

Rapid One-Step Immunofluorometric Assay for Measuring Soluble Transferrin Receptor in Whole Blood,

Riikka Vikstedt,^{1†} Piia von Lode,^{1*} Timo Takala,² Kerttu Irjala,³ Olli Peltola,² Kim Pettersson,¹ and Pauli Suominen³ (¹ Department of Biotechnology, University of Turku, Turku, Finland; ² Department of Clinical Chemistry, Turku University Central Hospital, Turku, Finland; ³ Mehiläinen Oyj, Turku, Finland; † current address: Department of Molecular Medicine, National Public Health Institute, Helsinki, Finland; * address correspondence to this author at: Department of Biotechnology, University of Turku, Tykistökatu 6A, 6th Floor, FIN-20520 Turku, Finland; fax 358-2-333-8050, e-mail piia.vonlode@utu.fi)

Iron deficiency is common, and understanding of it is advancing (1). The clinical investigation of body iron metabolism now extends beyond the demonstration of iron deficiency (e.g., by bone marrow examination) and estimation of iron storage status (by, e.g., measurement of serum ferritin). An important aim now is to determine whether a patient would benefit from iron supplementation (2–4). Soluble transferrin receptor (sTfR) measurements (5–14) have enabled efficient detection of early iron-deficiency anemia (IDA), the treatment of which is essential, especially during phases of psychomotor development, rapid growth, and pregnancy (15–18). Furthermore, the distinction between IDA and anemia of chronic disease (ACD) has become less complicated, as has the characterization of functional iron deficiency (FID) in cases of multimorbidity in the elderly or in chronic or persisting inflammatory diseases (5–14).

sTfR is a broadly applicable tool to identify individuals who are likely to benefit from iron supplementation and, consequently, is a candidate for point-of-care (POC) testing. Although most of the above-mentioned conditions are highly relevant in the outpatient setting, the current commercially available sTfR methods are not suitable for extra-laboratory testing, and none use unprocessed whole blood as sample (7, 19, 20). We describe a novel, 5-min POC immunoassay for sTfR based on the all-in-one dry-reagent assay concept (21) and time-resolved fluorescence detection.

The assays were performed in individual, assay-specific dry-reagent cups that were prepared similarly to the method described by von Lode et al. (22). In short, the monoclonal anti-sTfR capture antibody (HyTest Ltd.) was biotinylated and bound (200 ng/well) to polystyrene wells coated with streptavidin (Innotrac Diagnostics Oy). We used 100 ng/well of the monoclonal anti-sTfR detection antibody (HyTest Ltd.) labeled with an intrinsically fluorescent europium chelate (22). All assay components were dried in the wells, with a protective layer separating the antibodies to prevent nonspecific binding of the label to the solid phase. When protected from humidity, the cups were stable (i.e., activity >90%) for 5 months at room temperature and for 12 months at 4 °C.

The one-step assays were performed on the fully automated Aio! Immunoanalyzer (Innotrac Diagnostics Oy).

To start the assay, 10 μ L of 50-fold-diluted sample and 20 μ L of universal buffer (22) were dispensed into the wells. The wells were incubated with shaking for 5 min at 36 °C, after which they were washed and dried, and the time-resolved europium fluorescence was measured.

The detection limit of the assay (mean of zero + 3 SD) was 0.0003 mg/L (for calibration curve, see the upper inset in Fig. 1A), and the assay was linear up to 1 mg/L. Considering the 50-fold dilution of clinical samples, the true measurement range of the assay was 0.015–50 mg/L of blood. The limit of quantification, i.e., the lowest concentration of sTfR that could be measured with a CV \leq 15%, was \sim 0.05 mg/L (see Fig. 1 in the Data Supplement that accompanies the online version of this Technical Brief at <http://www.clinchem.org/content/vol50/issue10/>). No high-dose hook effect was detected at the sTfR concentrations tested (up to 150 mg/L).

Within-assay CVs ($n = 12$), determined with heparin-plasma pools, were 2.6%, 4.1%, and 4.6% for 1.3, 2.2, and 3.4 mg/L sTfR, respectively, with corresponding between-assay CVs (1 replicate run twice a day for 25 subsequent runs) of 6.2%, 5.3%, and 8.1%. Within-assay CVs for whole blood with added sTfR calibrator (HyTest Ltd.) were 6.1%, 5.8%, and 5.6% for 0.5, 1.1, and 3.0 mg/L sTfR, respectively. The measured concentrations of endogenous sTfR were 103% of the expected value in whole blood ($n = 12$) and 99% in plasma ($n = 12$).

To further study the suitability of whole blood as a sample material for the new assay, we compared sTfR concentrations in 50 EDTA-whole-blood samples with corresponding plasma samples. Linear regression analysis was performed before (slope, 0.725; y -intercept, -0.204 mg/L; $S_{y|x} = 0.106$ mg/L; $r = 0.984$; $P < 0.0001$) and after (slope, 0.994; y -intercept, -0.002 mg/L; $S_{y|x} = 0.093$ mg/L; $r = 0.993$; $P < 0.0001$) hematocrit (Hct) correction (mean Hct, 0.40; range, 0.29–0.48). As expected, the Hct-corrected values agreed more closely with the plasma concentrations. Difference analysis showed that concentrations measured in plasma were slightly higher than in whole blood, but with a mean difference of only 0.009 mg/L (95% confidence interval, -0.164 to 0.182 mg/L; measured sTfR range, 0.9–5.6 mg/L).

With the current assay and detection technology, samples with a very wide range of Hct values can be measured without any interference from erythrocytes, providing that individual Hct values are used for correction (23). Because of the very low volume (0.2 μ L) of undiluted whole blood used relative to the assay volume (30 μ L), variations in the number of blood cells also have very little effect on the viscosity of the reaction mixture. However, the use of a predetermined mean Hct value for correction may also be feasible especially in POC settings (23, 24). Hct values are, however, known to be decreased both by IDA and ACD. The use of a mean Hct value for patients with microcytosis (decreased Hct) would therefore lead to overestimated sTfR concentrations irrespective of the concurrent iron status. In cases of severe ACD, for example, this could potentially lead to misclassification of ACD as either IDA or FID, depending on the

clinical data available. However, the clinical significance regarding the need for individual Hct correction remains to be studied.

We assessed the agreement of the 5-min assay with the Orion Diagnostica immunoturbidimetric IDeA® sTfR IT assay in 70 serum samples. The results of linear regression analysis and difference analysis are shown in Fig. 1A. The mean difference between methods was negligible (-0.002 mg/L), but the area between the lines of agreement was wide (-1.055 to 1.051 mg/L). The relatively poor comparability between the sTfR assays here and in general is an acknowledged problem and is mostly attributable to the lack of international standardization material and the wide variety of antibodies used (7, 19, 20).

We evaluated the clinical performance of the new assay in serum samples from 72 patients from Turku University Central Hospital. Anemia was defined as a hemoglobin concentration <128 g/L in men and <117 g/L in women (lower reference limits in Turku University Central Hospital). The patients were divided into three groups on the basis of bone marrow examination and clinical data. The 28 nonanemic controls included 8 apparently healthy bone marrow donor candidates and 20 patients who had undergone bone marrow examination for nonanemic, nonmalignant hematologic conditions (other cytopenias). The IDA group consisted of 32 anemic patients who had no stainable iron in the bone marrow. Twelve anemic patients who presented with stainable iron in the bone marrow, were classified as the ACD group. The distribution patterns of the patient groups in the POC assay are shown in Fig. 1B. The mean sTfR concentrations were 1.36 mg/L (range, 0.71–1.96 mg/L) in the control group, 1.74 mg/L (range, 0.91–4.04 mg/L) in the ACD group, and 3.99 mg/L (range, 1.37–10.92 mg/L) in the IDA group.

To assess the clinical agreement between the two methods, we compared their capabilities to classify patients according to two criteria: (a) the ability to identify patients likely to benefit from supplemental iron (14, 25); and (b) the ability to differentiate between patients with IDA and ACD. Patients were first classified as likely to benefit from iron supplementation if their IDeA sTfR concentration was above the cutoff of 2.3 mg/L (14). The ROC analysis (26) showed that identical classification was achieved by the POC test when a cutoff of 1.96 mg/L was used (Table 1). We next performed ROC analyses to derive the optimum cutoff values for both methods to detect depleted iron stores irrespective of the hemoglobin concentration ($n = 72$) and to differentiate between the IDA and ACD groups ($n = 44$). The results from the ROC analyses (Table 1) showed that both methods were able to clearly distinguish between patients with and without stainable iron in their bone marrow. However, the optimum cutoff points for these distinctions were somewhat lower and less efficient than reported previously (7, 14).

The reason for the lower diagnostic accuracy of both tests evaluated in this study may be that we studied individuals with a broad range of both iron status and coexisting clinical conditions. This better simulates the

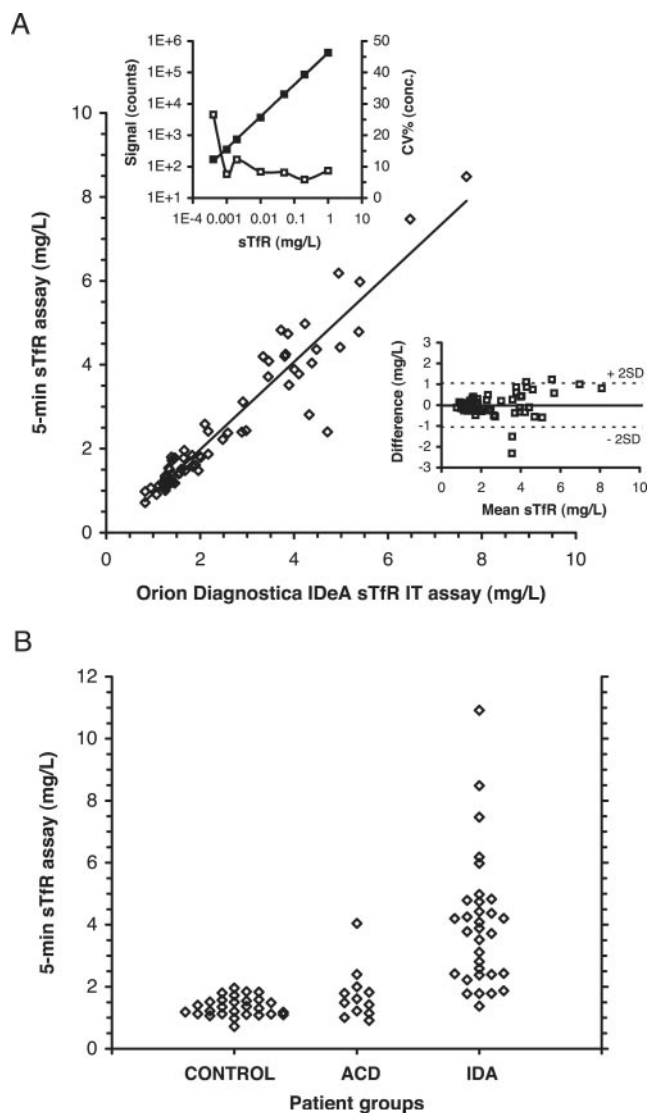


Fig. 1. Comparison of the 5-min and the Orion Diagnostica IDeA IT sTfR assays (A), and distribution profile of the 5-min sTfR assay (B).

(A), regression analysis. The equation for the line is: $y = 1.045x - 0.115$ ($r = 0.949$; $P < 0.0001$; $n = 70$). The upper inset shows the calibration curve (■) and precision profile (□) for the 5-min assay. The lower inset shows the absolute difference between the two assays (mean, -0.002 mg/L; 95% confidence interval, -1.055 to 1.051 mg/L). (B), sTfR concentrations measured in nonanemic controls ($n = 28$), in patients with ACD ($n = 12$), and in patients with IDA ($n = 32$).

population in an outpatient clinic and, consequently, extends the stainable-iron distinction analysis to patients with nonanemic iron-deficient erythropoiesis and recovering hemoglobin concentrations in addition to strictly isolated cases of IDA and ACD (Fig. 1B) (6, 7, 14, 25, 27). Furthermore, FID frequently occurs as a result of prolonged ACD because the mobilization of iron from storage becomes increasingly restricted by the reticuloendothelial system. Because individuals with FID present with a combination of increased sTfR and normal to increased iron stores, the presence of FID compromises the distinc-

Table 1. Evaluation of clinical classification by ROC analysis.

| Comparison criteria | Cutoff, mg/L | AUC ^a (95% CI) | Sensitivity (95% CI) | Specificity (95% CI) |
|--|--------------|---------------------------|----------------------|----------------------|
| Likelihood of benefit from iron supplementation using 5-min assay (n = 72) | 1.96 | 0.9909 (0.976–1.006) | 1.00 (0.90–1.00) | 0.95 (0.86–0.99) |
| Detection of depleted iron stores | | | | |
| IDeA (n = 72) | 2.01 | 0.8977 (0.822–0.974) | 0.88 (0.74–0.96) | 0.93 (0.81–0.98) |
| 5-min assay (n = 72) | 1.99 | 0.9332 (0.873–0.993) | 0.84 (0.70–0.94) | 0.95 (0.85–0.99) |
| Differentiation of IDA from ACD | | | | |
| IDeA (n = 44) | 2.01 | 0.7630 (0.582–0.944) | 0.88 (0.73–0.96) | 0.75 (0.47–0.93) |
| 5-min assay (n = 44) | 1.61 | 0.8880 (0.797–0.979) | 0.93 (0.79–0.99) | 0.58 (0.27–0.85) |

^a AUC, area under curve; CI, confidence interval.

tion between IDA (increased sTfR; no iron stores) and ACD (normal sTfR; normal iron stores). In effect, an increased sTfR in this setting should be considered indicative of FID rather than a false positive for IDA. However, this shortcoming in classification affected both methods similarly.

In conclusion, we have developed a new assay for rapid and reliable measurement of sTfR in whole blood, plasma, and serum specimens. Comparison of the POC assay with the routine IDeA method showed encouraging results regarding the POC setting. The observed analytical performance together with the capability of the test to identify individuals likely to benefit from iron supplementation, and to distinguish patients with isolated IDA from those with ACD, supports the implementation of the new assay into routine clinical practice.

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