Methods in Molecular Biology (2016) 1433: 85–91
DOI 10.1007/7651_2015_296
© Springer Science+Business Media New York 2015
Published online: 13 December 2015

# Islet Autoantibody Detection by Electrochemiluminescence (ECL) Assay

Liping Yu

## Abstract

Two fundamental aspects for precisely predicting the risk of developing type 1 diabetes by islet autoantibodies are assay sensitivity and disease specificity. We have recently developed electrochemiluminescent (ECL) insulin autoantibody (IAA) and GAD65 autoantibody (GADA) assays. ECL assays are sensitive, able to identify the initiation of islet autoimmunity earlier in life among high-risk young children before clinical onset of diabetes and are more disease specific because they are able to discriminate high-affinity, high-risk diabetes specific islet autoantibodies from low-affinity, low-risk autoantibodies.

**Keywords:** Autoantibodies, Assay, Prediction, Diabetes

## 1 Introduction

Islet autoantibodies (iAbs) play an essential role in prediction of type 1 diabetes (T1D). They can appear as early as at 6 months of life and usually precede clinical diabetes by years allowing a window of opportunity to intervene. Accurate detection of the first iAb as an important biomarker for the initiation of islet autoimmunity is required to pinpoint the time of exposure to candidate environmental factors investigated by cohort studies, e.g., The Environmental Determinants of Diabetes in the Young (TEDDY) (https://teddy.epi.usf.edu/). In addition, iAbs are used extensively to stage diabetes risk and as the inclusion criteria for trials to prevent T1D. The risk of developing T1D is strongly associated with the number of iAbs among the relatives of T1D patients and the general populations. Children with two or more persistent iAbs are at high risk—70 % will progress to diabetes in less than 10 years [1]. In contrast, children with a single persistent iAb are at a much lower risk; only 14 % have developed T1D by 10 years of follow-up. In screening for the Diabetes Prevention Trial—Type 1 (DPT-1), the majority of subjects found to be iAb positive by radioimmunoassay had single iAb to insulin (IAA) or GAD65 (GADA). In most cases, single iAb was present at low levels with low affinity. None of the 407 DPT-1 participants expressing only IAA progressed to diabetes during the

initial observation period [2], casting doubt whether the presence of a single iAb is diabetes-specific. Exclusion of these "low-risk" iAb by using more specific assays would greatly enhance staging of diabetes risk for clinical trials. Recently, we developed electrochemiluminescent (ECL) IAA and GADA assays [3–5], which are more sensitive; ECL-IAA antedated the onset of islet autoimmunity by a mean of 2.3 years (range: 0.3–7.2 years) in high-risk young children followed to clinical diabetes (Diabetes Autoimmunity Study in the Young, DAISY); and more disease-specific, ECL-IAA and GADA assays are able to discriminate high affinity, high-risk iAbs in prediabetic children from those "low-risk," low-affinity signals appearing in children with single IAA or GADA.

## 2 Materials

1. Human proinsulin.
2. Human GAD65.
3. Biotinylation kit (ThermoScience).
4. Sulfo-TAG (MSD).
5. 96-well PCR plate (Fisher).
6. Streptavidin coated MSD plate (MSD).
7. Zeba sizing spin column (ThermoScience).
8. Blocker A buffer (MSD).
9. Reader buffer (MSD).
10. 96-well Plate Shaker (Perkin Elmer).
11. Sector 2400 or Sector 6000 (MSD).
12. Benchtop centrifuge with bucket rotary (Beckman).
13. Antigen buffer: 1× PBS with 5 % BSA.
14. PBST: 1× PBS with 0.05 % Tween 20.

## 3 Methods

### 3.1 ECL-IAA Assay

#### 3.1.1 General Principle

Given the need for improved IAA assays and the hypothesis that the binding of insulin to solid phases obscures a key determinant for recognition by human autoantibodies as seen also for specific murine monoclonals [6], we set out to immobilize "insulin" to a solid phase in a manner that preserved critical determinants recognized by human insulin autoantibodies. We had previously failed to develop a plate capture assay for human insulin autoantibodies though we have developed such an assay (nonradioactive) for the insulin autoantibodies of the NOD mouse [7]. The two fundamental components for our recent success were utilization of proinsulin

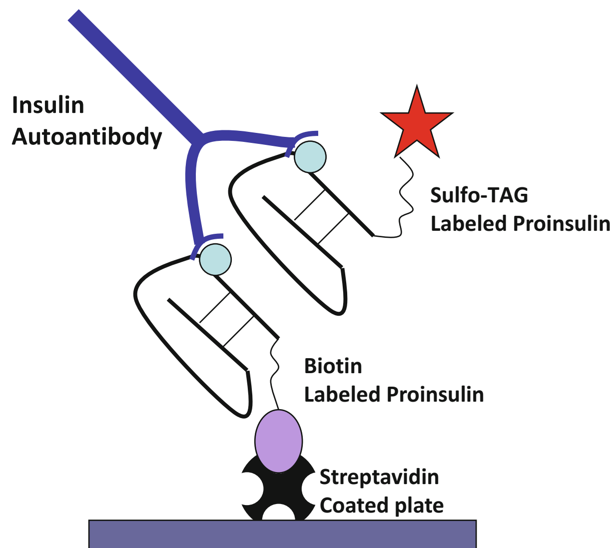


**Fig. 1** Electrochemiluminescence (ECL) assay

rather than insulin and use of streptavidin rather than avidin for plate capture of biotinylated proinsulin (Fig. 1). The ruthenium assay system of the Meso Scale Discovery (MSD) Company is a third important component. In the previous studies, all human insulin autoantibodies reacted with proinsulin in fluid phase radioassays [8].

#### *3.1.2 Part I: Label Human Proinsulin with Biotin and Sulfo-TAG Respectively*

1. Calculate the molar ratio of proinsulin with biotin or sulfo-TAG. **Note 1**. We recommend using 1:5 M ratio for proinsulin labeling. **Notes 2** and **3**.
2. Mix proinsulin with biotin or sulfo-TAG respectively, cover the reaction tubes with aluminum foil to avoid light, and incubate them at room temperature for 1 h.
3. During the 1 h incubation, prepare Zeba sizing spin column by washing the column three times with 1× PBS buffer, 1000 × *g* for 2 min each time in a centrifuge.
4. Stop the labeling reaction and purify labeled proinsulin by passing the reaction mixture through the prepared Zeba sizing spin columns with 1000 × *g* for 2 min in a centrifuge.
5. Measure the protein concentration.
6. Aliquot the labeled proinsulin and store at −80 °C freezer.

*3.1.3 Part II: Plate Capture Assay (2 Days)*

Day 1.

1. Acid treatment of serum samples: mix 15 μL of serum with 18 μL of 0.5 M acetic acid and incubate at room temperature for 45 min.
2. To antigen buffer add 100 ng/mL of biotin and 100 ng/mL sulfo-TAG labeled proinsulin. Add 35 μL of solution per well in a new PCR plate.
3. Just before the completion of the 45 min incubation (**step 1**), add 8.3 μL of 1 M Tris pH 9.0 buffer per well into the antigen plate of **step 2** (try to add onto the side wall of each well, avoid completely mixing at this time), immediately followed by transferring 25 μL of acid treated serum from **step 1** into the well, and then mix entirely. Cover the plate with sealing foil to avoid light.
4. Shake at room temperature on low setting for 2 h.
5. Put in 4 °C refrigerator to incubate overnight (18–24 h)

Prepare the streptavidin plate.

1. Let MSD streptavidin plate(s) come to room temperature.
2. Add 150 μL of 3 % Blocker A (in PBS) per well.
3. Cover with foil.
4. Incubate in 4 °C refrigerator overnight.

Day 2.

1. Remove streptavidin plate from refrigerator. Dump out buffer and tap upside down on paper towel to dry.
2. Wash three times with 150 μL PBST.
3. Transfer 30 μL of serum/antigen mixture into the MSD streptavidin plate.
4. Cover with foil to avoid light and shake at room temperature for 1 h on low speed setting.
5. Dump out incubates and wash three times with 150 μL PBST.
6. Add 150 μL/well of 2× Read buffer (avoid any bubbles).
7. Count on MSD machine.

*3.1.4 Standardization, Quality Control, and Quality Assurance*

1. The mouse anti-human proinsulin monoclonal antibody will be used as an internal standard positive control. The monoclonal antibody should be diluted with a normal human serum and treated identically as a human serum in the assay.
2. The laboratory should keep enough volume of the positive and negative control sera for long-term use. Each of these serum samples should be aliquoted and stored at −20 °C.

3. All the assays are run in duplicate and must include positive/negative control samples for quality control purposes and for lab index calculation. In our assay, IAA positive is any value greater than an index of 0.006.
4. The results of analysis of negative and positive controls from each assay should be plotted in a preestablished Shewart Plot with mean ±3 SD to monitor the assay drift and evaluate assay performance. Our positive control range should be within 3 SD. The negative control must be ≤0.006 for results of the assay run to be utilized.
5. Every positive sample in the first run is confirmed by repeating that sample in a different assay. If the second confirmatory assay comes out negative, a third assay is necessary. The results of two assays, which agree (e.g., +,+ or −,−), will be the final determination of positive or negative result.

### 3.2 ECL-GADA Assay

#### 3.2.1 General Principle

The format of ECL-GADA assay is adapted from ECL-IAA assay except for acid treatment of serum. In a similar way to the ECL-IAA assay, a full length human GAD65 protein was set out to be immobilized to a solid phase in a manner that preserved all critical determinants recognized by human GAD65 autoantibodies.

#### 3.2.2 Part I: Label Human GAD65 with Biotin and Sulfo-TAG Respectively

1. Calculate the molar ratio of GAD65 with biotin or sulfo-TAG. **Note 1**. We recommend using 1:20 M ratio for GAD65 labeling. **Notes 2** and **3**.
2. Mix GAD65 with biotin or sulfo-TAG respectively, cover the reaction tubes with aluminum foil to avoid light, and incubate them at room temperature for 1 h.
3. During 1 h incubation, prepare Zeba sizing spin column by washing the column three times with 1× PBS buffer, 1000 × *g* for 2 min each time in a centrifuge.
4. Stop labeling reaction and purify labeled GAD65 by passing the reaction mixture through the prepared Zeba sizing spin columns with 1000 × *g* for 2 min in a centrifuge.
5. Measure the protein concentration.
6. Aliquot the labeled GAD65 and store them in a −80 °C freezer.

#### 3.2.3 Part II: Plate Capture Assay (2 Days)

Day 1.

1. Dilute 4 μL of serum with 16 μL of PBS in a PCR plate.
2. Prepare antigen buffer containing both biotin and sulfo-TAG labeled GAD65 with the concentration of 32 ng/mL for sulfo-TAG labeled GAD65 and 1000 ng/mL for biotin labeled GAD65.
3. Add 20 μL of labeled antigen solution.

4. Cover the plate with sealing foil to avoid light.
5. Shake at room temperature on low setting for 1–2 h.
6. Put in 4 °C refrigerator to incubate overnight (18–24 h)

Prepare the streptavidin plate.

1. Let MSD streptavidin plate(s) come to room temperature.
2. Add 150 μL of 3 % Blocker A (in PBS) per well.
3. Cover with foil.
4. Incubate in 4 °C refrigerator overnight.

Day 2.

1. Remove streptavidin plate from refrigerator. Dump out buffer and bang on paper towel to dry.
2. Wash three times with 150 μL PBST.
3. Transfer 30 μL of serum/antigen mixture into the MSD streptavidin plate.
4. Cover with foil to avoid light and shake at room temperature for 1 h on low speed setting.
5. Dump out incubates and Wash three times with 150 μL PBST.
6. Add 150 μL/well of 2 × Read buffer (avoid any bubbles).
7. Count on MSD machine.

#### 3.2.4 Standardization, Quality Control, and Quality Assurance

1. The mouse anti-human GAD65 monoclonal antibody will be used as an internal standard positive control. The monoclonal antibody should be diluted with a normal human serum and treated identically as a human serum in the assay.
2. The laboratory should keep enough volume of the positive and negative control sera for long-term use. Each of these serum samples should be aliquoted and stored in −20 °C.
3. All the assays are run in duplicate and must include positive/negative control samples for quality control purpose and for lab index calculation. In our assay, GAA positive is any value greater than an index of 0.023.
4. The results of analysis of negative and positive controls from each assay should be plotted in a preestablished Shewart Plot with mean ±3 SD to monitor the assay drift and evaluate assay performance. Our positive control range should be within 3SD. The negative control must be ≤0.023 for results of the assay run to be utilized.
5. Every positive sample in the first run is confirmed by repeating that sample in a different assay. If the second confirmatory assay comes out negative, a third assay is necessary. The results of two assays, which agree (e.g., +,+ or −,−), will be the final determination of positive or negative result.

## 4 Notes

1. Biotin and sulfo-TAG powder should be dissolved just before labeling procedure every time.
2. The proinsulin and the GAD65 must be stored at −80 °C and thawed on ice before use.
3. The protein in either tris or glycine buffer systems should be exchanged to 2× PBS buffer with pH 7.9 by sizing spin column.

## Acknowledgement

This study was supported by NIH grant DK32083, Diabetes Autoimmunity Study in the Young Grant DK32493, the Immune Tolerance Network AI15416, the Juvenile Diabetes Research Foundation Grant 11-2005-15, DERC Clinical Core DK57516. We thank Dr. Tom Thomas of Vanderbilt for the gift of an insulin monoclonal antibody. We thank Eli Lilly Company for supplying proinsulin and thank MSD Company for helpful discussions during assay development.

### References

1. Ziegler AG, Rewers M, Simell O et al (2013) Seroconversion to multiple islet autoantibodies and risk of progression to diabetes in children. JAMA 309:2473–2479
2. Orban T, Sosenko JM, Cuthbertson D et al (2009) Diabetes Prevention Trial-1 Study Group. Pancreatic islet autoantibodies as predictors of type 1 diabetes in the diabetes prevention trial-type 1 (DPT-1. Diabetes Care 32:2269–2274
3. Yu L, Miao D, Scrimgeour L, Johnson K, Rewers M, Eisenbarth GS (2012) Distinguishing persistent insulin autoantibodies with differential risk: non-radioactive bivalent proinsulin/insulin autoantibody assay. Diabetes 61:179–186
4. Miao D, Guyer KM, Dong F, Jiang L, Steck AK, Rewers M, Eisenbarth GS, Yu L (2013) GAD65 autoantibodies detected by electrochemiluminescence (ECL) assay identify high risk for type 1 diabetes. Diabetes 62:4174–8
5. Yu L, Dong F, Fuse M, Miao D, Fouts A, Hutton JC, Steck AK (2013) Proinsulin/Insulin Autoantibodies Measured with Electrochemiluminescent Assay Are the Earliest Indicator of Prediabetic Islet Autoimmunity. Diabetes Care 36:2266–2270
6. Rojas M, Hulbert C, Thomas JW (2001) Anergy and not clonal ignorance determines the fate of B cells that recognize a physiological autoantigen. J Immunol 166:3194–3200
7. Babaya N, Liu E, Miao D, Li M, Yu L, Eisenbarth GS (2009) Murine high specificity/sensitivity competitive europium insulin autoantibody assay. Diabetes Technol Ther 11:227–233
8. Castano L, Ziegler AG, Ziegler R, Shoelson S, Eisenbarth GS (1993) Characterization of insulin autoantibodies in relatives of patients with type 1 diabetes. Diabetes 42:1202–1209