

**Table 1. Inhibition of TSH binding by sera (from 48 patients with Graves disease) containing TSH antibodies.**

Inhibition of TSH binding (range), %	No. of patients in range	
	PEG method	ELISA
>15	0/48	30/48
10–14	0/48	8/48
<10	48/48	10/48

effectiveness of TRAb measurement by inhibition of TSH-binding assay and by bioassay].

In terms of TRAb ELISA precision, typical variations in absorbances at 450 nm (in the same assay run) were  $1.67 \pm 0.05$ ,  $1.1 \pm 0.04$ ,  $0.76 \pm 0.025$ , and  $0.51 \pm 0.028$  (means  $\pm$  SD;  $n = 28$ ).

Intraassay imprecisions (CVs) were 9.0%, 4.5%, and 2.3% at mean inhibitions of TSH binding of 30%, 42%, and 71%, respectively ( $n = 28$ ), and interassay CVs were 6.8%, 3.3%, and 2.3% at mean inhibitions of TSH binding of 24% ( $n = 10$ ), 45% ( $n = 6$ ), and 69% ( $n = 10$ ), respectively. These values of intra- and interassay imprecision are similar to those observed for the radioactive assay (5).

Occasional sera from patients with Graves disease contain TSH antibodies (9, 10) that form complexes with labeled TSH in the PEG-based receptor assay for TRAb. These TSH-antibody complexes are precipitated with PEG in addition to TSHR-TSH complexes, giving an increase in precipitated  $^{125}\text{I}$  compared with healthy blood donor sera. Consequently, TRAb measurements with the PEG method are difficult to make in samples containing TSH antibodies. To investigate the effects of TSH antibodies in the ELISA, measurements were made in sera from 48 treated Graves disease patients selected for the presence of antibodies reactive with labeled bovine TSH. The effects of anti-TSH antibodies clearly evident in the PEG method (mean inhibition of TSH binding,  $-33.3\%$ ; range,  $-13\%$  to  $-87\%$ ) were not observed in the ELISA (mean inhibition of TSH binding,  $18.7\%$ ; range,  $-0.4\%$  to  $47\%$ ). This was presumably attributable to the removal of the test serum samples containing TSH antibodies after incubation in the receptor-coated wells. Consequently, TRAb concentrations were measurable in samples containing TSH antibodies with the ELISA, and in the series studied, 38 of 48 sera showed  $\geq 10\%$  inhibition (Table 1).

Our results indicate that porcine TSHR immobilized on ELISA plates, using a MAbs to the receptor's COOH terminus, and TSH-biotin can be used to create a TRAb ELISA. The ELISA has similar sensitivity and precision to the current radioactive test but has some major advantages, including easy automation and absence of interference from antibodies to TSH.

## References

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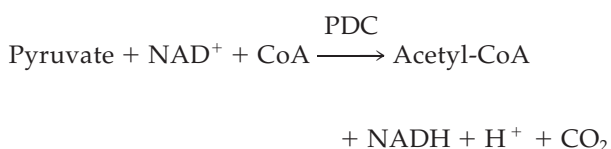
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**Evaluation of an Automated Enzyme Inhibition Assay for the Detection of Anti-Mitochondrial M2 Autoantibodies, Patrick Schmit,\* Georges Gilson, and René L. Humbel** (Laboratoire de Biochimie et d'Immunopathologie, Centre Hospitalier de Luxembourg, Rue Barblé 4, L-1210 Luxembourg, Luxembourg; \* author for correspondence: fax 00352-457794, e-mail schmit.patrick@chl.lu)

Primary biliary cirrhosis (PBC) is a chronic disease characterized by portal inflammation and necrosis of small intrahepatic bile ductules (1). PBC is an irreversible condition, and destruction of the bile ductules leads to progressive cholestasis and fibrosis, and may eventually lead to the development of cirrhosis. PBC is most likely an autoimmune disorder (2), and anti-mitochondrial antibody (anti-M2) has been a diagnostically very useful marker: several studies have reported a positivity rate for anti-M2 of  $>95\%$  in biopsy-confirmed PBC patients (3). The major mitochondrial antigen was identified as the 74-kDa E2 subunit of the pyruvate dehydrogenase complex (PDC), a member of the 2-oxoacid dehydrogenase complex family (4). The traditional technique for the detection of anti-M2 is immunofluorescence (5), but recently new serological assays, such as ELISA, immunoblotting, and enzyme inhibition (EI), have been developed (6, 7). A miniaturized EI assay for performance on microtiter plates has already been described (8, 9). In this study we evaluated a new commercially available and completely automated EI assay manufactured by Trace Scientific (Victoria, Australia) and compared it to the immunofluorescence and ELISA techniques performed in our laboratory.

The TRACE enzymatic procedure is a unique method based on the PDC inhibitory properties of the principal

anti-74-kDa antibody. PDC catalyzes the following reaction:



In the absence of anti-M2, this reaction will proceed at the maximal rate after the addition of a serum sample. If a sample contains anti-M2, the PDC activity will be inhibited, greatly reducing the rate of reaction, typically to <70%. The rate of reaction can be simply monitored by measuring the rate of increase in absorbance at 340 nm as NADH is formed as an end product of the catalytic degradation of pyruvate. We performed the TRACE enzymatic anti-M2 assay on a COBAS Mira (Roche Diagnostics) analyzer, using the program recommended by the manufacturer.

With a selected serum presenting a moderate degree of inhibitory activity close to the cutoff value of 70% of residual PDC activity, replicate testing for intraassay variation ( $n = 10$ ) gave a mean (SD) inhibition of activity of 76.1% (1.7%) with a CV of 2.2%, and replicate testing for interassay variation (10 determinations performed on 10 days with a new calibration for each day) gave a mean (SD) inhibition of activity of 74.5% (2.4%) with a CV of 3.2%.

Our ELISA method used polystyrene microtiter plates (Nunc Maxisorp) coated with porcine heart pyruvate dehydrogenase (cat. no. P-7032; Sigma) at 1.56 mU per well in 0.01 mol/L bicarbonate buffer, pH 9.6. Plates were left overnight at 4 °C, washed twice with distilled water, and blocked with SuperBlock (Pierce) for 5 min at room temperature. Duplicate samples for each of the sera were diluted 1:100 in phosphate-buffered saline, pH 7.4, with 1 mL/L Tween 20; 100  $\mu$ L was dispensed per well and incubated for 1 h under constant shaking at room temperature. Plates were washed three times in 150 mmol/L NaCl, 10 mmol/L Tris, 1 mL/L Tween 20, pH 7.4, and antibody binding was detected with 100  $\mu$ L of peroxidase-labeled rabbit anti-human IgG (cat. no. P0406; Dako) and anti-human IgM (cat. no. P322; Dako) diluted 1:5000 in SuperBlock containing 0.5 mL/L Tween 20 and incubated

for 1 h at room temperature under constant shaking. The reactivity was visualized with ABTS (Roche-Boehringer Mannheim) at 405 nm in a microplate reader (MIOS; Dynatech). The absorbance cutoff value of the assay (mean + 3 SD) was established on a blood donor population ( $n = 70$ ), and a reference curve using an anti-M2-positive PBC serum was established to assign arbitrary units (AU), with 10 AU being the cutoff value. With a selected serum presenting a weakly positive value, replicate testing for intraassay variation ( $n = 10$ ) gave a mean (SD) of 50.6 AU (6.3 AU) with a CV of 12%, and replicate testing for interassay variation (10 determinations performed on 10 days with a calibration for each day) gave a mean (SD) of 48.8 AU (7.3 AU) with a CV of 15%.

Indirect immunofluorescence (IIF) for anti-M2 was performed on 5- $\mu$ m cryostat sections from rat liver, kidney, and stomach (Sanofi Diagnostics Pasteur). The screening dilution of the sera was 1:20 in phosphate-buffered saline with 20 g/L bovine serum albumin. Fluorescein isothiocyanate-labeled anti-human IgG H + L (Scimedex) was used as a second antibody.

Our clinically well-characterized patient population was composed of 23 histologically demonstrated PBC patients, 20 cases of other autoimmune hepatic disorders (autoimmune hepatitis type I and II), 40 cases of non-autoimmune hepatic disorders (hepatitis B and C), and 32 healthy control subjects. Blood was drawn after an overnight fast and allowed to clot at room temperature; serum was obtained by centrifugation at 1500g for 10 min and was stored at -24 °C until analyses were performed. All PBC samples were positive with the TRACE EI assay, the ELISA, and the immunofluorescence technique. The discordant results (Table 1) obtained between the EI assay and the ELISA may be explained by the fact that the EI assay specifically measures the inhibitory properties of the 74-kDa E2 subunit of the PDC, whereas we used in our ELISA the commercially available purified intact PDC for coating, a preparation known to contain minor amounts of 2-oxoacid dehydrogenase complex enzymes other than PDC. Moreover, antibodies that bind PDC are not necessarily inhibitory. It has already been reported that with ELISA techniques, the choice among recombinant PDC-E2 (10), commercially purified PDC (11), and

**Table 1. Positivity rates for anti-M2 detection.**

Samples	EI	ELISA	IIF
Healthy controls ( $n = 32$ )	0	0	0
PBC ( $n = 23$ )	23	23	23
Non-autoimmune hepatic disorders			
Type B hepatitis ( $n = 20$ )	0	1	0
Type C hepatitis ( $n = 20$ )	0	1	0
Autoimmune hepatic disorders			
Anti-LKM1 <sup>a</sup> (autoimmune hepatitis type II; $n = 10$ )	0	1	LKM pattern
Anti-actin (autoimmune hepatitis type I; $n = 10$ )	0	0	Actin pattern

<sup>a</sup> LKM, liver kidney microsomes.

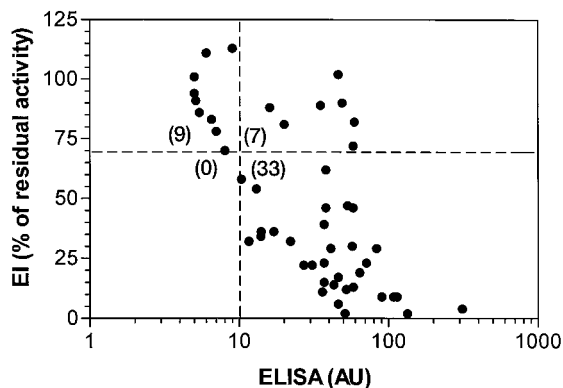


Fig. 1. Anti-M2 results obtained by TRACE EI assay and in-house ELISA for 49 patients without clinical evidence of liver disease but presenting a typical anti-M2 staining pattern by IIF.

The *dashed lines* indicate reference limits. Increased values are positive for ELISA; decreased values are abnormal by EI. The number of results in each quadrant is indicated in *parentheses*.

in-house-purified PDC (12) could influence the results obtained for anti-M2 antibodies.

We also performed a comparative study of the three techniques (IIF, ELISA, and EI) with 70 samples that were referred to our laboratory for diagnostic testing by immunofluorescence and gave a specific anti-M2 staining pattern. Available clinical data for these 70 samples showed 19 patients (27%) for whom PBC could be established as the diagnosis, 2 (3%) with another liver disease (1 acute hepatitis and 1 chronic ethylism), and 49 (70%) for whom there was no clinical evidence of liver disease. The high percentage of sera from patients with no evidence of liver disease presenting a typical anti-M2 pattern in IIF may be surprising, but similar data have already been presented in a larger study (13). The long asymptomatic phase in PBC is probably the main reason for this situation because in 33 of these 49 cases (67%), the anti-M2 pattern observed in immunofluorescence could be confirmed by a positive result obtained by the ELISA and EI techniques (Fig. 1). In seven cases (14%), only the ELISA gave a positive result, illustrating the difference in performance of the ELISA and the EI assay already described above. In the remaining nine cases (18%) of serum from patients with no evidence of liver disease presenting an anti-M2 pattern, neither the ELISA nor the EI assay gave a positive result, confirming that the immunofluorescence assay identifies mitochondrial reactants other than PDC.

In conclusion, our study on a limited number of samples showed that the TRACE EI assay, run on a COBAS Mira automated analyzer, is a very promising test for the determination of anti-M2 antibodies, having the advantage of few procedural steps, rapid turnaround time, nonsubjective read-out, and low costs. The analytical performance was better than that obtained for the ELISA. All of the PBC patients in our populations were correctly detected by the EI assay, and the specificity of the assay was higher than with IIF and ELISA using commercially purified PDC. We feel that the TRACE EI assay is a valuable tool that could provide considerable help in the interpretation of anti-M2-positive patterns in IIF and could replace labor-intensive ELISA testing for anti-M2 antibodies.

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