [3]. ADAP technology leverages the exponential amplification of PCR to achieve 100 to 10,000-fold improved analytical sensitivity as compared to either enzyme-linked immunosorbent assay (ELISA) or radio-binding assay (RBA) [10,11]. Notably, ADAP is a pure solution-phase assay that prevents antigen denaturation or epitope masking that may be found when using solid supports. Results from the multiplex ADAP T1D assay are highly concordant and correlated with those of the gold-standard RBA in multiple blinded cohort studies [3]. In one international and blinded study, for example, ADAP showed the highest sensitivity for GAD and IA2 autoantibodies at 95% specificity (AS95) and top-tier performance for the detection of insulin autoantibodies [3,12].

While the manual T1D ADAP assay is operationally simple and suitable for wide deployment for near point-of-care testing, large-scale manual testing can be labor intensive, costly and subject to human error. For example, recent pilot T1D screening programs typically process 10,000 to 20,000 samples per year [13], and it is extremely challenging to perform manual testing of this many samples consistently and quickly. Therefore, an automated multiplex ADAP T1D assay is needed to help deliver results in a reliable, timely and cost-effective manner as demonstrated in the recent nationwide screening program called TIDetect (https://www.jdrf.org/t1d-resources/tidetect/).

Here we describe a validated automated ADAP assay for high-throughput T1D screening on the Hamilton Microlab STAR liquid handling system that fulfills these needs.

Materials and methods

Human specimens

Serum samples from 20 patients with type 1 diabetes were obtained from Stanford University’s Lucile Packard Children’s Hospital with Institutional Review Board (IRB) approval. An additional 123 serum samples (33 new-onset type 1 diabetes and 90 negative controls) were obtained from the 2018 Islet Autoantibody Standardization Program (IASP 2018) via the IASP Committee and the University of Florida. The IASP sample set was previously analyzed by more than 40 participating laboratories in 20 different countries. The Enable Biosciences laboratory received all of the samples as de-identified coded samples, which were determined to be IRB-exempt by Western IRB (IRB #20180015).

Reagents and consumables

Molecular biology grade water was purchased from Corning, Inc., (Corning, NY; P/N 46 000-CM), and all other chemical supplies were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Multiplex ADAP reagent kits were manufactured by Enable Biosciences (South San Francisco, CA; P/N DK1-100 (T1D)) [3,11]. Reagent reservoirs were purchased from Agilent Technologies, Santa Clara, CA (P/N 201256 100). FramePlates in 96-well and 384-well PCR format (P/N 814302 and P/N 814305 respectively) as well as 50 μL and 300 μL conductive non-sterile filter tips (P/N 235948 and P/N 235903 respectively) were obtained from Hamilton Company, Reno, NV.

Instrumentation and accessories

The Hamilton Microlab STAR liquid handling system included eight motorized independent pipette channels, an autoload barcode reader, plate sealer (Hamilton Company, Reno, NV), and 96-well and 384-well On-Deck Thermal Cyclers (ODTC) and Cold Plate Air Cooled (CPAC) devices from Inheco (Inheco Industrial Heating and Cooling GmbH, Martinsried, Germany). The liquid handling systems were operated by Hamilton Venus software version 4.0 with the Total Aspiration and Dispense Monitoring (TADM) feature activated. A CFX384 Touch real-time PCR detection system (Bio-Rad, Hercules, CA) was used for quantitative PCR analysis.

Principle and workflow of multiplex ADAP T1D assay

ADAP is a solution-phase immunoassay that detects antibodies through conversion of analyte identities into distinct PCR amplifiable DNA barcodes. ADAP harnesses the agglutination power of antibodies to aggregate DNA-labelled antigens (Fig. 1) [3,10,11]. The agglutination process condenses the antigen-DNA conjugates into close proximity with one another, permitting hybridization with an added “connector” oligonucleotide (also known as “bridge oligonucleotides”), and creating a nicked double-stranded segment. Addition of a DNA ligase joins the single strands to produce a PCR-amplifiable double stranded DNA (dsDNA) sequence, which is then detected with qPCR to quantify the abundance of antibody in the original sample. The antigen-DNA conjugates are present at very low concentrations, such that a double-stranded amplicon is only formed upon binding of autoantibodies to their antigens. This is in sharp contrast to similar methods such as immuno-PCR, where full length DNA reporters are attached directly to the probe. This innovation precludes the need for washing or centrifugation steps to remove excess unbound reporters, thus simplifying the procedure and improving assay specificity.

For the detection of T1D, three of the islet-cell antigens (GAD, IA-2 and insulin) are chemically conjugated to pairs of DNA barcodes that each bear distinct primer-binding sites, permitting multiplex detection of GAD, IA-2 and insulin autoantibodies in a single assay.
Experimental procedures

Manual analysis of multiple autoantibodies was conducted as previously described [3]. Scripted protocols for the automated analysis of 80 samples, including blank and control samples, were developed based on the Hamilton Venus software. Reagents were prepared from components in the Multiplex ADAP reagent kits according to manufacturer instructions [3]. The sources of proteins and conjugate synthesis were the same as the previous report [3]. The DNA sequences used in this study were previously published [11]. DNA sequences used for p24, gp41 and gp120 were now used for insulin, IA-2 and GAD65 proteins respectively [11]. The reagents, together with the molecular biology grade water, blank solution, ADAP conjugate mixture, ligation mixture, pre-amplification mixture and qPCR mixture were loaded onto their designated deck or CPAC locations that had been pre-chilled to 4°C. Then, pipette tips and sample plates were manually loaded onto designated areas of the instrument deck. Finally, 6 μl volumes of neat serum were aliquoted into a Bio-Rad Hard Shell 96-well plate and loaded onto the deck. In an alternate sample input routine, 10 μl of neat serum was aliquoted into 2D-barcoded Micronic 0.3 ml tubes and loaded in place of a hard shell 96-well plate. The automated ADAP protocol began by mixing 4 μl of samples with 8 μl of ADAP conjugate mixture that contains 500pM of islet antigen-DNA conjugates. The solution was then incubated on the 96-well ODTC at 37°C for 30 minutes. Then, 4 μl of the incubated conjugate mixture was mixed with 116 μl of ligation mixture and incubated on the ODTC at 30°C for 15 minutes. Next, 25 μl of the incubated ligation mixture was mixed with 25 μl of pre-amplification product and subjected to thermal cycling on the ODTC (12 PCR cycles alternating between 95°C and 56°C, for approximately 30 minutes). After thermal cycling, the amplified products were diluted 20-fold with molecular biology grade water. The diluted PCR products were then added to 384-well qPCR plates for subsequent real-time qPCR quantification. Notably, the qPCR mixtures with distinct primers were added to different wells in the 384-well plates for multiplex autoantibody detection. Thus, an input plate of 96 specimens could be quantified for 4 targets each on a 384-well plate. Owing to the fact that additional diluted PCR product volume was available, a second 384-well plate containing additional primers may be added to achieve greater discriminatory power. The qPCR-ready plate was sealed using an on deck Hamilton Plate Sealer, and the user was prompted to transfer the sealed plate onto a CFX 384-well RT-qPCR detection system for off deck analysis. The next automated ADAP assay could then be set up while the qPCR analysis of the first plate was running.

The value of the qPCR ΔCT was calculated as the difference in CT values between the blank samples and unknown samples [3]. This is proportional to the initial amplicon concentrations in the PCR plate well, which correlates to the amount of target antibodies present in the samples.

Data analysis

Prism and XLSTAT software (GraphPad Software, San Diego, CA and Addinsoft, Paris, France, respectively) were used for data analysis, and a P value with an alpha of 0.05 was used as the cutoff for significance.

Results

Strategy for automated procedures of multiplex ADAP T1D assay

Despite its strong performance, a manually-performed multiplex ADAP takes approximately 4 hours to analyze 20 samples with controls. In addition, given the small volumes involved, the technical operators need to be highly focused to prevent human operational errors. As such, employment of manual multiplex ADAP to analyze large numbers of samples for type 1 diabetes general population screening (e.g. more than 20,000 samples per year) is challenging.

To address the limitations of assay throughput and the potential for human error, we sought to integrate the multiplex ADAP procedures onto a robotic liquid handling system (Fig. 2). An automated multiplex ADAP should show low sample consumption, minimal human intervention, high-throughput, and reliability. First, in order to achieve low sample consumption, we specifically used sample vials with V-shape bottoms (e.g. 96-well qPCR plates, Micronic 96-1 0.3 ml tubes). The V-shape bottom allowed small liquid volumes to possess enough depth for reliable pipetting by the robotic liquid handling system. It was noteworthy that after systematic adjustments, the platform permitted the use of 4 μl serum sample with only 2 μl dead volume in a 96-well qPCR plate. Alternatively, Micronic 96-1 0.3 ml tubes with 2D barcodes also allowed dead volume as small as 6 μl. Second, to minimize human intervention, we have designed a custom Hamilton Microlab STAR to encompass liquid transfer, plate movement, incubation thermocycling, and plate sealing. In particular, the custom automated ADAP workstation included two PCR thermocyclers (i.e. 384-well and 96-well). The former was for small liquid volumes in the early step of ADAP processes, and the latter used to incubate a larger volume of solution (e.g. ligation step 120 ul) at later stages. This design permits the user to simply input samples and reagents onto the system deck. The workstation then completes all necessary steps and outputs a sealed qPCR ready plate for multiplex quantification. Third, the ADAP workstation analyzes 80 samples with blanks and quality controls materials (e.g. duplicate of positive and negative controls) for 3-plex in 3 hours. Thus, in a typical 8–9 h shift, a user can analyze up to 160–240 samples for multiple antibodies. The annual throughput (e.g. 48,000–72,000 samples per instrument) well exceeds the testing demands of current T1D risk screening program.

Finally, the reliability of the automated system was secured by the liquid sensing and pressure monitoring capabilities of the liquid handling systems. Briefly, the Hamilton Microlab STAR used conductive pipette tips to sense liquids using change of conductance to verify proper loading of required reagent solutions. The pressure monitoring functions tracked the pressure changes during aspiration and dispense processes to confirm successful pipetting without bubbles or clogging. Taken together, the multiplex ADAP workstation is configured to minimize human intervention by streamlining and automating the testing procedures with all necessary components on deck. The various monitoring functions allow users to capture unusual or potentially problematic pipetting behaviors and flag the data as needed. These advantages over manual processes allow continuous and reliable testing of large numbers of type 1 diabetes clinical specimens with high reliability.

Pilot validation of automated multiplex ADAP T1D assay with specimens from T1D patients

To validate the automated multiplex ADAP T1D system, we obtained 20 established T1D patient serum samples from Lucile Packard Children’s Hospital at Stanford University. We analyzed the sample using multiplex ADAP with both manual and automated procedures (Fig. 3). The data showed that the readout from automated and manual procedures were highly correlated. The Pearson’s coefficient R were 0.97, 0.98, and 0.94 for GAD, IA-2, and insulin autoantibodies respectively (p<0.05). The overall agreements between manual and automated procedures are 95%, 95%, and 100% for GAD, IA-2, and insulin autoantibodies. Therefore, the data demonstrated that the automated ADAP workstation can reliably perform ADAP analysis concordant with manual procedures.

Validation of automated multiplex ADAP T1D assay with specimens from an international standardization program

Then, to further ensure the automated multiplex ADAP T1D assay performed as expected, we obtained a panel of 33 new onset and 90 negative control samples from the IASP 2018. The same sample sets have been analyzed by more than 40 participating laboratories from...
20 countries. ADAP analysis results of new onset patients from manual and automated procedures were compared (Fig. 4). The data that the Pearson’s correlation coefficient R between manual and automated procedures were 0.97, 0.99, and 0.99 for GAD, IA-2, and insulin autoantibodies respectively (p<0.05). The overall agreements are 100%, 98%, and 95%.

We have previously reported that the manual multiplex ADAP has highest sensitivity for GAD and IA-2, and top tier performance for insulin [3]. Here we further showed that the automated assay can generate results in strong agreement with the manual assay, affirming the applicability of the automated procedures.

**Reproducibility of automated multiplex ADAP T1D assay**

To assess the reproducibility of the automated procedures, we further studied the intra- and inter-assay variations. Intra-assay variations were determined by measuring 20 replicates of samples on the same plates, whereas inter-assay variations were tested by repeatedly measuring the
samples on 3 different days. The results showed that for low positive samples, the intra-assay variations were 11.6%, 11.4%, and 19.8% and the inter-assay variations were 7.0%, 5.6%, and 9.9% for GAD, IA-2, and insulin autoantibodies respectively. For high positive samples, the intra-assay variations were 8.5% and 4.8%, while the inter-assay variations were 1.9% and 2.5% for GAD and IA-2 autoantibodies. The data affirmed that the automated procedure could reliably detect islet autoantibodies with acceptable variations [14].

Discussion

ADAP is a pure solution-phase assay, the islet autoantigens remain intact and free from epitope masking and denaturation on solid surfaces. Hence, even challenging anti-insulin autoantibodies can be faithfully detected. Moreover, ADAP leverages the exponential amplification of PCR to improve analytical sensitivity 100 - 10,000 fold above traditional methods like ELISA. Thus, ADAP permits the use of small sample volume (manual assay: 1µL and automated assay: 4µL) and enables detection of low quantities of islet autoantibodies at the early stages of disease. The strong analytical performance of manual ADAP T1D assay has been reported previously. Most notably, ADAP achieved top tier performance for all three autoantibodies in an internationally blinded study [3]. These results pave the ground for further automation efforts in this report.

The ability to achieve high-throughput and cost-effective analysis of multiple islet autoantibodies is essential for large-scale population screening for type 1 diabetes risk. Current RBA methods use hazardous radioactive materials, rendering the assay lower throughput and expensive. While ELISA can be automated to increase throughput, ELISA is not a multiplex assay and omits critical insulin autoantibodies, one of the earliest markers for type 1 diabetes risk [15]. New methods such as ECL and nanoplasmonic assay have been developed to address some of these limitations, but requires dedicated readers for assay readouts [4–9] current screening programs rely on a combination of RBA and ELISA for testing [13]. The reflex between testing methods not only increases program complexity but also demands a larger volume of blood, which could be challenging for targeted pediatric populations.

The ADAP T1D assay enables multiplex analysis of islet autoantibodies in small sample volumes using commonly available instruments. In addition, the use of small amounts of reagents also reduces assay costs. Such assays address many of the unmet needs described above for population screening of T1D risk. The automated procedure established in the report further strengthens the power of the multiplex ADAP T1D assay for large-scale screening efforts.

The data demonstrate that the automated assay generates results that are consistent with high-performing manual procedures. The automated assay shows higher throughput and high performance independent of user pipetting skills while reducing hand-on time, thus allowing the user to perform other critical tasks (e.g. sample accessioning) while the instrument is running.

It should be noted that the method presented herein is not without limitations. Firstly, the method requires the user to transfer the qPCR ready plate from the Hamilton MicroLab STAR to the RT-qPCR analyzer. Additional process improvements are underway to automate plate transfer steps in order to minimize human interventions. Secondly, the current method only automates the analytical steps. Users must still perform pre-analytical (e.g. sample accessioning) and post analytical steps (e.g. result analysis) in order to complete clinical specimen testing. Thirdly, this study was conducted with limited number of samples. Future studies are currently underway to evaluate automated method performance on a large scale in order to confirm the finding presented herein.

The implementation of automated multiplex testing systems may improve the economic feasibility of screening trials. In particular, current screening trials are largely funded by government and non-profit organizations. To empower self-sustainable screening trials, the cost of screening should be offset by the benefits of the screening program (e.g. reduce DKA onset and disease complications, prevent fatality, improve quality of life). The automation system has the potential to reduce labor costs, in that a single operator can operate 2-3 systems simultaneously. Considering a system has the throughput about 50,000 samples annually, a single operator can analyze up to 150,000 samples annually, which far exceeds any current screening program. In addition, the multiplex system consumes a minute amount of input samples, as low as 6 µL of total sample. As such, even a small volume finger prick or capillary blood is sufficient for analysis.

Population screening for type 1 diabetes risk identifies asymptomatic patients, enabling physicians to carefully monitor and educate patients to prevent severe disease onset and associated complications. Furthermore, the early-stage patients typically still have a high level of islet cell function and are ideal candidates for clinical trials for intervention trials. Taken together, the automated multiplex ADAP T1D assay may facilitate diabetes patient care and prevention treatment development from multiple perspectives [16–18].

Notably, the system established herein is not only applicable to type 1 diabetes but also applicable to other diseases such as other autoimmune diseases (e.g. thyroid, celiac disease), infection diseases (e.g. COVID19, Lyme, Zika, dengue infection) and food allergy (e.g. IgE against food components) [19,20]. In addition to serum samples, the system has been shown to be compatible with plasma, whole blood, oral fluids and cerebrospinal fluids. Thus, the ADAP workstation may have diverse clinical diagnostic applications.

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Declaration of Competing Interest

Felipe de Jesus Cortez, David Gebhart, Devangkumar Tandel, Peter V. Robinson, David Seftel, and Cheng ting Tsai and are employees of Enable Biosciences, which sells ADAP reagent kit chemistries and analytical services related to T1D testing. Kevin W.P. Miller is employed of Hamilton Company, which manufactures and sells the Microlab ADAP STAR liquid handling platform.

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