Improved Specificity of Glutamate Decarboxylase 65 Autoantibody Measurement Using Luciferase-Based Immunoprecipitation System Assays

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Autoantibodies to glutamate decarboxylase (GADA) are widely used in the prediction and classification of type 1 diabetes. GADA radiobinding assays (RBAs) using N-terminally truncated antigens offer improved specificity, but radioisotopes limit the high-throughput potential for population screening. Luciferase-based immunoprecipitation system (LIPS) assays are sensitive and specific alternatives to RBAs with the potential to improve risk stratification. The performance of assays using the Nanoluc luciferase (Nluc)-conjugated GAD_{65} constructs, Nluc-GAD65(96–585) and full length Nluc-GAD65(1–585), were evaluated in 434 well-characterized serum samples from patients with recent-onset type 1 diabetes and first-degree relatives. Nonradioactive, high-throughput LIPS assays are quicker and require less serum than RBAs. Of 171 relatives previously tested single autoantibody positive for autoantibodies to full-length GAD_{65} by RBA but had not progressed to diabetes, fewer retested positive by LIPS using either truncated (*n* = 72) or full-length (*n* = 111) antigen. The Nluc-GAD65(96–585) truncation demonstrated the highest specificity in LIPS assays overall, but in contrast to RBA, N-terminus truncations did not result in a significant increase in disease-specificity compared with the full-length antigen. This suggests that binding of nonspecific antibodies is affected by the conformational changes resulting from addition of the Nluc antigen. Nluc-GAD65(96–585)

ARTICLE HIGHLIGHTS

- New nonradioactive, low-blood-volume methods for detection of autoantibodies to glutamate decarboxylase (GADA) are needed for population screening.
- We evaluated two Nanoluc luciferase (Nluc)-GAD constructs (full-length and truncated) using the luciferase-based immunoprecipitation system (LIPS) against the current gold-standard radioactive method for full-length GADA.
- In this study the LIPS methods were faster, required less serum, and were more specific than the current gold-standard radiobinding assay method.
- LIPS assessment using Nluc-GAD truncated construct represents an improved and easy-to-establish method for GADA assessment in the general population.

LIPS assays offer low-blood-volume, high-specificity GADA tests for screening and diagnostics.

Approximately 80% of patients with type 1 diabetes (T1D) are positive for autoantibodies to glutamate decarboxylase (GADA) (1). This test is widely used in prediction and classification of diabetes. Commonly used methods for measuring

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GADA include radiobinding assays (RBA) (2) and ELISAs (3). Despite efforts to improve GADA measurement, many patients who test single GADA positive are unlikely to develop diabetes (4). There is consensus that diabetes-associated GADA primarily recognizes epitopes in the middle and C-terminal regions of GAD_{65}, whereas autoantibodies specific to the N-terminal have little association with progression (5). We previously showed that the first 142 amino acids of GAD_{65} do not contribute to epitopes recognized by disease-associated autoantibodies (6). Using N-terminally truncated GAD_{65} radiolabels ^{35}S-GAD_{65}(98–585) and ^{35}S-GAD_{65}(143–585) improved the specificity of RBAs without affecting sensitivity. In first-degree relatives (FDRs) of patients, autoantibodies measured with these constructs were more closely associated with diabetes risk (7).

However, the use of radioactive tracers in RBAs is costly and time consuming, and has environmental implications; moreover, their future availability is in question, which potentially limits the long-term sustainability of RBAs. Alternative methods, including electrochemiluminescence, antibody detection by agglutination-PCR, bridge ELISAs, chemiluminescence immunoassays, and luciferase-based immunoprecipitation system (LIPS) assays are increasing in popularity (8). Good performance of LIPS assays to measure T1D-associated insulin (9) and islet-antigen 2 (IA-2A) (10) autoantibodies has been reported.

Using well-characterized samples from the Bart's Oxford (BOX) family study (11), we evaluated how GADA levels measured by LIPS, using full-length and truncated antigens, compared with the equivalent RBAs.

RESEARCH DESIGN AND METHODS

Population

Screening Cohort

To assess sensitivity and specificity of the Nanoluc luciferase (Nluc)-GAD_{65} constructs, we selected 11 patients with recent-onset T1D (n = 5 male patients [45.5%]; median duration, 43 days [range, 1–84 days]) and 25 low-risk GADA(96–585)-positive FDRs of patients (n = 8 male FDRs [32%]). FDRs were considered low risk because they had not developed diabetes during follow-up (median, 19 [range, 1.2–29.7] years) and did not have additional islet autoantibodies (IAAs). These samples had been tested previously by RBA using full-length GAD_{65} and five truncated GAD_{65} constructs (6).

Evaluation Cohort

Samples were selected for detailed evaluation of the LIPS assay, based on previous studies (6,7) including 154 patients with recent-onset T1D and 732 FDRs followed for disease development by questionnaire (Table 1).

Of 732 FDRs, 271 (37.6%) were previously found GADA positive by RBA. Of these, 64 (25%) developed diabetes and 254 (91%) had follow-up data for survival analysis. The remaining 461 FDRs previously tested GADA negative. This population was enriched with 32 FDRs (6.9%) who developed diabetes (excluding one with insufficient sera samples; Table 1).

All samples were previously tested for GADA using the harmonized RBA protocol with the full-length ^{35}S-GAD_{65} (1–585) and N-terminally truncated ^{35}S-GAD_{65}(96–585) antigens (7). Data on additional autoantibodies (IA-2A, IAA, and zinc transporter 8 autoantibodies [ZnT8A]) were also available.

Recombinant Luciferase–Tagged GAD_{65} Antigen Production

Comparable to the harmonized RBA protocol (12), recombinant Nluc-tagged GAD_{65} antigens (Fig. 1*A*) were encoded in a pCMVTnT plasmid (Promega) and synthesized using the

Table 1—Characteristics of participants in the evaluation cohort

Characteristic	Patients (n = 154)	GADA-positive relatives		GADA-negative relatives	
		Progressors (n = 64)	Nonprogressors (n = 207)	Progressors (n = 32)	Nonprogressors (n = 429)
Male sex, n (%)	94 (60)	31 (48)	100 (48)	23 (72)	205 (48)
Age, median (range), years	11.7 (1.3–20.9)	33.3 (5.7–52.9)	30.6 (1.33–57.4)	39.2 (1.4–56.2)	32.6 (1.7–57.3)
Age at diagnosis, median (range), years	15.3 (2.7–100)	37.5 (11.7–69.8)	—	48.3 (5.1–68.5)	—
Follow-up, median (range), years; or diabetes duration, median (range), days	1 (−7 to 90)	7.56 (0.2–27.8)	17.6 (0.6–30.8)	12.8 (1.6–23.8)	17.6 (0–31.0)
Additional autoantibodies, n (%)*	140 (91)	34 (53)	37 (18)	4 (13)	11 (3)†
IA-2A	120 (78)	19 (30)	13 (6)	1 (3)	1 (0.2)
IAA	71 (46)‡	19 (30)	23 (11)	3 (9)	10 (2)
ZnT8A	111 (72)	19 (30)	17 (8)	0 (0)	—

Data are given as number of FDRs (%) or median (range). GADA-positive relatives: n = 271; GADA-negative relatives: n = 461. —, data unavailable or not applicable. *Additional autoantibodies are from IA-2A, IAAs, and ZnT8A. †Not tested for ZnT8A. ‡57 patients not tested for IAAs, because the sample was taken >2 weeks after diagnosis and any antibody to insulin may derive from insulin therapy induction.

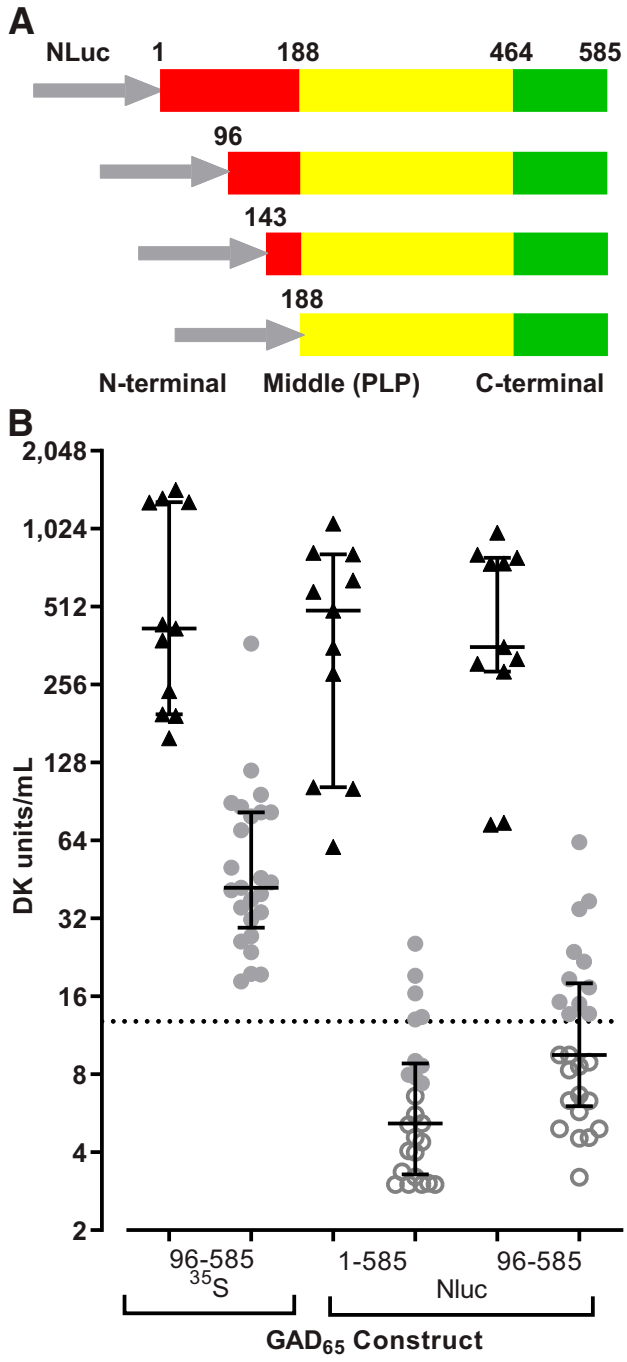


Figure 1—*A*: Diagram of the Nluc GAD_{65} constructs, which were assessed for the sensitivity and specificity of GADA measurement by the LIPS assay. PLP, pyridoxal 5′phosphate. *B*: A plot of 25 low-risk, single GADA(96–585)–positive relatives who had not developed diabetes during follow-up (grey circle) and 11 patients with recent-onset T1D (black triangle) who were measured for GADA using ^{35}S-GAD_{65} (96–585) in radioimmunoassay and Nluc-GAD_{65}(1–585) and Nluc-GAD_{65}(96–585) in LIPS assays. Filled triangles and circles indicate positives by that construct by individual assay thresholds. Dotted line indicates the positivity threshold for ^{35}S-GADA(96–585). In patients, the median antibody levels (DK units/mL) for ^{35}S-GADA(96–585), Nluc-GADA(1–585), and Nluc-GADA(96–585) were 341.29 (range, 157.2–1267.3), 494.4 (range, 60.4–1072.5), and 357.8 (range, 73.8–988.1), respectively. In relatives, the median antibody levels were 42.0 (range, 18.3–368.5), 5.16 (range, 0.39–25.6), and 9.5 (range, 3.2–63), respectively.

SP6 TnT quick coupled in vitro transcription/translation kit (Promega) using 1 μg of antigen and a 1.5-h incubation at 30°C. The antigen was purified using a NAP5 desalting column (packed with Sephadex G-25 – illustra; VWR) and Tris-buffered saline with Tween-20 buffer (TBST; 20 mmol/L Tris, 150 mmol/L NaCl, pH 7.4, and 0.5% Tween-20), by collecting three fractions (400, 200, and 500 μL). Luciferase activity was quantified in light-unit equivalents (LU) by measuring the emitted bioluminescence of 2 μL of antigen mixed with 40 μL of Nano-Glo substrate (per manufacturer instructions; Promega) in a Centro XS3 luminometer (Berthold Technologies GmbH & Co.) for 2 s/well. A typical reaction yielded between 10^6 and 10^7 LU/μL antigen in a total volume of 600 μL pooled from the first two fractions. The antigen was divided into aliquots and stored at −70°C.

GADA LIPS Assay

The Nluc-GAD_{65} antigen was diluted in TBST plus 0.1% BSA to a concentration of 4.0×10^6 LU/25 μL (±200,000 LU). Sera (1 μL; n = 2 replicates) were pipetted into a 96-well plate (Sarstedt, Nümbrecht, Germany) and incubated with 25 μL of diluted Nluc-GAD_{65} antigen for 2.5 h at room temperature protected from light. Immunocomplexes were precipitated using a 25% Protein A Sepharose 4 fast flow suspension ([6.25 μL/well washed four times in TBST plus 0.1% BSA]; GE Healthcare Life Sciences, Amersham, UK) and a 1-h incubation with orbital shaking (∼700 rpm) at 4°C. Unbound Nluc-GAD65(96–585) was removed by five serial washes with TBST, each including centrifugation (500*g* at 4°C for 3 min) and automatic buffer removal and dispensing (BioTek Elx405; Agilent). Resin pellets were transferred into 96-well OptiPlates (Perkin-Elmer), centrifuged (500*g* at 4°C for 3 min) and aspirated to a final volume of 30 μL. Nano-Glo substrate (40 μL) was injected into each well immediately before LU determination, using a standardized protocol on the Centro XS3 luminometer (inject, shake for 5 s/well, detect for 2 s/well).

In the 2020 Islet Autoantibody Standardization Program workshop, the adjusted sensitivity at 95% specificity for GADA(1–585) and GADA(96–585) measured by radioimmunoassay was 78% and 84%, respectively. For GADA(1–585) and GADA(96–585) measured by LIPS, the adjusted sensitivity at 95% specificity was 76% and 86%, respectively.

Antibody Quantification and Thresholds

Logarithmic standard curves were generated from standards established by the National Institute of Diabetes and Digestive and Kidney Diseases harmonization program, allowing quantification of autoantibody levels (digestive and kidney [DK] units/mL) (12). Thresholds for GADA by RBA and LIPS were set at the 97.5th percentile of 221 healthy schoolchildren. This was equivalent to 13.5, 12.8, 7.3, and 10.7 DK units/mL for ^{35}S-GAD_{65}(1–585),^{35}S-GAD_{65} (96–585), Nluc-GAD_{65}(1–585), and Nluc-GAD_{65}(96–585), respectively.

Statistical Analysis

Wilcoxon matched-pairs signed-rank tests were used to compare antibody levels, and McNemar's test with Yate's correction to compare antibody status with different GAD_{65} constructs and assay formats. Kaplan-Meier curves with Mantel-Cox log-rank test were used to compare survival between groups. For all analyses, a two-tailed $P < 0.05$ was considered significant. The partial area (90th percentile) under the curve of the receiver operating characteristic with 95% CIs was calculated assuming a nonparametric distribution of results, using R software version 3.2.2 (R Foundation). Other statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software).

Ethics Statement

The BOX study is currently approved by the South Central–Oxford C. National Research Ethics Committee. Participants provided informed, written consent and the study was performed according to the principles of the Declaration of Helsinki.

Data and Resource Availability

The data sets generated during and/or analyzed during this study are available from the corresponding author upon reasonable request. No applicable resources were generated or analyzed during this study.

RESULTS

Construct Screening

GADA measured in sera from the screening cohort, using LIPS assays with the four Nluc-GAD_{65} constructs (Fig. 1*A*), was compared with that for GADA(96–585) obtained by RBA. Median antibody levels with the Nluc constructs were similar to ^{35}*S*-GAD_{65}(96–585) in patients ($P > 0.05$ for all comparisons) (Fig. 1*B*). In 25 low-risk, single GADA-positive (by either RBA) relatives, median GADA levels were lower when measured by LIPS than when measured by RBA with ^{35}*S*-GADA(96–585) ($P < 0.01$ for all comparisons) (Fig. 1*B*).

Additional constructs tested (Nluc-GAD_{65}(143–585) and Nluc-GAD_{65}(188–585) did not improve discrimination further (data not shown). Therefore, Nluc-GAD_{65}(1–585) and Nluc-GAD_{65}(96–585) were selected for detailed evaluation compared with the highly diabetes-specific RBA using ^{35}*S*-GAD_{65}(96–585).

Assay Evaluation

The sensitivity of Nluc-GAD_{65} constructs were comparable to the higher specificity ^{35}*S*-GAD_{65}(96–585) construct in patients and high-risk relatives. Of 154 patients, 125 (81%) were positive for ^{35}*S*-GADA(1–585), and 125 (81%) were positive for ^{35}*S*-GADA(96–585). More of these patients were positive for Nluc-GADA(1–585) than for Nluc-GADA(96–585) (129 [84%] vs. 116 [75%]; $P = 0.0036$). There was very good correlation between ^{35}*S*-GADA (96–585) and Nluc-GADA(1–585) ($r = 0.96$; 95% CI 0.94–0.97; $P < 0.0001$) and Nluc-GADA(96–585) ($r = 0.93$; 95% CI 0.90–0.95; $P < 0.0001$) (Fig. 2*A*).

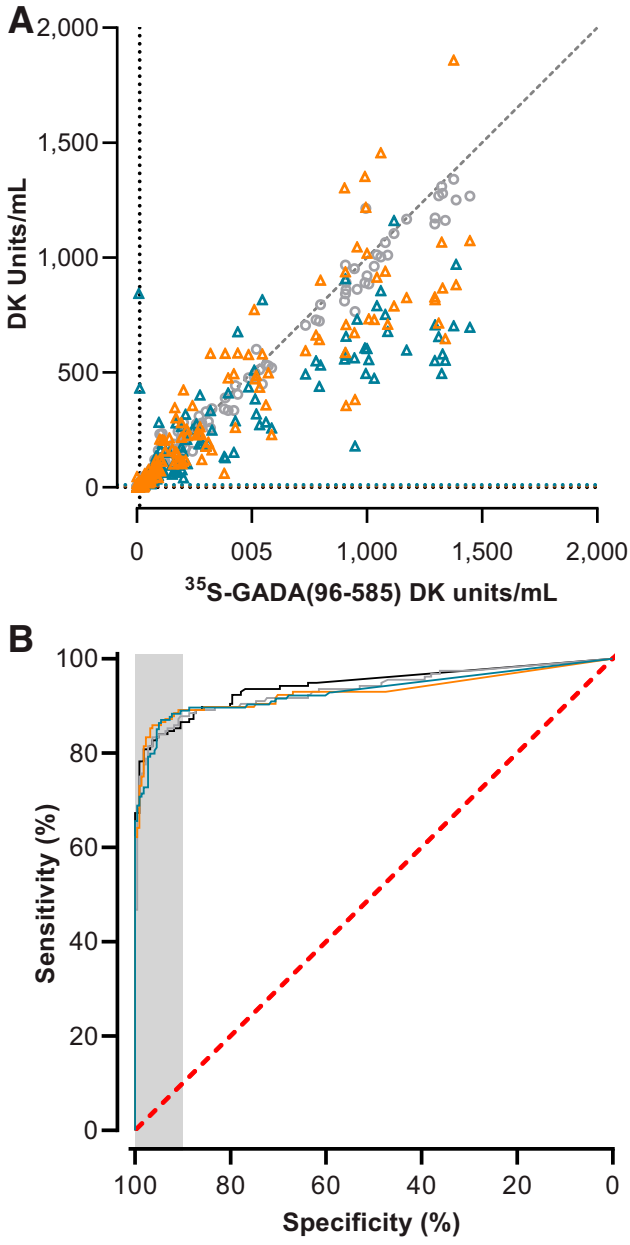


Figure 2—*A*: A plot of ^{35}*S*-GADA(1–585) (grey triangles), Nluc-GADA(1–585) (orange triangles), and Nluc-GADA(96–585) (teal triangles) levels against ^{35}*S*-GADA(96–585) levels in 156 patients with recent-onset T1D. Overall, correlation of ^{35}*S*-GADA(1–585), Nluc-GADA(1–585), and Nluc-GADA(96–585) with ^{35}*S*-GADA(96–585) was excellent ($r = 0.99$, 0.91, and 0.87, respectively; $P < 0.0001$ for all). *B*: Receiver operator characteristic curve for ^{35}*S*-GADA(1–585) (black line), ^{35}*S*-GADA(96–585) (grey line), Nluc-GADA(1–585) (orange line), and Nluc-GADA(96–585) (teal line) measured by radioimmunoassay or LIPS based on data from 156 patients with newly diagnosed T1D and 221 healthy schoolchildren. The area under the curve (AUC) was 0.94 for ^{35}*S*-GADA(1–585), 0.93 for ^{35}*S*-GADA(96–585), 0.93 for Nluc-GADA(1–585), and 0.93 for Nluc-GADA(96–585). The partial AUC (at specificities >90%; within the grey box) was 0.082 for ^{35}*S*-GADA(1–585), 0.081 for ^{35}*S*-GADA(96–585), 0.084 for Nluc-GADA(1–585), and 0.082 for Nluc-GADA(96–585).

Receiver operating characteristic analysis showed that Nluc-GADA(1–585) and Nluc-GADA(96–585) could discriminate between patients and healthy schoolchildren with

comparable sensitivity and specificity to ^{35}S-GADA(1–585) and ^{35}S-GADA(96–585). Partial area under the curve values (at specificities >90%) were 0.082 (95% CI 0.075–0.088), 0.081 (95% CI 0.074–0.087), 0.084 (95% CI 0.077–0.089), and 0.082 (95% CI 0.076–0.088) for ^{35}S-GADA(1–585), ^{35}S-GADA(96–585), Nluc-GADA(1–585) and Nluc-GADA(96–585), respectively (Fig. 2*B*). Of 100 relatives who progressed to diabetes during follow-up and/or had autoantibodies to additional islet antigens, 84% were positive for all four specificities. Of these, 93% were positive for ^{35}S-GADA(1–585), 90% for ^{35}S-GADA(96–585), 94% for Nluc-GADA(1–585), and 88% for Nluc-GADA(96–585) (Supplementary Table 1*A*).

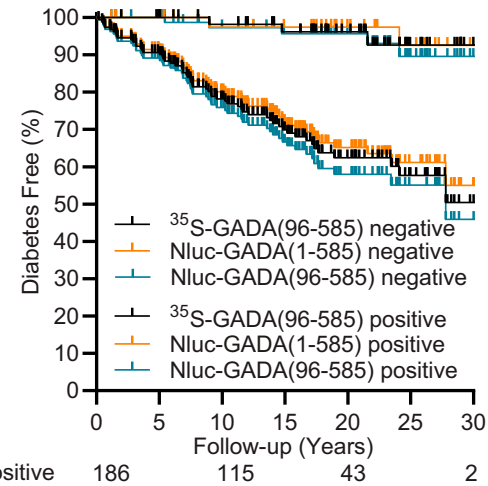
Fewer Single GADA-Positive Relatives Without Diabetes Were Positive for GADA Measured Using LIPS Assays

Of 171 GADA-positive relatives who had no additional autoantibodies and had not progressed to diabetes during follow-up, 74 (43%) had antibodies to Nluc-GAD_{65}(96–585). This was a lower proportion than for ^{35}S-GADA(1–585) (n = 156 [90%]; $P < 0.0001$), ^{35}S-GADA(96–585) (n = 108 [62%]; $P < 0.0001$), or Nluc-GADA(1–585) (n = 112 [65%]; $P < 0.0001$), respectively (Supplementary Table 1*B*).

Of the 430 relatives who originally tested GADA(1–585) negative and remained diabetes-free during follow-up 12 (3%) were found positive for ^{35}S-GADA(1–585), 6 for ^{35}S-GADA(96–585) (1%; $P > 0.05$), 17 for Nluc-GADA (1–585) (4%; $P > 0.05$), and 10 (2%; $P > 0.05$) for Nluc-GADA(96–585) (Supplementary Table 1*B*).

Nluc-GAD65 Antigens Offer Improved Discrimination of Risk in ^{35}S-GADA(1–585)–Positive Relatives

In 254 relatives with follow-up data who tested GADA positive using the harmonized RBA with ^{35}S-GAD_{65}(1–585), the 15-year risk of diabetes was 25% (95% CI 20%–31%). Within this group, positivity for ^{35}S-GADA(96–585), Nluc-GADA(1–585), and Nluc-GADA(96–585) further stratified risk of diabetes ($P < 0.0001$ for all comparisons). Individuals positive for ^{35}S-GADA(96–585) had a 32% risk of diabetes within 15 years (95% CI 25%–40%), and individuals positive for Nluc-GADA(1–585) had a 30% risk (95% CI 24%–38%), and those positive for Nluc-GADA(96–585) had a 30% risk (95% CI 23%–39%) (Fig. 3).



^{35}S-GADA positive	186	115	43	2
(96-585) negative	62	55	32	2
Nluc-GADA positive	200	131	52	2
(1-585) negative	48	39	24	2
Nluc-GADA positive	159	101	35	2
(96-585) negative	89	69	41	2

Figure 3—Kaplan-Meier survival curve for FDRs positive for ^{35}S-GADA(1-585) according to positivity for ^{35}S-GADA(96-585) (black lines), Nluc-GADA(1-585) (orange lines), and Nluc-GADA(96-585) (teal lines). ^{35}S-GADA(96-585), Nluc-GADA(1-585), and Nluc-GADA(96-585) identified relatives at increased risk of diabetes progression. Individuals positive for ^{35}S-GADA(96-585) had a 32% risk of developing diabetes within 15 years, and individuals positive for Nluc-GADA(1-585) or Nluc-GADA(96-585) had a 30% risk.

DISCUSSION

LIPS assays provide a high-performance alternative to well-established RBAs, which are widely used in diabetes studies. We compared LIPS Nluc full-length GAD_{65}(1–585) and truncated GAD_{65}(96–585) assays with the equivalent RBAs, particularly the truncated assay, which demonstrated improved specificity in T1D and latent autoimmune diabetes in adults (13,14). Unexpectedly, the full-length LIPS assay performed almost as well as the truncated assays and much better than the equivalent RBA.

The sensitivity of GADA measurement by LIPS using Nluc-GAD_{65}(1–585) and Nluc-GAD_{65}(96–585) was comparable to RBAs using ^{35}S-GAD_{65}(1–585) and ^{35}S-GAD_{65}(96–585) in patients and high-risk relatives. The specificity of the LIPS assay using Nluc constructs was also improved compared with the harmonized RBA using ^{35}S-GAD_{65}(1–585) and/or ^{35}S-GAD_{65}(96–585). Relatives positive for GADA using LIPS were at increased risk of developing diabetes within 15 years versus those who were positive for GADA(1–585) by RBA, but they had a similar risk to those positive using ^{35}S-GAD_{65}(96–585). The sensitivity of GADA measurement was maintained using ^{35}S-GAD_{65}(143–585) compared with ^{35}S-GAD_{65}(1–585) and ^{35}S-GAD_{65}(96–585). Specificity for diabetes was improved compared with ^{35}S-GAD_{65}(1–585), similar to ^{35}S-GAD_{65}(96–585). Therefore, we focused on GAD_{65}(96–585) for comparison with LIPS assays.

The large population, comprising patients with recent-onset T1D and high- and low-risk FDRs from the well-characterized BOX study, with up to 30 years of follow-up, strengthened this study. Previous research using this population explored the relationship between truncated GADA epitopes and the presence of other islet autoantibodies (6). Although samples were prescreened by RBA, a large cohort of GADA-negative FDRs was included to overcome this selection bias.

Overall, fewer low-risk relatives were positive for GADA(1–585) when measured by LIPS compared with RBA, although future studies in an independent cohort are merited. Nluc-GAD_{65} antigens were fused to the N-terminus of the GAD_{65} constructs, which may explain why assay

performance was improved in the Nluc-GAD_{65}(1–585) compared with the ^{35}S-GAD_{65}(1–585) test. Primary diabetes-associated epitopes of GAD_{65} are located in the middle and C-terminal domains (15–20), whereas minor N-terminal reactivity results from epitope spreading (21,22). Nluc enzyme fusion may have obscured nonspecific, linear epitopes and supported the stability and/or solubility of the antigens, accounting for the enhanced performance observed.

RBAs have dominated islet autoantibody measurement for two decades but are disadvantaged by their high cost, short shelf life, tight regulation, and environmental impact. Although alternative, sensitive, and specific, nonradioactive methods are available for GADA measurement, these too have limitations, including large serum-sample requirements and/or the need for specialist equipment and reagents (7,23). The LIPS format was designed to be a simple replacement for the harmonized fluid-phase GADA RBA, using common techniques based on the precipitation of autoantibodies bound to cognate tracer antigens. Laboratories already set up to perform RBAs can easily adopt this method using widely available equipment and reagents. Luciferase-tagged antigens are safe and can be produced in-house with potentially long half-lives, giving greater control over label variability and eliminating reliance on radiolabels. The protocol can be completed within one working day and has the lowest serum requirement of all the widely available tests (2 μL for testing in duplicate). This is critical for high-throughput testing of low-volume samples, for instance, capillary blood collection for general population screening (24,25). We also demonstrated the flexibility of LIPS, which can use a range of GAD antigens to facilitate with epitope analysis. Currently, we have similar LIPS assays validated for IA-2A and ZnT8A assessment (in preparation for publication), as well as a triplex assay to screen for GADA, IA-2A, and ZnT8A and a competitive displacement assay for IAA measurement (9). These will facilitate future analysis of risk of multiple islet autoantibodies assessed via LIPS.

These assays were among the top performers in the 2017 Islet Autoantibody Standardization Program workshop. Future intervention trials and natural history studies may benefit from using this method and/or truncated antigens to measure GADA to identify high-risk individuals.

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Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. R.C.W., A.J.K.W. (deceased), and K.M.G. contributed to the original idea and designed the study. R.C.W., S.L.G., B.T.G., A.E.L., and K.C. designed and performed the data collection. I.M., C.B., D.K.S., and V.L. created the luciferase-tagged GAD constructs. R.C.W., S.L.G., A.E.L., K.M.G., and A.J.K.W. performed the data analysis and wrote the manuscript. R.C.W., S.L.G., C.B., I.M., B.T.G., D.K.S., K.C., P.A., L.P., A.E.L., K.M.G., V.L., and A.J.K.W. contributed to data interpretation, manuscript revision, and approved the final manuscript. A.E.L. and K.M.G. are responsible for the integrity of the work as a whole. A.E.L. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Prior Presentation. This work was presented as a poster at The Diabetes UK Professional Conference, London, U.K., 14–16 March 2018, and at the Immunology of Diabetes Society 15th International Congress, San Francisco, CA, 19–23 January 2017.

APPENDIX

The BOX Study Group comprises Isabel Wilson, Rachel Aitken, Ilana Kelland, and Clare Megson at the University of Bristol as well as local consultants in the BOX region including Chitrabhanu Ballav, Atanu Dutta, and Michelle Russell-Taylor (Bucks Healthcare Trust); Rachel Besser (Oxford University Hospitals Trust UK); James Bursell and Shanthi Chandran (Milton Keynes University Hospital); Sejal Patel (Wexham Park Hospital); Anne Smith and Manohara Kenchaiah (Northampton General Hospital); Gomathi Margabanthu (Kettering General Hospital); and Foteini Kavvoura and Chandan Yaliwal (Royal Berkshire Hospital).

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