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Development of a Simple Multiplex Electrochemiluminescence (ECL) Assay for Screening Pre-Type 1 Diabetes and Multiple Relevant Autoimmune Diseases

Zhiyuan Zhao, Yong Gu, Jeremy Cheng and Liping Yu

Additional information is available at the end of the chapter

Abstract

The presence of islet autoantibodies (iAbs) is currently the most reliable biomarker for type 1 diabetes (T1D). The current "gold" standard radio-binding assays that measure four major iAbs to insulin, IAA, GAD65, IA-2A and ZnT8, are laborious and do not fit for large-scale screenings. Around 40% of patients with T1D develop other autoimmune diseases like celiac disease, autoimmune thyroid disease, and so on. It is highly recommended to screen these closely related autoimmune diseases during T1D screening; however, there is no method available. Recently, on the platform of extensively validated high-sensitive and high-specific electrochemiluminescence (ECL) assay, we developed a multiplex ECL assay to combine up to 10 autoantibody assays into one single well with 5 μl of blood sample. It not only allows us to combine multiple iAbs into one but also makes it possible to simultaneously screen T1D and other multiple autoimmune diseases, which in turn facilitates large-scale screenings in the general population.

Keywords: autoantibody, autoimmune disease, type 1 diabetes, electrochemiluminescence, multuplex assay

1. Introduction

Type 1 diabetes (T1D), the immune-mediated form of diabetes [1], is increasing worldwide, 3–5% annually [2], with rates doubling every 20 years [3, 4], especially in young children.

In the USA, 1.4 million people have T1D and as many have multiple islet autoantibodies (iAbs) or pre-T1D with normal glucose homeostasis. Of the latter, 84% will progress to clinical diabetes in 15 years with a remarkable consistency across populations [5]. Although T1D is a T-lymphocyte-mediated autoimmune disease with specific destruction of pancreatic islet β-cells, autoantibodies directed against proteins in insulin-producing beta cells [1] are the best biomarkers for risk prediction and clinical diagnosis. These iAbs usually appear years before overt clinical disease, and the presence of ≥2 iAbs [antibodies-directed against insulin (IAA), glutamic acid decarboxylase (GADA), islet antigen 2 (IA-2A) or zinc transporter 8 (ZnT8A)] predicts the development of clinical T1D in nearly all affected children [5]. Children at risk for T1D need to be identified prior to the onset of symptoms to: (1) prevent life-threatening diabetic ketoacidosis, (2) identify individuals for current and upcoming trials to prevent T1D and (3) define the onset of islet autoimmunity and its triggers.

Appearance of iAbs is currently the most reliable marker of the autoimmune process leading to T1D, and it determines the disease risk and marks the onset of autoimmune beta cell destruction. The presence of iAbs, their number [6–8] and titer [9–11], has being used extensively to stage diabetes risk and as inclusion criteria into T1D prevention trials [12]. Immunoassays to detect iAbs can be classified into four generations. The first generation is the indirect immunofluorescence on cryostat sections of the pancreas for islet cell antibodies (ICA) [13, 14] present in patients' sera to a variety of autoantigens in islet β-cells. This assay requires the pancreas tissue to conduct the measurement and is very limited in the regular screening. The second generation of iAbs immunoassays is a well-established radio-binding assay (RBA) which is based on various biochemically defined autoantigens and plays a major role as the current gold standard assay for all four major islet autoantibodies IAA, GADA, IA-2A and ZnT8A and ELISAs. Traditional ELISA has been proved not to work well for any iAbs with less sensitivity and less specific in multiple workshops of Islet Autoantibody Standardization Program (IASP, previously DASP). However, a modified bridging ELISA with semifluid-phase interaction between the antibody and antigen has achieved sensitivity and specificity equivalent to the RBA for the measurement of GADA, IA-2A and ZnT8A [15–19]. The third generation is a recently developed and extensively validated nonradioactive iAb assay using electrochemiluminescence (ECL) detection with a higher sensitivity and higher disease specificity compared to the RBAs [20–25]. The ECL assay has been demonstrated to be more sensitive and is able to identify the first iAb of "seroconversion" earlier than RBA by years in young children followed from the birth on pre-T1D who were followed to clinical diabetes. More remarkably, ECL assay is able to discriminate high-affinity, high-risk autoantibodies from those "low risk," low-affinity signals generated by RBA in subjects with single iAb who are less likely to progress to T1D. The fourth generation of autoantibody immunoassays is to develop a simple multiplexed assay to fit for the needs of ongoing and future clinical trials to simultaneously screen multiple iAbs and other autoantibodies in one single well, which will facilitate high-throughput autoantibody screening simultaneously for T1D and other multiple relevant autoimmune diseases in large scale of populations. ECL assay has been illustrated as an excellent platform for a simple multiplex assay with a superior advantage of high sensitivity and disease specificity. In this chapter, we mainly focus on the discussion of: (1) two currently most popular assays for iAbs, a gold standard RBA and a modified bridging ELISA; (2) ECL assay and its comparison with RBA and (3) development of a simple multiplex ECL assay with all advantages considered.

2. Radio-binding assay (RBA) and enzyme-linked immunosorbent assay (ELISA)

At present, four major biochemically defined β-cell autoantigens were well characterized including IAA [26], GAD65 [27], IA-2 [28] and ZnT8 [29]. Through laboratory proficiency programs [30, 31] and harmonization efforts [32], RBA, a fluid-phase assay using radiolabeled antigens and precipitating antibody-antigen complex by Protein A/G Sepharose, has been well established as a current "gold" standard assay for autoantibodies to all four major antigens [29, 32, 33].

In RBA, recombinant human insulin is labeled with I-125, while GAD, IA-2 and ZnT8 antigens are expressed from their corresponding recombinant human cDNA and labeled with 35S-methionine by coupled in vitro transcription/translation. To assess general implementation of assay methods and to standardize the iAb assays, the Center for Disease Control and Prevention (CDC) and Immunology and Diabetes Society (IDS) started organizing the Diabetes Autoantibody Standardization Program (DASP) workshop in the year of 2000 [34]. Forty-six laboratories in 13 countries participated in the program. The first proficiency evaluation of DASP showed a high concordance in measurement of GADA (r = 0.96; p < 0.0001) and IA-2A (r = 0.89; p < 0.0001) using the new WHO international reference reagent. In contrast, the workshop demonstrated wide variation among IAA assays, with poor overall performance and low sensitivity. IAA assays were improved after the first proficiency evaluation and the median laboratory assigned sensitivity was 26, 36 and 45% in 2002, 2003 and 2005, respectively (p < 0.0001). However, the IAA assay has proven the most difficult to standardize with relatively wide discrepancies between laboratories in the IASP workshop and has not yet achieved a satisfactory level of sensitivity and specificity. There was still remarkable variation between laboratories and the ranking of IAA levels in patient serum samples was concordant to AUC (p < 0.001), and the AUC ranged from 0.36 to 0.91 [30]. To facilitate comparison of quantitative islet autoantibody results between studies, the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) set up an Islet Autoantibody Harmonization Committee in 2007 to align measurement and reporting of iAbs in all NIDDK-sponsored studies and several different central laboratories are used [32]. With a few years of combined efforts, both GADA and IA-2A assays were successfully harmonized with great concordance as positive or negative between participating laboratories. The IA-2A assay, using a common threshold of 5 DK units/ml, achieved 64% sensitivity with specificity greater than 99% in all laboratories. For GADA, using thresholds equivalent to the 97th percentile of 974 control samples in each laboratory, 1051 (97.9%) of 1074 samples were concordant. ZnT8 is a most recently identified new islet β-cell autoantigen, and ZnT8A assay has been well established through IASP workshop. In combination of current four iAb assays by the standard RBA, the overall sensitivity is able to cover around 95% in newly diagnosed patients with T1D [29]. To lead to more reproducible identification of individuals at risk of type 1 diabetes and improve monitoring in long-term prospective studies, some recommendations by the Islet Autoantibody Harmonization Committee have been applied to current national and international clinical trials for T1D: (1) confirmation of positive results in a second laboratory is likely to be valuable to identify discrepancies; (2) laboratories should use common calibrators against reference standards; (3) the methods used in multiple centers should be compared

and harmonized to eliminate potential factors that will contribute to discrepancies; (4) a common set of samples which can provide knowledge of concordance, sensitivity and specificity including a large number of samples from healthy controls, and patients should be identified to establish working thresholds of positivity that are similar between the participating laboratories.

T1D is now able to be predicted by measuring iAbs present in the peripheral blood, and these iAbs have been used to determine disease risk and onset of autoimmune beta cell destruction. Almost all children with two or more of these iAbs will progress to clinical diabetes with a remarkable consistency across populations, while progression to diabetes in children persistently positive for a single iAb is only 15% in 15 years [30], as most of these children have a low-affinity iAb not associated with disease [35–37].

In summary, the current method most commonly used for screening iAbs is the "gold" standard RBA, a fluid-phase assay using radiolabeled antigens and immunoprecipitation, to quantify four major iAbs named IAA, GADA, IA-2A and ZNT8A. These four RBAs are currently able to cover 95% of sensitivity in newly diagnosed patients with T1D. Through the efforts of iAb proficiency programs and harmonization consortia, current standard RBA for iAbs have been greatly improved, but there are still work to be done, especially (1) IAA is not achieved its sensitivity and specificity and (2) there is a low risk prediction in subjects with single iAb positivity.

A direct enzyme-linked immunosorbent assay (ELISA) format (binding of antigen to plate and detection of bound autoantibody with labeled anti-antibodies) has proven difficult to develop. To date, only one ELISA-based ElisaRSR™ for GADA, IA-2A and ZnT8A, distributed by Kronus that utilizes capture of solution-phase antigen by one chain of immunoglobulin (Ig) while being bound by its other chain to plate-bound antigen has demonstrated sensitivity and specificity similar to the fluid-phase RBA (www.rsrltd.com). But there is no any ELISA-based assay that works well for IAA measurement according to IASP workshop [30]. ELISA assays were found only to detect insulin antibodies induced by exogenous insulin injection [38], but not natural insulin autoantibodies. Our group reported a high sensitive ELISA-based competition Europium IAA assay in 2009 [39], and it worked very well for mouse IAA assay, but unfortunately it did not work at all for human samples, although RBA-IAA assay works equally well for both human and mouse samples [33].

3. Electrochemiluminescence (ECL) assays

While the current standard RBA for iAbs has been greatly improved through the laboratory proficiency programs and harmonization efforts, there are still works in progress. The IAA assay, especially, has not yet achieved a satisfactory level of sensitivity and specificity. Importantly, IAA has a high prevalence among young children [36] and is usually the first iAb to appear in young children [37]. The ability of prospective clinical studies, for example, The Environmental Determinants of Diabetes in the Young (TEDDY), which aims to identify triggers of islet autoimmunity, depends on accurate detection of the timing of appearance of the first iAb to mark the very beginning of islet autoimmunity. We have recently developed

and extensively validated a new generation of nonradioactive iAb assay using ECL detection, as we described in the previous section, with an excellent sensitivity and specificity [20–25] for both IAA and GADA. The assay is based on the principle that interaction of autoantibodies with antigen molecules is in liquid phase, and the detection signals are directly from the labeled antigen molecules bound to specific autoantibodies without applying the labeled second antibody (labeled anti-human IgG antibody usually used in conventional ELISA) for detection, which usually causes a very high background in autoantibody assays with human samples. As illustrated for ECL assay protocol in **Figure 1**, autoantibodies in serum help bridge the Sulfo-tagged antigen molecule to the biotinylated antigen molecule, which will be captured on the solid phase of the streptavidin-coated plate. Detection of plate-captured Sulfo-tagged antigen is accomplished with electrochemiluminescence. The fact that the ECL assay is able to capture all immunoglobulin subgroups no matter IgG, IgM, IgA or IgE allows this assay a higher sensitivity than current standard RBA based on IgG detection. With the new ECL assays, we have analyzed thousands of participants in Diabetes Autoimmunity Study in the Young (DAISY) [21], TrialNet Pathway to Prevention subjects [23] and very recently TEDDY subjects (unpublished data). Compared with gold standard RBA, the ECL assays, especially ECL-IAA assays, were demonstrated more sensitive. In DAISY longitudinally follow-up study, we analyzed 427 sequential samples from 63 pre-T1D who were closely followed to clinical T1D. Nearly all of these children (62/63) were detected ECL-IAA years before disease onset, including 10 children who were completely IAA negative by RBA-IAA during the follow-up. Remarkably, 25% of these early longitudinally followed samples during
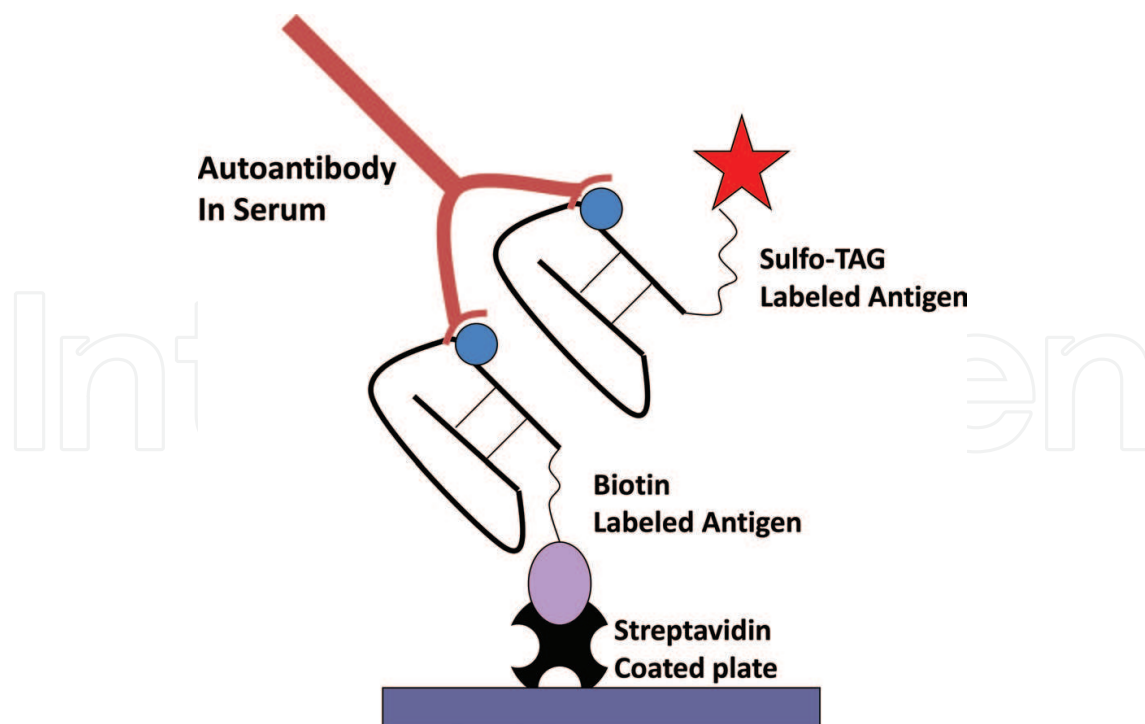

Figure 1. Illustration of the bivalent plate capture ECL assay. The antibody in serum will link the Sulfo-tagged antigen to the biotinylated antigen, which will be captured on the solid phase of the streptavidin-coated plate. Detection of plate-captured Sulfo-tagged antigen is accomplished with electrochemiluminescence.

pre-diabetes stage which were positive for ECL-IAA were negative for RBA-IAA (**Figure 2**). ECL-IAA were found to antedate the onset of islet autoimmunity in these children by a mean of 2.3 years (range: 0.3–7.2 years) [21]. The earlier identification of IAA among young children was validated from a later TEDDY study cohort (unpublished data). Results from these studies indicate that this novel ECL-IAA assay is not only more sensitive but also defines the timing of the initial autoantibody appearance earlier than the previously used RBA-IAA. This earlier detection and accuracy in timing of onset of islet autoimmunity in young children followed from the birth is important to find potential environmental causes of diabetes and our understanding of the etiology of T1D as the ability to identify triggers of islet autoimmunity, for example, TEDDY study depends on the sensitivity and validity of iAb assays used to pinpoint the "seroconversion" to islet autoimmunity.

Remarkably, both ECL-IAA and ECL-GADA assays were selectively detecting the positivity from the high-risk subjects who were followed to progression of clinical T1D or the subjects who were multiple iAbs positive. In the study of DAISY children [20, 22], we analyzed all pre-T1D children who were followed to clinical diabetes and all non-diabetic children who were persistently iAbs positive, either multiple iAbs or single iAb, and compared them with RBA. Almost all samples positive by RBA (IAA or GADA) were ECL assay positive in children on pre-T1D or children with presence of multiple iAbs. In contrast, only around 25% of non-diabetic children with either single IAA or single GADA positive by RBA were ECL positive. **Figure 3** shows positivity of IAA among some children in the DAISY study as an example, persistently expressing IAA with other iAbs (multiple iAbs) or as a single iAb. Panel A: ECL-IAA
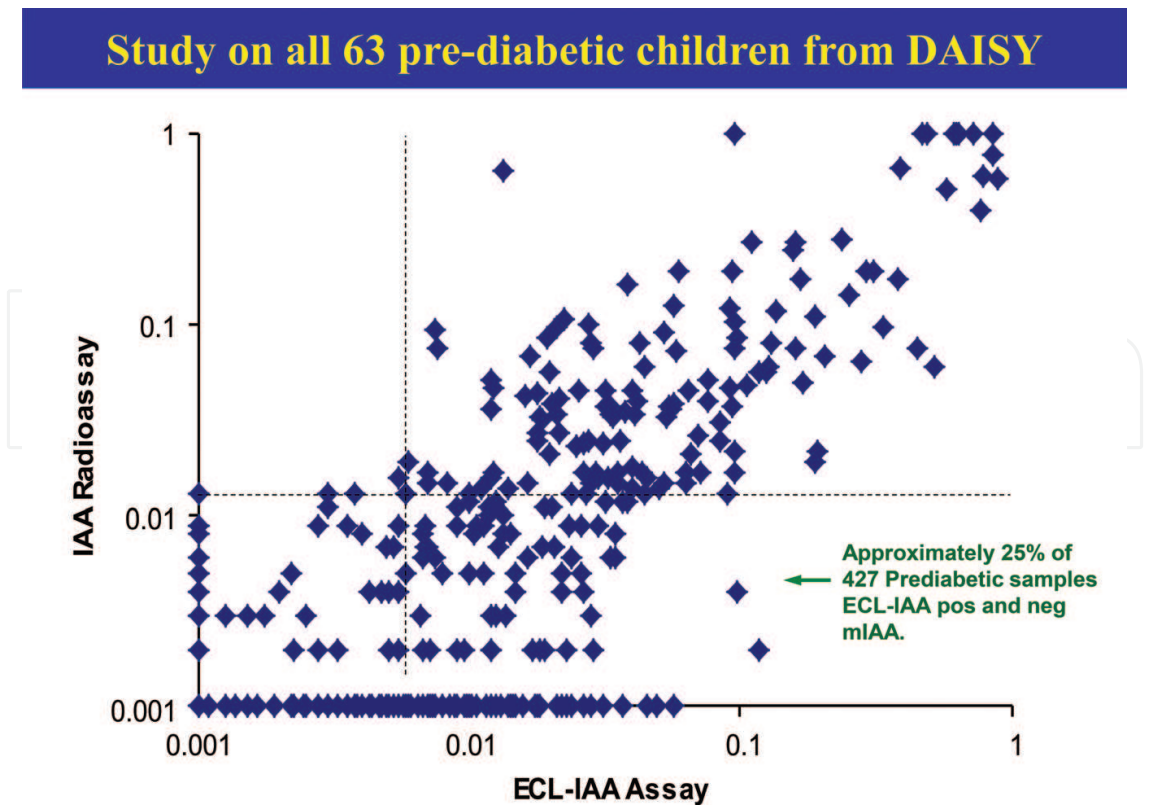

Figure 2. IAA identification in pre-diabetic children by ECL-IAA. The x-axis represents results of ECL-IAA assay and the y-axis represents results of mIAA RIA assay. About 25% of pre-diabetic samples were positive for ECL-IAA but negative for mIAA RIA assay.
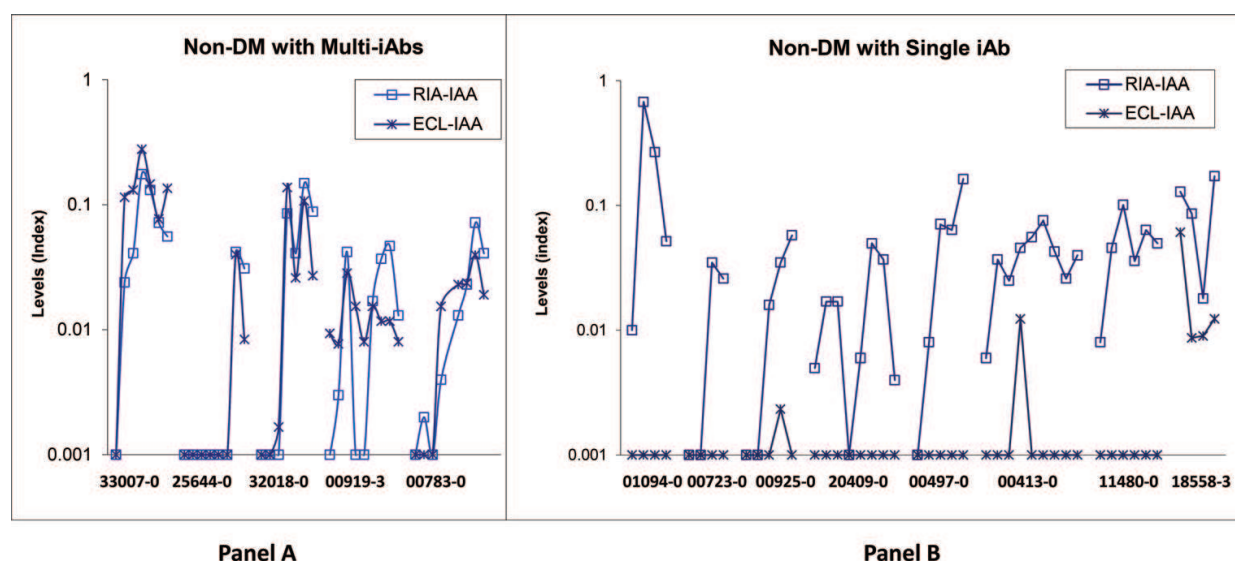
Figure 3. Sera from children in the DAISY study persistently expressing IAA as a single or with multiple iAbs were analyzed with the ECL-IAA assay. Panel A: ECL-IAA was well correlated with RBA-IAA for all 5 subjects (multiple follow-up positive for both assays) who were multiple iAbs positive. Panel B: 7/8 subjects with single iAb (IAA only) were consistently ECL-IAA negative.

was well correlated with RBA-IAA for all five subjects (multiple follow-up positive for both assays), who were multiple iAbs positive. Panel B: 7/8 subjects with single iAb (IAA only) were consistently ECL-IAA negative. The antibody affinity study was performed to compare these IAA or GADA detected by RBA, but differentiated by ECL assay as positive or negative. The results of affinity analysis discovered that IAA or GADA detected by RBA, but negative with ECL assay, in children who were absent of other iAbs had low affinity, while IAA or GADA positive by both RBA and ECL assays had high affinity. **Figure 4** illustrates the IAA (panel A) or GADA (panel B) competition assays with unlabeled proinsulin or GAD65 protein. The IAA or GADA not detected by the ECL assay required a 10- to 100-fold higher concentration of unlabeled insulin/proinsulin or GAD65 protein for 50% inhibition of binding of IAA or GADA to labeled insulin or GAD65 protein. These results demonstrated that both ECL-IAA and ECL-GADA were able to discriminate high-affinity, high-risk iAbs from those "low risk," low-affinity signals generated by RBA in subjects who are less likely to progress to T1D. In our later validation study with a large TrialNet cohort of Pathway to Prevention, identical results were obtained [23] by characteristics of disease specificity with both ECL-IAA and ECL-GADA. The study analyzed 3500 subjects in blind with 571 multiple iAbs, 1727 single iAb and 384 pre-T1D who were followed to clinical T1D later in the study. The ECL assay and RBA were found congruent in pre-diabetics and in the subjects with multiple autoantibodies, but only 24% of single RBA-IAA ($p < 0.0001$) and 46% of single RBA-GADA ($p < 0.0001$) were confirmed by the ECL-IAA and ECL-GADA assays, respectively. With the prospective following up for the subjects with single IAA or GADA, 51% of RBA-IAA and 63% of RBA-GADA subjects not confirmed by ECL were found lost their iAbs and became iAb negative after a mean follow-up time of 2.4 years, behaving as "transient" iAb positivity. Only a few subjects converted to multiple iAbs or progressed to clinical diabetes. In contrast, only small percentages of RBA-IAA and RBA-GADA confirmed by ECL assays became negative ($p < 0.0001$ for both IAA and GADA) during the follow-up as significant number of subjects progressed to clinical diabetes or multiple iAbs. In the study, 2944 subjects were studied with their very first initial screening samples
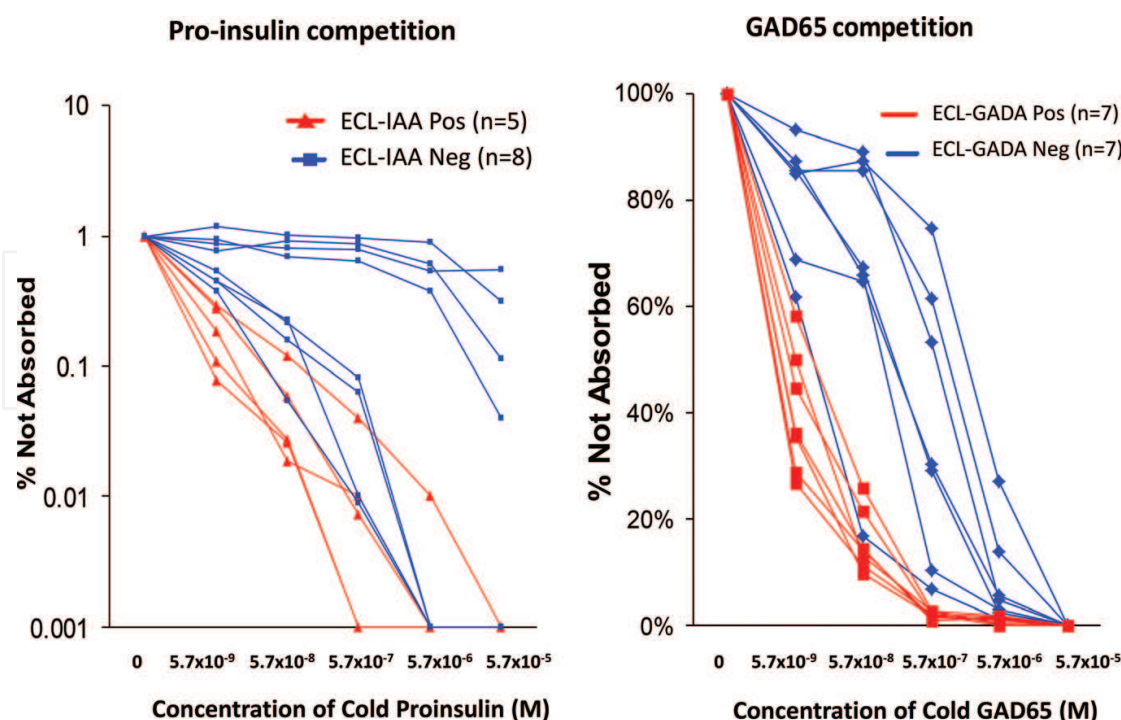
Figure 4. Illustration of the IAA (Panel A) or GADA (Panel B) competition assays with unlabeled proinsulin or GAD65 protein. IAA /GADA negative by ECL-IAA / ECL-GADA assay (blue line), compared with IAA /GADA positive by ECL-GADA/ECL-IAA assay (red line), and required higher concentrations of unlabeled insulin/proinsulin or GAD65 protein for 50% maximal inhibition, which is consistent with low affinity.

and prospectively followed to clinical T1D during the study period. The positive and negative predictive values of RBA and ECL assays, in terms of progression to T1D, were analyzed and compared, and the data are illustrated in **Figure 5**. The positive predictive values for ECL-IAA and ECL-GADA were 32 and 24%, significantly higher than those RBA-IAA and RBA-GADA (21 and 16%, respectively; both $p < 0.0001$) (panel A). Similarly, the negative predictive values for ECL-IAA and ECL-GADA were 94 and 96%, significantly higher than those for RBA-IAA and RBA-GADA (92%; $p < 0.05$ and 94%; $p = 0.007$, respectively) (panel B). Compared with HLA analysis, the highest risk genotype for T1D, HLA-DR3/4, DQB1*0302 was significantly higher in subjects with IAA or GADA confirmed by ECL than those not confirmed by ECL assays ($p < 0.0017$ and $p < 0.0001$, respectively). The frequency of this high-risk HLA genotype in subjects with either single IAA or GADA not confirmed by ECL assays was found identical to subjects with negative results for all iAbs, a very low-risk population who are very unlikely to progress to T1D.

Autoantibody affinity in sequential follow-up samples from their initial positive screening to their later follow-up with a mean follow-up of 5.3 years were investigated [25] in a subset of subjects in TrialNet Pathway to Prevention study who were persistent single RBA-IAA or single RBA-GADA positive confirmed or not confirmed by ECL assays. Among either single IAA or single GADA subjects, all subjects who were confirmed by ECL assays were found to have high affinity autoantibodies at their very first initial positive visit. Affinity results stayed consistent over time. Similarly, those who were negative for ECL assays showed lower affinity at initial visit and affinity stayed low over time. No converting events from low to high or high
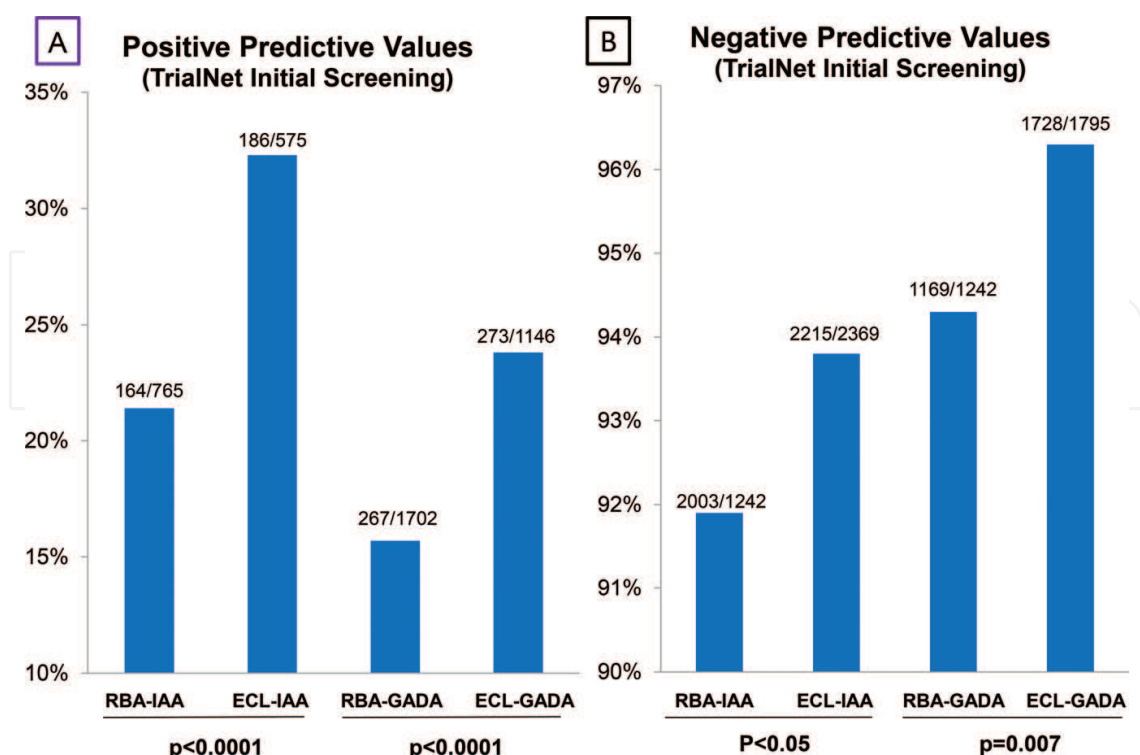
Figure 5. Predictive values of IAA and GADA assays in the TrialNet Initial Screening study. A: Positive predictive values of RIA-IAA and ECL-IAA assays, RIA-GADA and ECL-GADA assays. B: Negative predictive values of RIA-IAA and ECL-IAA assays, RIA-GADA and ECL-GADA assays. Positive and negative predictive values of ECL-IAA and ECL-GADA were significantly higher than those of RIA-IAA and RIA-GADA assays, respectively.

to low affinity were seen over time. There were 14 subjects in the group who progressed to clinical T1D during the study period. All except one were ECL-positive and had a high affinity for IAA or GADA. These results implicate that a more disease-specific iAb measurement like ECL assay can identify high affinity iAbs with high disease risk on the early stage of initial screening. It is generally assumed that the very low risk of those with only a single iAb, either single IAA or single GADA, spreading of autoimmunity to other autoantigens is needed to increase risk or marks a stage closer to overt diabetes, and multiple studies of both relatives of T1D patients and general populations have documented that expression of multiple iAbs are associated with extreme risk of progression to T1D. The pattern of expression of iAbs detected by ECL assays predicts both epitope spreading and diabetes risk. Differences in iAbs reactivity can be assessed prior to epitope spreading consistent with early determination of both

	RBA positive, GADA only			RBA positive, IAA only		
	ECL+ (n = 107)	ECL− (n = 78)	p-value	ECL+ (n = 24)	ECL− (n = 63)	p-value
PS6M (mg/dl)	22 ± 90	−19 ± 71	0.001	36 ± 75	−9 ± 70	0.009
Progressed to T1D	14.0% (n = 15)	1.3% (n = 1)	0.002	16.7% (n = 4)	0% (n = 0)	0.005

PS6M is a 6-month progression scale based on worsening of the 30–120-min OGTT glucose.

Table 1. ECL iAb identify progressors to T1D among children and adults with single iAb by RBA.

epitope spreading and diabetes risk. This is of particular value in children and young adults who are positive for a single iAb, as demonstrated by TrialNet Pathway to Prevention (**Table 1**, J Sosenko, manuscript submitted). Among TrialNet participants who were positive for a single iAb by RBA, 52% (141/272) were not confirmed by ECL. These ECL-negative subjects showed no worsening of glycemia and little progression to T1D during a median follow-up of 4.7 years. In contrast, OGTT glycemia worsened significantly in the ECL-positive single iAb participants, comparably with the worsening in 90 multiple iAb + subjects (PS6M 23 ± 96 mg/dl); the latter group had a high risk for progression to T1D (30%). The ECL assay can substantially refine the selection of single iAb positive individuals at high risk, who possibly could be recruited for participation in T1D prevention trials.

Presently, clinical prevention trials in T1D TrialNet study are only selecting relatives of patients with T1D who have multiple iAbs. It is generally agreed that some clinical intervening on early stages of islet autoimmune processes could result in a better outcome as some evidence in animal model for prevention [40], but the clinical trial studies using subjects with single iAb, either single IAA or GADA, are not available since the risk is too low. However, the subjects with single iAb (IAA or GADA) detected by more disease-specific assays like ECL assay with the nature of ability to detect high affinity antibodies may qualify for enrollment into prevention trials as their risk for diabetes is much higher and those with low affinity, low-risk signals generated by RBA will be removed [20, 22, 23, 25]. On the other hand, subjects found to be negative for ECL assays may benefit from less intensive monitoring in these longitudinal prospective studies [41] to save the efforts and costs of these studies.

In summary, islet autoimmunity of T1D can be identified at the very beginning of the disease process by measurements of iAbs. Two major iAbs, IAA and GADA, usually appear earlier than other iAbs and are often detected in isolation as single iAb in the screening of relatives and general population. Most of these single iAbs detected by current gold standard RBA are at low risk, low affinity and non-disease relevant as "biologically" false positives while part of these single iAbs does represent the early stage of islet autoimmunity in T1D progression. With more disease-specific assays like an ECL assay method, high-risk and disease-relevant iAbs are able to be identified at the very beginning of the disease process in subjects with single iAb before the development of multiple iAbs closer to overt clinical diabetes, which will be greatly appreciated for clearing the current confusions of single iAb positivity and aid the T1D clinical trials for both identifying environmental triggers of islet autoimmunity and intervening with islet autoimmune process on very beginning stage to prevent the disease. The ECL assay was demonstrated its superiority to RBA in sensitivity and especially ECL-IAA was able to antedated the onset of islet autoimmunity by years than RBA, which will be very important to accurately pinpoint the very beginning of islet autoimmunity for identifying the environmental triggers to cause the T1D.

4. Development of a multiplex assay for large-scale screening

At present, many large-scale national and international clinical trials for T1D are in progress, and multiple candidate interventions are being proposed to abrogate or slow progression of T1D among iAb positive subjects. A wider screening of iAbs in the general population, especially in young children, is perspectively in progress or in planning. Currently, four biochemically

defined iAbs including IAA, GADA, IA-2A and ZnT8A are equally important in prediction and evaluation of risk of progression to T1D in both relatives of patients with T1D and general population. The screening methods using current standard RBA with single iAb measurement are laborious and inefficient for such a large scale of screening. While significant progress has been made in standardization of iAb assays and high-throughput technologies, the cost and logistic complexity of currently used methods preclude their widespread use in population-based screening. The determination of initial iAb positivity is very important, which may represent the initiation of islet autoimmunity. However, the results of iAb measurement at this early stage in subjects with a single iAb are not reliable with current standard RBA since the majority of these single iAb subjects identified by RBA have low affinity antibodies, most of them transient, and therefore biologically appear to be "false positives" with respect to T1D development as we discussed earlier. Poor assay specificity is likely to be more problematic for screening process in general population with lower frequency of risk for T1D than high risk relative cohort as in TrialNet Pathway to Prevention we studied. A high-throughput assay technology with improved disease specificity will be important and necessary.

One in four children at risk for T1D develops islet, celiac, thyroid or rheumatoid autoimmunity in the DAISY study. Interestingly, there is little overlap of these phenotypes in an individual child, but this overlap increases with age. The incidence of autoantibodies to thyroid peroxidase (TPOA) surges after the age of 12. Importantly, up to 40% of patients with T1D have an additional autoimmune condition [42–44]. It is important and urgent to screen biomarkers of other autoimmune diseases when screening diabetes, simultaneously. Unfortunately, there is no easy and inexpensive tool to screen for these conditions. With a big effort, all DAISY and TEDDY study participants are screened for autoantibodies to tissue transglutaminase (TGA) for celiac disease autoimmunity. Persistent TGA positivity and celiac disease are secondary endpoints in both studies [45, 46]. DAISY data suggest that, by age 18, at least 7% of the general population persistently express one or more of the nine autoantibodies: IAA, GADA, IA-2A, ZnT8A, TPOA, TGA and three other autoantibodies for rheumatoid arthritis, Addison's disease and autoimmune gastritis. If confirmed, this would argue for a universal screening. We have carried out a pilot of such screening for iAbs and TGA, in children of 2–6 years old attending general pediatric care offices in Denver [47]. Participating parents and providers ranked the combined screening for iAbs and TGA as more valuable than screening for iAbs alone.

To fit the purpose of large-scale screening in national clinical trials and the general population, people are starting to seek a possible method of a multiplexed assay combining multiple autoantibody assays together in one single well. Recently, a few studies of multiplex antibody assays were reported with different technologies [48–51], but none of these assay platforms has neither compared with currently used "gold" standard RBA for its sensitivity and specificity in T1D study, especially in subjects with risk to T1D, nor validated in an international Islet Autoantibody Standardization Program (IASP) workshop or in a large cohort of clinical trial. From previous multiple studies, none of the conventional ELISA methods worked well for any iAb measurements, especially for IAA according to multiple IASP workshop [30]. Interaction of iAbs with their corresponding antigen proteins in liquid phase will still be necessary in a multiplex assay setting to achieve a proper sensitivity and specificity and it is a particularly essential condition for IAA assay. The capacity of specific autoantibody-antigen binding might be a new consideration with our recent experiences in a multiplex assay setting, which has never been an issue in any single antibody assay format. Multiple

autoantibody-antigen interactions share the space within one single well in a multiplex assay setting, and each of these interactions might need enough number of their own specific antibodies binding to their corresponding antigen proteins to generate a signal strong enough to be detected, which is particularly important when these autoantibodies are at low levels.

Very recently, a modified ELISA-based ElisaRSR™ 3-Screen ICA™ is now available for research from the RSR Limited (3-Screen ICA™ ELISA; www.rsrltd.com). It is a combination assay for measuring GADA, IA-2A and ZnT8A. Its single assay platform was validated at multiple IASP workshops for its sensitivity and specificity. The 3 Screen ELISA assay measures three autoantibodies either in three separate wells consuming large volume of serum or in one single well with three assays mixed not able to distinguish which of the three beta cell autoantibodies are present. The biggest disadvantage of the 3 Screen ELISA is its inability to include IAA measurement in the assay. IAA has a very high rate of positivity in young children and is considered as the first iAb on early stages of islet autoimmunity as we discussed earlier. Compared with current single iAb RBA or original single ELISA-based ElisaRSR™ assays, the 3 Screen ELISA assay definitely has its advantage of higher efficiency [52], but screening with its own without including IAA measurement will be a big defect and will not be the best way to persume.

With the platform of our newly established ECL assay technology, we recently published our study of a multiplex assay to accurately measure four autoantibodies in one single well [53] with MesoScale Discovery (MSD) QuickPlex 4-Spot plate as illustrated in
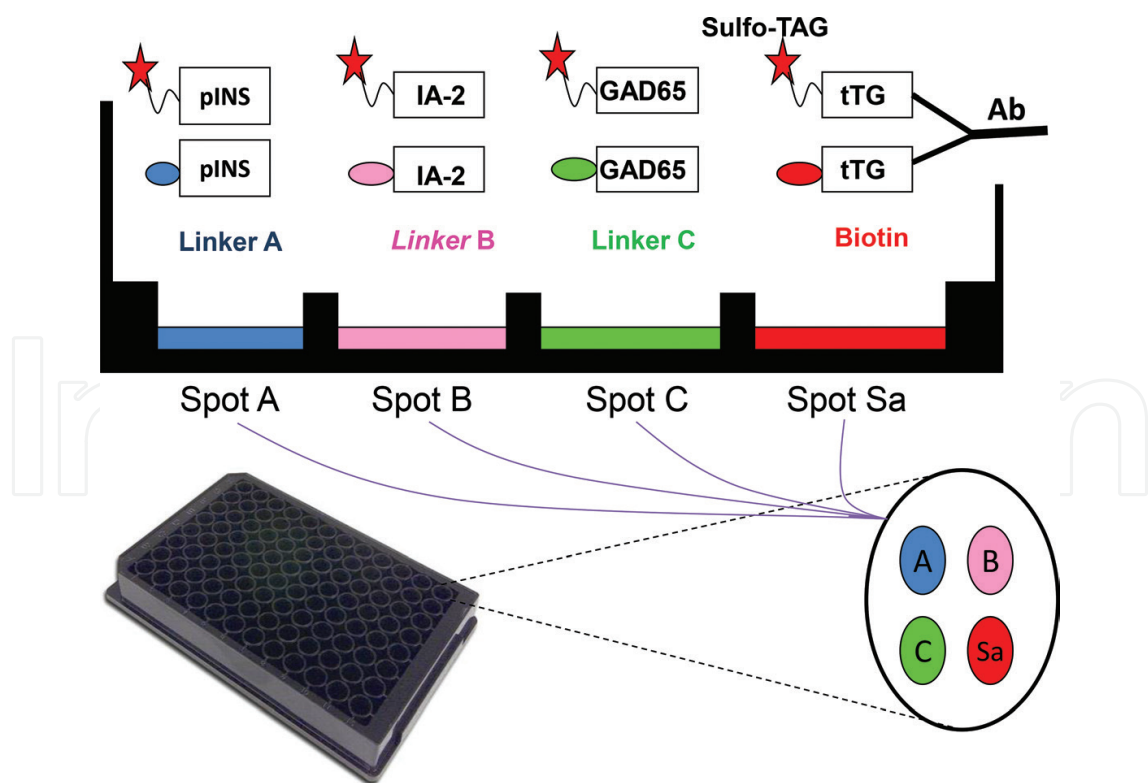

Figure 6. Illustration of ECL 4-spot assay. The 4-spot assay is based on the same mechanism of single ECL assay. Each biotinylated antigen will be linked by its corresponding linker which will be captured on the solid phase of the pre-coated plate. Detection of plate-captured Sulfo-tagged antigen is also accomplished with electrochemiluminescence. 4-spot assay is able to accommodate up to 4 autoantibody assays in one well.

Figure 6. The 4-plex assay was based on the same mechanism of our single ECL assay, but linker system was introduced. Four interactions of antibody-antigen are, respectively, restrained on each of four specific linker spots within the same well, and the camera is able to catch the signals from four different sources of spots, respectively. With the limitation of four spots able to accommodate maximum four autoantibody assays in one well, we selected, on purposely, IAA, GADA, IA-2A and TGA. We included TGA instead of ZnT8A because (1) ZnT8A is almost always present with other iAbs and ZnT8A alone is only 1% in subject followed to T1D (8). In two large national clinical trials of TrialNet and TEDDY, ZnT8A is not included in intial screening, and the ZnT8A assay is only performed if any of other three iAbs is positive. (2) We want to screen both T1D and celiac disease as we rationaled earlier. The 4-plex assay retained 100% sensitivity and 100% specificity for all four autoantibodies in terms of positivity identified in patients versus normal controls compared to the corresponding standard RBA and our single ECL assays. In early 2015, MSD company released the new U-Plex™ Development Packs system for creating custom multiplex panels of analytes to replace the QuickPlex 4-Spot system. With a similar principle of multiplex assay mechanism of QuickPlex 4-Spot system, this new system expanded multiplexing up to 10, combining 10 different autoantibody assays in one single well with the same amount of 6 μl serum sample used for a single ECL assay. The UPlex plate assay system is illustrated in **Figure 7** as we currently used it as an UPlex 8-plex assay. With this new system, we have successfully combined eight autoantibody assays within one single well including all four iAbs and four other
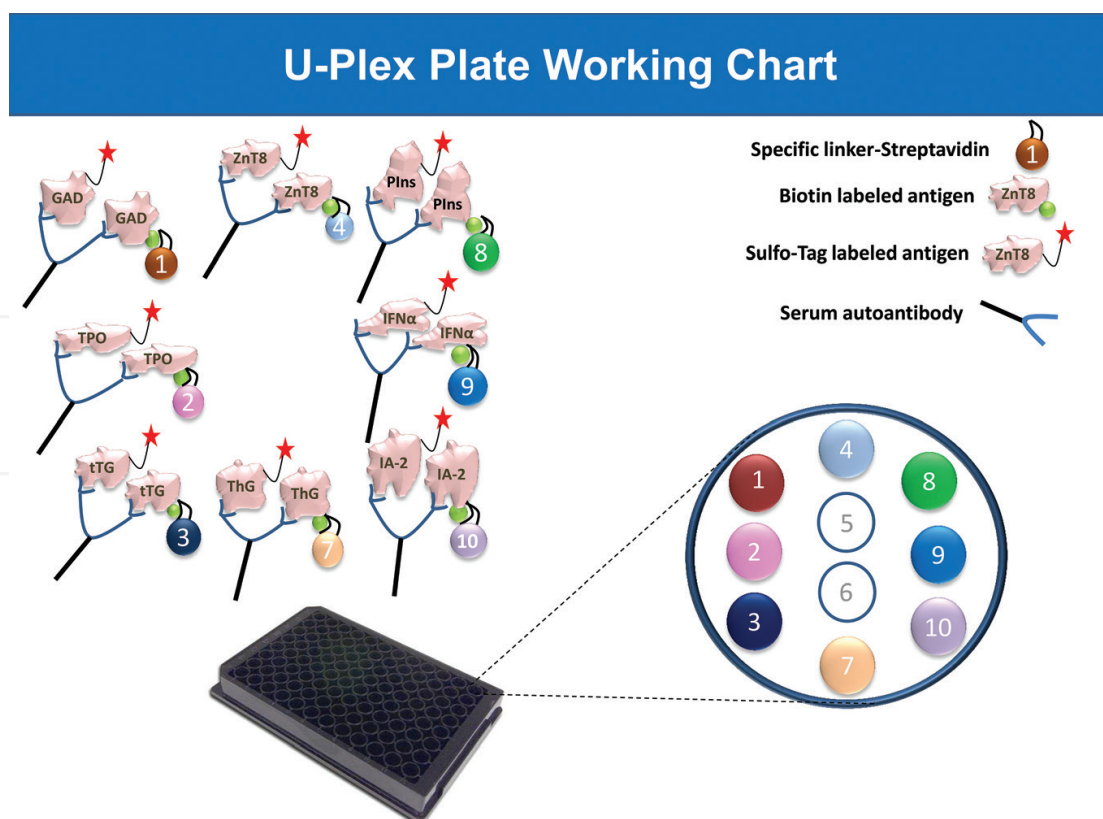

Figure 7. Illustration of UPlex 8-plex plate working chart. 8-plex assay is working on the same mechanism of 4-spot assay. Uplex can combine up to 10 antibody assays in one well.

autoantibody assays, TGA for celiac disease, TPOA and autoantibodies to thyroid globulin (ThGA) for autoimmune thyroiditis, autoantibodies to interferon alpha (IFNaA) for autoimmune polyglandular syndrom-1 (APS-1). With 100th percentile of specificity in 118 healthy normal controls, the 8-plex assay was able to retain 100% sensitivity for all autoantibodies, and the levels of autoantibodies in 8-plex assay were well correlated with their corresponding single RBA or ELISA (for IFNaA) in 168 T1D patients. The further work of assay optimization needs to be done to minimize the interferences of cross-talking between spots, especially an extreme high signal on one spot overspilled to a neighboring spot when it should be negative. The UPlex system made it possible to customize a multiplexed assay according to the needs for screening. It is capable to screen children simultaneously for T1D and other multiple autoimmune diseases often happening in childhood. It is also capable to screen adults simultaneously for T1D and other multiple autoimmune diseases usually seen in adulthood. Such a multiplex ECL assay technology will retain high assay sensitivity and disease specificity as we discussed earlier and provide a great tool for a large scale of screening in the general population with high efficiency and low cost using only a tiny amount of blood sample. We expect these multiplex assays with new technologies, be available in clinic and easily applied for population screening in the near future.

In conclusion, T1D is now predictable by measuring major iAbs. The ECL assay for iAbs is superior to the current gold standard RBA and other methods in terms of assay sensitivity and specificity for disease risk prediction. With a rapid increasing rate of disease, large scales of population screenings are becoming important for the public health. Large percent of patients with T1D develop other autoimmune diseases, and it has been recommended to screen other relevant autommimune diseases when screening diabetes autoimmunity simultaneously. With the advantages of the ECL assay in its nature of high assay sensitivity and high disease specificity, a simple multiplex assay built on the platform of ECL technology will provide an excellent tool to not only screen multiple iAbs in one single well, but also screen multiple autoimmune diseases simultaneously in large scale of populations efficiently and economically.

Acknowledgements

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