Video Article

Electrochemiluminescence Assays for Human Islet Autoantibodies

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Abstract

Pinpointing islet autoantibodies associated with type 1 diabetes (T1D) leads the way to project and deter this disease in the general population. A novel ECL assay is a nonradioactive fluid phase assay for islet autoantibodies with higher sensitivity and specificity than the current 'gold' standard radio-binding assay (RBA). ECL assays can more precisely define the onset of presymptomatic T1D by distinguishing the high-risk, high-affinity autoantibodies from the low-risk, low-affinity autoantibodies generated in RBAs, and conventional enzyme-linked immunosorbent assays (ELISA). The antigen protein used in this ECL assay is labeled with Sulfo-tag and Biotin, respectively. Each ECL autoantibody assay that uses a particular antigen protein needs an optimization step before it can be used for laboratory application. This step is especially vital in determining the requirements for serum acid treatments, concentrations, and ratios of the two different antigens labeled with Sulfo-tag and Biotin. To perform the assay, serum samples are mixed with Sulfo-tag-conjugated and biotinylated capture antigen protein in phosphate buffered solution (PBS), containing 5% Bovine Serum Albumin (BSA). Afterwards, the samples are incubated overnight at 4 °C. The same day, a streptavidin-coated plate is prepared with blocker buffer and incubated overnight at 4 °C. On the second day, wash the streptavidin plate and transfer the serum-antigen mixture onto the plate. Place the plate on the plate shaker, set it at low speed, and incubate at room temperature for 1 h. Subsequently, the plate is washed again, and reader buffer is added. The plate is then counted on the plate reader machine. The results are conveyed through an index, which is generated from internal standard positive and negative control serum samples.

Video Link

The video component of this article can be found at https://www.jove.com/video/57227/

Introduction

A recent staging classification system has been created to assist with the diagnosis of initial stages of T1D in patients. Exact detection of human islet autoantibodies plays an important role in identifying and staging presymptomatic type 1 diabetes, as the presence of islet autoantibodies indicates the presence of β-cell autoimmunity. The rate at which diabetes affects patients from the initial occurrence of β-cell autoimmunity to the symptomatic disease, associated with the number and type of islet autoantibodies, is variable[2,3].

The age of autoantibody seroconversion, titer, and affinity of islet autoantibodies can affect the rate of the progression to symptomatic type 1 diabetes[4,5,6,7,8,9,10]. Recently, developed ECL assays have been extensively validated, have demonstrated increased sensitivity, and are more disease-specific[10,11,12,13]. These assays enhance the prediction and staging of diabetes risk through earlier detection of islet autoantibodies. They more precisely mark the initiation of islet autoimmunity and ignore the low-affinity and low risk signals not relevant to diabetes.

In an ECL assay, the autoantibodies in the serum, if present, bridge the Sulfo-tag-conjugated antigen to the biotinylated capture antigen in the fluid phase. After bridging, the Biotin linker is caught in the solid phase and detected through ECL by the Sulfo-tag on the streptavidin coated plate (**Figure 1**).

In this review, single antibody ECL assays with human islet autoantibodies are primarily utilized. Briefly, multiplexed antibody assays based on single ECL assays will be discussed. The multiplex assay can be used to identify multiple, up to 10, autoantibodies within one single well, using 15 µL of serum. This simple high throughput assay can be used to screen, simultaneously, multiple autoantibodies for multiple relevant autoimmune diseases in the general population.

Protocol

1. Buffer Preparation

1. Labeling buffer (2x PBS, pH 7.9): In 400 mL of distilled deionized (DD) water, add 100 mL of 10x PBS, adjust pH to 7.9 with NaOH.
2. 3 mM of Biotin: Dissolve 1 mg of Biotin in 588 µL of labeling buffer.

3. 3 mM of Sulfo-tag: Dissolve 150 nmol of Sulfo-tag in 50 µL of labeling buffer.
4. Antigen buffer (5% BSA): In 500 mL of 1x PBS, add 25 g of Bovine Serum Albumin (BSA).
5. Prepare 0.5 M acetic acid solution.
6. 1.0 M of Tris-HCl buffer pH 9.0: Prepare 1 M Tris-HCl buffer, and adjust pH to 9.0 with HCl.
7. Coating buffer (3% Blocker A): In 500 mL of 1x PBS, add 15 g of Blocker A.
8. Washing buffer (0.05 % Tween 20, PBST): In 5000 mL of 1x PBS, add 2.5 mL of Tween 20.
9. Reading buffer (2x Read Buffer T with surfactant): In 500 mL of DD water, add 500 mL of 4x Read Buffer T with Surfactant (**Table of Materials**).
10. Store Biotin and Sulfo-tag in a -20 °C freezer.
    NOTE: Both Biotin and Sulfo-tag solutions should always be freshly prepared just before the labeling procedure.

2. Label the Human Islet Autoantigen with Biotin and Sulfo-tag

Note: A high concentration of antigen, ≥0.5 mg/mL, is recommended for a more efficient labeling reaction.

1. Determine the molar ratio of human islet autoantigen for Biotin and Sulfo-tag.
    1. Obtain the antigen molar number by dividing the antigen weight by the molecular weight.
    2. Use the molar ratio of 1:5 for the antigen with smaller molecular weight (≤10 kd), and the molar ratio of 1:20 for the antigen with larger molecular weight (>50 kd).
    3. Calculate the volume for Biotin or Sulfo-tag by dividing the molar number by its concentration.
2. Mix the human islet autoantigen with Biotin or Sulfo-tag with the molar ratio of 1:5.
    NOTE: The protein in either tris or glycine buffer systems should be exchanged to 2x PBS buffer with pH 7.9 using the sizing spin column.
3. Since Biotin and Sulfo-tag are light sensitive, cover the reaction tubes with aluminum foil. Incubate the reaction at room temperature (RT) for 1 h.
4. During the incubation, prime the spin column by dispensing 2x PBS buffer into the column three times. Centrifuge the solution at 1000 x g for 2 min each time.
    NOTE: Currently the labeling reaction, through bridging, is still occurring and needs to be stopped.
5. Stop the labeling reaction by purifying the labeled human islet autoantigen. To purify, pass the autoantigen through the spin column and centrifuge the column at 1000 x g for 2 min.
6. Determine the labeled antigen concentration (µg/µL) using the amount of antigen protein and the final volume.
    NOTE: Roughly, there will be 90 - 95% retention of labeled antigen after every spin column pass.
7. Aliquot the labeled antigen and store labeled antigen at -80 °C.

3. Define the Best Concentrations and Ratios for the Two Labeled Antigens for the Assay (Checker Board Assay)

1. Prepare two serum samples, one high positive and one negative, with a total volume of 200 µL for each.
2. Aliquot 4 µL of serum and add 1x PBS until the final volume is 20 µL per well for a 96-well PCR plate. Use half of a plate for the high positive sample and the other half for the negative sample, as shown in **Figure 2A**.
3. Add 10 µL of Biotin and 10 µL of Sulfo-tag labeled antigen and conduct serial dilutions for the two differently labeled antigens, as shown in **Figure 2A**. Run a horizontal serial dilution for one labeled antigen and a vertical serial dilution for the other labeled antigen.
4. Continue the rest of the assay steps described in 5.3 to 9.1.
5. Calculate the ratio of signals from the high positive sample against each of the corresponding signals from the negative sample shown in **Figure 2B**.
6. Identify the best concentrations for Biotin and Sulfo-tag labeled antigens by selecting a point with the highest or near highest ratio of positive to negative. Consider the low background signal from the negative sample to identify the ratio.
    Note: Assay Day 1 includes Step 4 to Step 6.

4. Prepare the Antigen Buffer Using the Correct Concentration of Biotin/Sulfo-tag Labeled Antigen

1. Select the rational concentration of each antigen based on the checker board assay.
2. Prepare 3 mL of antigen solution per plate, using the rational concentration of Biotin/Sulfo-tag labeled antigen for the antigen buffer.

5. Incubate Serum Samples with Labeled Antigen

Note: There are two protocols in this section, one without serum acid treatment and one with serum acid treatment. All islet autoantibody assays except IAA assay use regular protocol without serum acid treatment from steps 5.1 to 5.5, whereas IAA assay skips these steps and uses protocol with serum acid treatment from steps 5.6 to 5.9.

1. Aliquot 4 µL of serum and add 1x PBS until the final volume is 20 µL per well for a 96-well PCR plate.
2. Add 20 µL of labeled antigen solution per well.
3. Cover the PCR plate with sealing foil to avoid light.
4. Put the plate on a shaker (low speed) at RT for 2 h.
5. Put the plate in the 4 °C refrigerator and incubate overnight (18 - 24 h).
    Note: The following protocol of steps from 5.6 to 5.9 is only designed for IAA assay and other autoantibody assays skip these steps.

6. Mix 15 µL of serum with 18 µL of 0.5 M acetic acid for each serum sample. Incubate this mixture at RT for 45 min.
7. Prepare the antigen solution with the rational concentration of Biotin and Sulfo-tag labeled antigen, based on the checker board assay, using antigen buffer. In each well, aliquot 35 µL of antigen buffer, containing Biotin and Sulfo-tag labeled antigen, into a new PCR plate.
8. Before the 45 min incubation (step 5.6) step is finished, add 8.3 µL of 1 M Tris pH 9.0 buffer to the side of each well on the antigen plate (step 5.6). It is important to limit the mixing between the Tris buffer and the antigen. Immediately transfer 25 µL of the serum, which is treated with acid, in step 5.6, into each well and agitate the solution. Cover the PCR plate with sealing foil to avoid light.
9. Put the plate on a shaker (low speed) at RT for 2 h. Put the plate in the 4 °C refrigerator and allow the plate to incubate overnight (18 - 24 h).

6. Prepare the Streptavidin Plate

1. Take a streptavidin plate from the 4 °C refrigerator and allow the plate to come to RT.
2. Once the streptavidin plate is at RT, add 150 µL of 3% Blocker A to each well.
3. Cover the PCR plate with sealing foil.
4. Incubate the plate in the 4 °C refrigerator overnight.
   Note: Assay Day 2 includes Step 7 to Step 9.

7. Transfer Serum/Antigen Incubates to the Streptavidin Plate

1. The next day, take out the streptavidin plate from the refrigerator and discard the buffer from the plate. Set some dry paper towels out on the table and tap the plate upside down until there is no more buffer inside of the wells.
2. Fill the empty streptavidin plate with 150 µL of PBST per well and discard the PBST from the plate for a total of three washes.
3. Transfer 30 µL of serum/antigen incubates per well into the streptavidin plate.
4. Cover the plate with foil to avoid light. Shake the plate, at a low setting, at RT for 1 h.

8. Wash the Plate and Add Read Buffer

1. Discard incubates from the plate. Add 150 µL of PBST per well and discard the PBST from the plate for a total of three washes.
2. After washing, add 150 µL per well of Reading buffer.
   NOTE: It is important to prevent air bubbles in the solution because this will affect how the plate is read on the plate reader machine.

9. Read the Plate and Analyze Data

1. Count the plate on the plate reader machine. Antibody values are shown as counts per second (CPS).
2. Convey the antibody levels received from the plate reader machine as a relative index. Calculate the index from the following equation: Index value = [CPS (sample) - CPS (negative control)] / [CPS (positive control) - CPS (negative control)].
3. Identify positive and negative antibody results using the cut-off for positivity, defined based on the 99th percentile of 100 healthy control subjects (non-diabetic individuals without any known autoimmune diseases and who have no family history of diabetes).

Representative Results

Figure 2 displays the checker board. It is shown that 250 ng/mL of Biotin and 250 ng/mL of Sulfo-tag labeled antigen are the most rational concentrations used in the assay considering the signals from the high positive control sample, the negative control sample as the assay's background, and the ratio of positive to negative signals**.** With the optimized concentrations of these two labeled antigens, the assay was performed with the serum samples from 100 newly diagnosed patients with T1D and 100 healthy controls. The index value of 0.023 was defined as the assay cut-off for positivity. This represents 85% sensitivity in patients and 99% specificity in healthy controls using the receiver operating characteristic (ROC) curve shown in **Figure 3A**. The assay for the unknown samples was conducted with internal standard high positives, low positives, and negative control samples. The results of the CPS counts are shown in **Table 2A.** The mean CPS of high and low positive controls are highlighted in red, 17903 [(19940+15866)/2] and 839 [(857+820)/2], and the negative controls are highlighted in green, 168 [(170+165)/2]. The index for unknown samples is calculated by "(CPS_{sample}-168)/(17903-168)." **Table 2B** shows the calculated index values for all samples. The index values that are greater than 0.023 are written in red, corresponding to the CPS values also written in red in **Table 2A**. These values will be defined as the positive results that are greater than the 99^{th} percentile of the healthy control population. When an irrational antigen concentration is used, the assay will have a high background, as shown in **Table 2C.** Low levels of positive antibodies will be missed like the values A2, A5, D1, and H4 highlighted in gray in **Table 2D**.

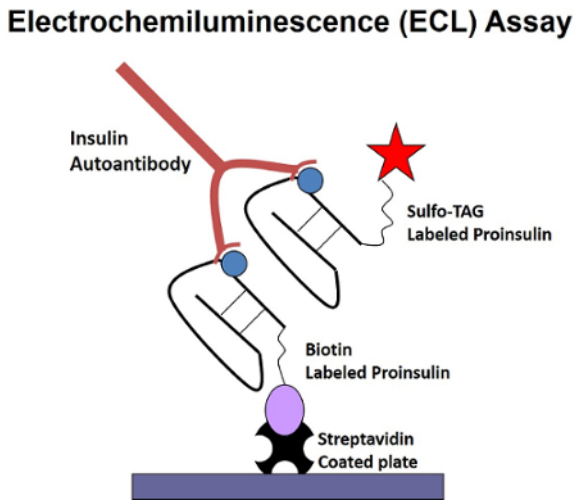


Figure 1: Illustration of a bivalent plate capture on a single ECL-IAA assay. The islet autoantibody in the serum bridges the Sulfo-tag conjugated antigen to the biotinylated capture antigen, which is captured in the solid phase on the streptavidin-coated plate. Detection of plate-captured Sulfo-tag conjugated antigen is accomplished through ECL. This figure has been modified from Yu, *et al.*[11]. Please click here to view a larger version of this figure.

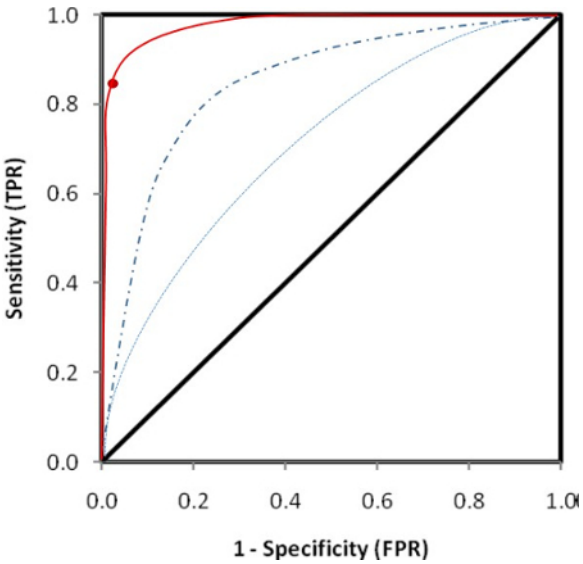
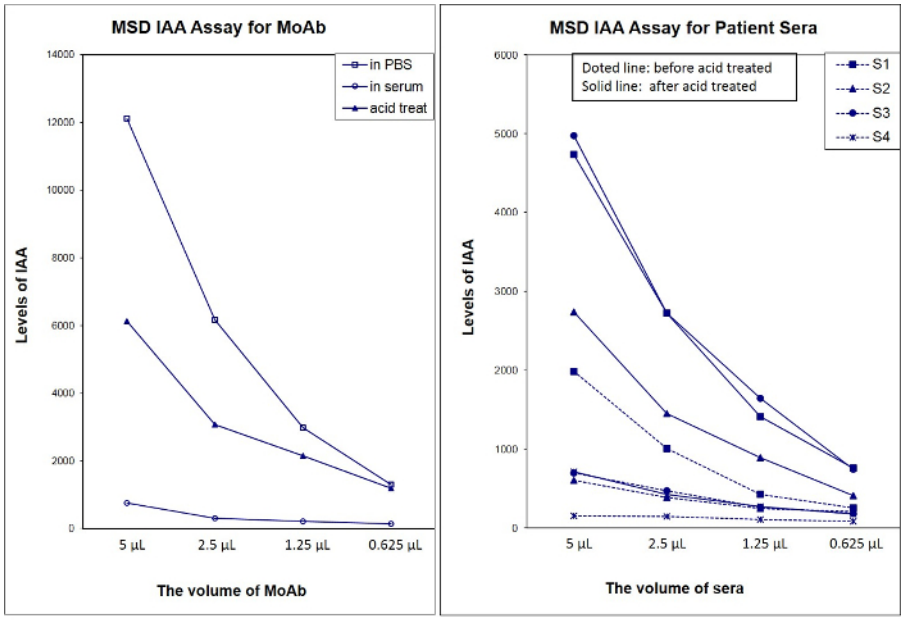


Figure 2: The ROC curve for determining the cut-off of assay positivity. The 99th percentile of specificity corresponding to 85% sensitivity was selected and is represented as an index value of 0.023. This upper limit of the assay was taken from 100 healthy controls. Please click here to view a larger version of this figure.



Panel A Panel B

Figure 3: Illustration of normal human serum blocking ECL-IAA signals. A: The signals from ECL-IAA assays with an insulin monoclonal antibody (MoAb) were drastically blocked by the addition of normal human serum. When comparing MoAb in PBS, the signal was partially restored when the human serum was treated with acid. B: Signals from an ECL-IAA assay with 4 patient sera were significantly enhanced with acid treatment. This figure has been modified from Yu, *et al.*[11]. Please click here to view a larger version of this figure.

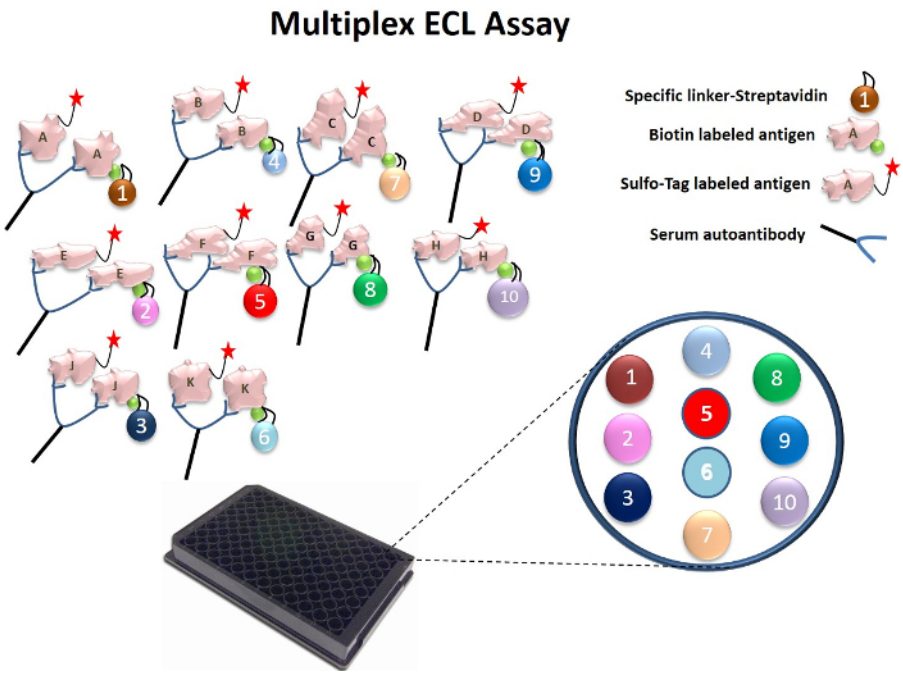


Figure 4: Illustration of the Mutiplex ECL assay. Antibody-antigen complexes are formed in the fluid-phase with specific linkers. The specific antibody-antigen-linker complexes are restrained on each of the specific linker-spots in the same well. The plate reader machine is able to distinguish the signals from different sources of spots and gives CPS counts on 10 different channels, respectively. Please click here to view a larger version of this figure.

A

		Positive control						Negative control					
Biotin (ng/mL)		1000	500	250	125	62.5	31	1000	500	250	125	62.5	31
SULFO-TAG (ng/mL)		1	2	3	4	5	6	7	8	9	10	11	12
2000	A	41271	24782	12430	6814	3394	1743	2799	1914	964	553	389	269
1000	B	36633	21364	11520	5274	2907	1992	1556	918	537	322	238	194
500	C	26318	14159	10721	3813	2279	1428	904	477	256	227	170	123
250	D	14777	10238	8086	3034	2008	868	543	293	191	152	131	102
125	E	8385	7572	4347	2015	1237	564	298	218	154	116	99	82
62.5	F	5119	3953	1809	1048	598	391	187	158	118	96	91	73
31	G	2398	1675	911	608	384	265	129	110	91	79	75	66
0	H	66	65	69	62	67	60	63	60	67	62	68	60

B

Ratio: PC/NC					
15	13	13	12	9	6
24	23	21	16	12	10
29	30	42	17	13	12
27	35	42	20	15	9
28	35	28	17	12	7
27	25	15	11	7	5
19	15	10	8	5	4
1	1	1	1	1	1

Table 1: Checker board assay determines the concentrations and ratios of Biotin and Sulfo-tag labeled antigens. A: Raw CPS counts for the checker board plate with high positive serum on half of the plate and negative serum on the other half. The concentration of biotinylated antigen was diluted horizontally in series and the concentration of Sulfo-tag labeled antigen was diluted vertically in series. B: The ratio values of the CPS counts from the high positive serum against each of their corresponding CPS counts from the negative serum. The yellow highlighted wells represent the best ratio of positive to negative in Panel B. This ratio corresponds to the 250 ng/mL Biotin and 250 ng/mL Sulfo-tag labeled antigen concentrations in Panel A with an acceptably low CPS count for negative serum. Please click here to view a larger version of this table.

A

	1	2	3	4	5	6	7	8	9	10	11	12
A	19940	15866	857	820	188	169	1655	1551	721	689	106	114
B	170	165	109	107	1010	1057	461	473	185	198	10850	12316
C	154	121	107	103	128	120	184	128	159	146	567	521
D	765	745	149	148	2684	2610	503	551	156	157	11542	11703
E	49686	43654	197	185	180	188	473	462	789	796	159	148
F	156	141	163	178	168	172	165	178	116	103	141	153
G	188	175	132	134	377	378	164	175	441	414	115	129
H	1452	1533	219	237	201	215	755	797	316	357	213	239

B

	1	2	3	4	5	6
A	1.000	0.039	0.001	0.081	0.030	-0.003
B	0.000	-0.003	0.049	0.017	0.001	0.644
C	-0.002	-0.004	-0.002	-0.001	-0.001	0.021
D	0.033	-0.001	0.140	0.020	-0.001	0.646
E	2.622	0.001	0.001	0.017	0.035	-0.001
F	-0.001	0.000	0.000	0.000	-0.003	-0.001
G	0.001	-0.002	0.012	0.000	0.015	-0.003
H	0.075	0.003	0.002	0.034	0.010	0.003

C

	1	2	3	4	5	6	7	8	9	10	11	12
A	19940	15866	857	820	688	669	1655	1551	921	889	506	514
B	505	495	609	607	1010	1057	861	873	585	598	10850	12316
C	554	521	507	503	728	720	884	828	659	546	867	821
D	765	745	649	648	2684	2610	703	751	556	557	11542	11703
E	49686	43654	597	585	580	588	673	662	989	996	559	548
F	656	641	563	578	568	572	565	578	616	603	541	553
G	588	575	432	434	677	678	864	875	541	614	615	629
H	1452	1533	619	637	501	515	755	797	516	557	315	559

D

	1	2	3	4	5	6
A	1.000	0.019	0.010	0.063	0.023	0.001
B	0.000	0.006	0.031	0.021	0.005	0.637
C	0.002	0.000	0.013	0.020	0.006	0.020
D	0.015	0.009	0.123	0.013	0.003	0.639
E	2.653	0.005	0.005	0.010	0.028	0.003
F	0.009	0.004	0.004	0.004	0.006	0.003
G	0.005	-0.004	0.010	0.021	0.004	0.007
H	0.057	0.007	0.000	0.016	0.002	0.002

Table 2: Analysis of assay results. A: Raw CPS counts from the assay plate with the standard high and low positive controls highlighted in red and the standard negative controls highlighted in green. Each sample was duplicated in the assay. B: Index values were calculated, as described in the assay protocol. Any index value that was greater than 0.023 was defined as a positive result, highlighted in red. C: Raw CPS counts from the assay plate with the same set of samples when an irrational antigen concentration was used. This resulted in a high background and some low positives normally detected, as shown in Panel B, were converted to negatives, highlighted in gray in Panel D. Please click here to view a larger version of this table.

Discussion

Islet autoantibodies are currently the most reliable biomarkers for autoimmunity of type 1 diabetes. They mark the onset of islet specific autoimmunity and determine overt disease risks. The ECL assay, for islet autoantibodies, has been extensively validated in multiple national and international type 1 diabetes clinical trials. The assay has shown increased sensitivity and specificity as compared to the current 'gold' standard RBAs. The ECL assay has shown its superior advantage for higher disease specificity by discriminating high-affinity and high-risk islet autoantibodies from low-risk, low-affinity signals generated by the RBA. This is especially noticed in subjects who are only single islet autoantibody positive and have never progressed to type 1 diabetes. Most of these low affinity autoantibodies were found to be lost during follow up testing done within months to years, behaving as a 'transient positive.' As previously hypothesized, these low affinity 'single' autoantibodies likely resulted from immunization with a cross-reactive molecule. While higher affinity, higher risk islet autoantibodies resulted from immunization with the islet antigens themselves. In addition, ECL-assays have demonstrated the ability to antedate the time of 'seroconversion' from current standard radio-binding assays (RBA) by years in children with pre-diabetes, who were followed to clinical diabetes from birth. An ongoing international clinical trial, The Environmental Determinants of Diabetes in the Young (TEDDY), depends on accurate detection to pinpoint the timing and appearance of the first islet autoantibody 'seroconversion' to mark the very beginning of islet autoimmunity and for identifying environmental triggers. A possible reason for increased sensitivity in the ECL assay is that the assay captures all immunoglobulin classes: IgG, IgM, IgA, or IgE, rather than the traditional RBA or ELISA which rely only on the detection of IgG.

In ECL assays, for some particular autoantibodies like IAA, the binding of autoantibodies to the antigen seems to be inhibited by some component present in normal human serum. To remove or release this inhibition, acid treatment of serum samples was necessary to do before the serum was incubated with antigen. As shown in [**Figure 5**], ECL-IAA assay, the binding activities of both the mouse insulin monoclonal antibody and patients' serum autoantibodies with the antigen was greatly enhanced. The acidification of serum samples, used in antibody assays, is usually applied to disassociate pre-existing bound complexes. The mechanism behind how IAA signals are inhibited in human serum samples and released by acidification of serum is not known, but this method has been used in other ECL based assays[14,15].

In a few cases, when labeling molecules, either Biotin or Sulfo-tag, the labeling positionsinside of the antigen protein molecules are at, or very close to key epitopes of antibody binding, which may interfere with the binding activity to antibodies. This will reduce assay sensitivity and can completely tear down the assay. For routine labeling procedure, maximization or saturation is desired for each antigen protein molecule to generate maximum activity or signal per labeled molecule by maximizing labeling capacity of every possible labeling position. Unsaturated labeling should be performed by reducing the molar ratio of labeling molecules (Biotin and Sulfo-tag) to the antigen protein if labeling on the antigen becomes a possible reason for interruption of antibody binding activity. Major epitopes can be better reserved and interruption of antibody binding activity can be released using the unsaturated labeling strategy in most cases.

Each assay should include an internal standard high positive and negative control for index calculations of unknown samples. A low positive control near the assay's upper limit of normal controls is important to include for the monitoring of assay sensitivity. The laboratory should keep enough aliquots of standard positive and negative controls for long-term use and all aliquots should be stored at -20 °C. For assay quality assurance, assays must be run in duplicates for each sample and every positive result should be confirmed by repeating the sample in a separate assay. A third assay is necessary when the second confirmatory assay does not agree with the first assay and the results of two assays, which agree (*e.g.*, +,+ or -,-), will be the final determination of a positive or negative result.

With the platform set for single ECL assays, a multiplexed assay can be expanded upon from these. It can simultaneously determine up to 10 different autoantibodies in one single well with a tiny amount of serum. Currently, four islet autoantibodies including IAA, GADA, IA-2A, and ZnT8A are equal in importance for the risk prediction of progression to T1D in both relatives of patients with T1D and the general population. The methods utilized for screening these 4 autoantibodies using current single autoantibody measurements are laborious and inefficient, especially for large scale population screening. Importantly, up to 40% of patients with T1D have an additional autoimmune condition[16,17,18]. Unfortunately, there is no easy and inexpensive tool to screen for these conditions. The multiplex ECL assay is not only capable of combining 4 current major islet autoantibody assays into one, but also is able to further combine more autoantibody assays from other relevant autoimmune diseases. This makes it possible to efficiently conduct high throughput screening for multiple autoimmune diseases simultaneously in large scale populations. In the multiplex ECL assay, as shown in [**Figure 6**], each antibody-antigen complex formed in the fluid-phase will be restrained to a specific linker source spot in the same well. The signal receiver on the plate reader machine is able to recognize the signals from 10 different sources of spots. However, the spots with an extremely high signal can generate a high assay background and cause interference to neighboring spots through cross-talk. For this reason, the upper limit signals for each autoantibody assay should be limited to fewer than 20,000 counts. In our experience, the autoantibodies with lower backgrounds should be placed relatively far away from those spots having higher counts when the spot map is designed. For long-term studies using multiplex ECL assays, it is recommended that the same linker be used for the same autoantibody assay to keep the assay consistent.

Disclosures

The authors have nothing to disclose.

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