European Thyroid Journal (2026) **15** ETJ260041
https://doi.org/10.1530/ETJ-26-0041

Received 13 October 2025
Accepted 3 March 2026
Available online 4 March 2026
Version of Record published 19 March 2026

RESEARCH

Measuring deiodinase activity: a need for standardization?

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Abstract

Thyroid hormones are produced in the thyroid gland and metabolized in the peripheral tissues. The major pathway of thyroid hormone metabolism is the removal of an iodine atom from the phenolic or tyrosyl ring by deiodinating enzymes, the so-called deiodinases. Three distinct types of deiodinase have been identified, namely type 1 (D1), type 2 (D2) and type 3 (D3), which differ in main function and expression profile. Measuring the activities of D1, D2 and D3 is an indispensable tool in research on thyroid hormone metabolism. At present, only a limited number of research laboratories worldwide measure deiodinase activity using a variety of assays and protocols. Unlike diagnostic labs, research labs rarely participate in external quality control due to limited availability for most analytes or enzymes. However, implementing a quality assurance program for deiodinase assays is crucial to ensure consistent enzyme activity across laboratories. The present study provides the results of a method comparison between five established laboratories with experience in measuring deiodinase activity. The results showed that there are considerable differences in deiodinase activity levels determined by participating laboratories, which could be partially explained by differences in techniques and protocols. We therefore concluded that in most cases, absolute deiodinase activities can only be compared within the same laboratory. External quality control only adds value when all the laboratories use the same technique with their own optimized protocols. The use of internal controls is recommended to ensure that the correct enzymatic activity is being measured over time in the same laboratory.

Keywords: deiodinase; assay; thyroid hormone metabolism; radioactive tracers

Introduction

Thyroid hormones are essential for growth and development and are the major regulators of energy homeostasis. The potency of thyroid hormones is regulated by three enzymes called deiodinases. These enzymes are located intracellularly and catalyze the activation or inactivation of thyroid hormones by

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removing an iodine atom at different positions of the iodothyronine molecule, namely type 1 (D1), type 2 (D2) and type 3 (D3) (1). Studying the activity of deiodinases has provided valuable insights into thyroid economy in health and disease.

To further advance research in this field, the American Thyroid Association, together with international leading experts, published an extensive guideline with recommendations for thyroid research a decade ago (2). This guideline was an important step toward achieving a consensus to obtain more reproducible and comparable results from different deiodinase assays and recommended that D1, D2 and D3 activity levels could be measured using radioactive iodothyronines as substrates, followed by quantitation of the radioactive products generated per unit time. However, this guideline does not suggest specific procedures for the measurement of deiodinase activity.

Differences in activity can occur at three levels: i) preparation of the sample, ii) assay conditions and iii) detection methods. Differences in sample preparation may include sample collection, storage, the number of freeze–thaw cycles and homogenization procedures. Assay conditions can differ in multiple ways, including buffers, concentrations of substrates and cofactors and controls. The latter includes a positive control sample (known deiodinase activity), a negative control sample (only buffer) and, when appropriate, a control sample that contains an excess of non-labeled substrate or inhibitor, thereby blocking the specific activity.

These controls are not standardized across the different laboratories, and the detection methods employ different principles. Given these factors, inter-laboratory differences in deiodinase activities could be expected although the occurrence and extent are unclear. Therefore, establishing inter-laboratory protocols for comparing deiodinase assays would be of great value to scientists in this field. However, there are no independent reference materials available for these assays and inter-laboratory controls in research laboratories are very rare compared to those in clinical laboratories, where external quality assurance is common practice. All these limitations, combined with the increasing demand for improving the reproducibility of research data and the desire of some research laboratories to adhere to quality standards similar to those used in clinical laboratories, such as ISO 15189 or provided by CLIA, have encouraged the present comparative study.

This article presents the findings of a worldwide effort consisting of two rounds of quality control exchange between five laboratories with extensive experience in performing a variety of deiodinase assays using radioactive tracers. We explored the feasibility of external quality assurance in deiodinase and thyroid economy research, aiming to investigate and improve the comparability of the results generated. Our results may represent a first step toward harmonization of assays used in basic thyroid hormone research.

Materials and methods

Materials

The following materials were distributed to participating laboratories for each of the two rounds of quality control. For the first round, we used homogenates from transfected HEK293 cell lines with deiodinase overexpression D1 (A), D2 (B), D3 (C) and D2 + D3 (D) (kindly provided by Kostja Renko (Institut fur Experimentelle Endokrinologie, Charite – Universitatsmedizin Berlin, Germany) and described previously (3)), fresh frozen pituitaries from untreated C57Bl6 mice for D2 and undiluted mouse liver homogenate for D1 and D3, all from a previous study (4). For the second round, we used mouse liver homogenates for D1 and D3 and kidney homogenates for D1; fresh frozen rat pituitary and mouse brown adipose tissue (BAT) for D2, all from previous studies. Human placenta homogenate for D3, provided by Dr Marcel Meima (Erasmus MC Rotterdam) and part of a study that received exemption for approval from the local institutional medical ethics committee according to the Dutch Medical Research with Human Subjects Law (MEC 2017-418). All patients gave written informed consent before donating their placentas. Healthy placentas of uncomplicated singleton pregnancies were collected immediately after delivery (via cesarean section) at Erasmus University Medical Center, Rotterdam, the Netherlands. In the first round, the laboratories were instructed to perform their assay according to their own protocols (see below and Table 1). In the second round, a consensus protocol was provided based on the method used to detect the radioactive tracers of the different thyroid hormone by-products (Tables 2, 3, 4).

For tissues, 50–100 mg of frozen tissue were homogenized in 10 volumes (w/v) of the indicated buffers (see below) using a Polytron PT2500E or PT3000 homogenizer (Kinematica, Germany) in the laboratory of the Amsterdam UMC, snap-frozen and stored at −80°C until shipment (D1 and D3). Tissues used for D2 analyses were shipped snap-frozen and homogenized shortly before the measurement in the specific laboratory as D2 activity must be measured in fresh homogenate. Protein concentration was measured with the Bio-Rad protein assay using bovine serum albumin as the standard following the manufacturer's instructions (Bio-Rad Laboratories, the Netherlands).

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Table 1 Overview of the participation of the laboratories for the different enzymes and the two rounds.

	D1		D2		D3	
	Round 1	**Round 2**	**Round 1**	**Round 2**	**Round 1**	**Round 2**
Hungary	+	+	+	+	–	–
Netherlands						
Amsterdam	+	+	+	+	+	+
Rotterdam	+	+	+*	+	+	+
USA						
Chicago	–	–	+*	+	+†	+‡
Maine	–	–	+	+	+	+

+ Indicates participation; – indicates assay not performed in this lab.
*Pituitary tissue could not be determined due to low signal. †One liver tissue could not be determined due to running out of material. ‡Two liver tissues could not be determined due to low signal.

Not all laboratories measure all deiodinase activities due to unavailability of the radioactive tracer needed, and some measurements could not be completed due to low signal and/or running out of material. This is summarized in Table 1.

Method

The activity measurements are based on the conversion of radioactive iodothyronines as a substrate followed by quantification of the radioactive products generated per minute per mg protein of the tissue or cell homogenate (2). These reactions require both a deiodinase enzyme and a cofactor, most likely a thiol such as dithiothreitol (DTT), to reduce the oxidized selenolyl group in the active center of each deiodinase. Type 1 deiodinase can be measured in snap-frozen tissue or cell homogenates using rT3 as the preferred substrate (outer ring deiodination) although T4 can also be used. Adding PTU as a D1 inhibitor blocks the activity and can be used as a tissue blank. Type 2 deiodinase activity can only be measured in fresh tissue homogenate using T4 as the preferred substrate (outer ring deiodination), and adding an excess of T4 blocks the activity and can be used as a tissue blank. Type 3 deiodinase can be measured in snap-frozen tissue or cell homogenates using T3 as the preferred substrate (inner ring deiodination). Adding an excess of T3 blocks the activity and can be used as a tissue blank.

The reaction mixture contains homogenate, radioactive tracer and, depending on the tissue analyzed, different combinations of iodothyronines and/or inhibitors to block the activity of the other enzymes that interfere with outcome. After incubation at 37°C for a certain period of time, the reaction is stopped using different strategies as given in Tables 2, 3, 4.

Radioactive tracers are labeled in the outer ring only, which implicates that type 1 and type 2 deiodinase activities result in the production of both ^{125}I and [^{125}I]-T2 or [^{125}I] and [^{125}I]-T3, respectively, while type 3 deiodinase only yields [^{125}I]-T2. The products can be quantified using different techniques; the reaction mixture can be applied to a resin column soaked in 10% acetic acid. The resin absorbs the iodothyronines, while the iodide passes through and can be quantified directly. It is also possible to precipitate the iodothyronines with horse serum and TCA and quantify the iodide in the supernatant. These two methods are not feasible for D3 activity as no free ^{125}I will be released during inner ring deiodination. Another way of quantification is separating the [^{125}I]-iodothyronines and ^{125}I by paper chromatography, high-performance liquid chromatography (HPLC) or ultra-high-performance liquid chromatography (UPLC) followed by detection of the products.

In general, the homogenates tested must be diluted to a protein concentration that ensures <30% substrate conversion to prevent underestimation of activities due to substrate depletion. For assay blanks, incubations were carried out with PED10 or PED20 buffer instead of homogenates.

Each laboratory analyzed the samples of round 1 following its standard protocol. Experimental details are given in Table 2. The samples in round 2 were assayed according to the steps given in the consensus protocol (Table 5), while assay stop and measurement was done according to own laboratory protocol.

Statistical analysis

Due to the small number of data points for each comparison and the large variance in absolute values, statistical analysis is limited. The coefficient of variation usually gives valuable insights in method comparison but cannot be used because of a small number of data points and/or because of outliers. For this reason, the median and fold change are given instead of the average value. The small number of data points also excludes the median absolute deviation as possibly valuable statistic.

Table 2 Original methods used by the laboratories participating in the study and used for measuring samples in round 1.

D1	Amsterdam UMC	Erasmus MC	Maine	Budapest	Chicago
Tissue	Cells (A) and liver 1	Cells (A) and liver 1	-	Cells (A) and liver 1	-
Duplo	Yes	Yes	-	Yes	-
Protein concentration			-		-
A	0.17 and 0.018 mg/mL (2 dilutions)	0.0077 mg/mL		0.08 mg/mL and 0.008 mg/mL (2 dilutions)	
Liver	0.27 mg/mL	0.076 mg/mL		5.5 mg/mL	
Buffer	PED20	PED10	-	PED10	-
Substrate	0.1 μM rT3	0.1 μM rT3	-	1 μM T4	-
Tracer	1.5 × 10^5 cpm [^{125}I]-rT3*	2 × 10^5 cpm [^{125}I]-rT3†	-	1 × 10^5 cpm [^{125}I]-T4	-
Buffer tracer	PE	PED10	-	PED20	-
Final volume (μL)	150	100	-	300	-
Assay blank (background)	PED20 + 0.1 μM rT3 + 1.5 × 10^5 cpm [^{125}I]-rT3*	PED10 + 0.1 μM rT3 + 2 × 10^5 cpm [^{125}I]-rT3†	-	PED20 + 1 μM T4 + 1 × 10^5 cpm [^{125}I]-T4	-
Tissue blank	Assay blank + 500 μM PTU	-	-	Assay blank + empty HEK293T cell homogenate	-
Positive control	Mouse liver	None	-	None	-
Incubation time (min)	30 at 37°C	30 at 37°C	-	60 at 37°C	-
Reaction stop (on ice)	150 μL ethanol, vortex, 5 min incubation, 2,370 ***g*** at 4°C for 20 min, 100 μL SN + 100 μL 0.02 M NH_4OAc (pH 4)	125 μL 0.1% HOAc in acetonitrile, vortex 5 s, 1 h incubation on ice, 2,868 ***g*** at 4°C, 100 μL SN + 125 μL 0.02 M NH_4OAc (pH 4)	-	100 μL 30% NHS followed by 200 μL 10% TCA. 10,000 ***g*** at 4°C for 4 min. ^{125}I counted in 360 μL SN	-
Column	4.6 × 250 mm symmetry C18	Acquity Premier BEH C18 (130 Å, 11.7 μm, 2.1 × 100 mm)	-	-	-
HPLC system	Shimadzu HPLC system	Acquity Premier UPLC System (Waters, USA)	-	-	-
Detector	Radiomatic 150 TR Flow Scintillation Analyzer (Revvity, USA)	Ramona star detector (Elysia-Raytest, Belgium)	-	Wizard2 2,470 Gamma Counter (Revvity, USA)	-
Calculation	Tissue blank-adjusted activity with 0.1 μM rT3	Assay blank-adjusted activity with 0.1 μM rT3	-	Tissue blank-adjusted activity with 1 μM T4	-
Formula	(2x ^{125}I-T2)‡/((2x ^{125}I-T2) + ^{125}I-rT3))* 100	(^{125}I + ^{125}I-T2)/((^{125}I + ^{125}I-T2) + ^{125}I-rT3))* 100		Sample cpm-background cpm/total cpm/0.6* substrate (300 pmol)/time (min)/protein (mg)	
Unit	T2 pmol/min/mg protein	T2 pmol/min/mg protein	-	^{125}I pmol/min/mg protein	-

PE buffer, 0.1 M sodium phosphate, 2 mM EDTA pH 7.2; PED10, 0.1 M sodium phosphate, 2 mM EDTA pH 7.2, 10 mM dithiothreitol (DTT); PED20 buffer, 0.1 M sodium phosphate, 2 mM EDTA pH 7.2, 20 mM DTT; PED50 buffer, 0.1 M sodium phosphate, 2 mM EDTA pH 7.2, 50 mM DTT; T/S 25 buffer, 20 mM Tris–base, 0.25 M sucrose, DTT 25 mM, pH 7.0–7.4; vtx, vortexing; NHS, normal horse serum; SN, supernatant. The Maine and Chicago laboratory did not measure D1 activity due to unavailability of the rT3 tracer.

*In-house single-labeled tracer according to Wiersinga *et al.* (5). †Single ^{125}I-labeled T3, T4 and rT3 were produced as described (9). ‡Factor 2 is needed as half of the formed T2 is radioactive and thus measurable.

Table 3 Original methods used by the laboratories participating in the study and used for measuring samples in round 1.

D2	Amsterdam UMC†	Erasmus MC	Maine	Budapest	Chicago§
Tissue	Cells (B + D) and pituitary	Cells (B + D)	Cells (B + D) and pituitary	Cells (B + D) and pituitary	Cells (B + D)
Duplo	Yes	Yes	Yes	Yes	Yes
Protein concentration					
B	0.0019 mg/mL	0.00047 mg/mL	0.003 mg/mL	0.001 and 0.0001 mg/mL	0.28 mg/mL
D	0.01 and 0.001 mg/mL	0.00031 mg/mL	0.018 and 0.0018 mg/mL	0.06 and 0.006 mg/mL	0.16 mg/mL
Pituitary	0.89 mg/mL		0.34 mg/mL	2.29 mg/mL	
Buffer	PED50 + 0.25M sucrose	PED20	T/S-25	PED10 (pH 6.9) + 0.25 M sucrose	PE
Substrate	1 nM T4	1 nM T4	1 nM T4	1 nM T4	1 nM T4
Tracer	1 × 10^5 cpm [^{125}I]-T4*	2 × 10^5 cpm [^{125}I]-T4‡	1 × 10^5 cpm [^{125}I]-T4 (NEX111X, Revvity)	∼1 × 10^5 cpm [^{125}I]-T4	2.5 × 10^5 cpm [^{125}I]-T4 (NEX111X, Revvity)
Buffer tracer	PE	PED20 + 0.1 mM PTU	T/S-25	PED20 + 100 nM T3, 1 mM PTU	PE
Final volume (uL)	150	100	100	300	300
Assay blank (background)	PED50 + 0.25M sucrose + 1 nM T4 + 1 × 10^5 cpm [^{125}I]-T4*	PED20 + 1 nM T4 + 2 × 10^5 cpm [^{125}I]-T4‡ + 0.1 mM PTU	T/S-25 + 1 nM T4 + 1 × 10^5 cpm [^{125}I]-T4	PED20 + 1 nM T4 + ∼1 × 10^5 cpm [^{125}I]-T4	PED20 + 1 nM T4, 2.5 × 10^5 cpm [^{125}I]-T4
Tissue blank	Assay blank + 0.5 μM T4	-	Assay blank + 1 μM T4	Assay blank + 100 nM T4	-
Positive control	Rat pituitary	None	Mouse neonatal BAT	None	None
Incubation time (min)	120 at 37°C	60 at 37°C	20–60 at 37°C	90 at 37°C	120 at 37°C
Reaction stop (on ice)	200 μL bovine serum + 300 μL 20% TCA, 3,162 ***g*** at 4°C for 30 min. ^{125}I counted in 2 × 200 μL SN	125 μL 0.1% HOAc in acetonitrile, vtx 5 s, 1 h on ice, 2,862 ***g*** at 4°C, 100 μL SN with 125 μL 0.02 M NH_4OAc (pH 4)	100 μL ethanol	100 μL 30% NHS followed by 200 μL 10% TCA. 10,000 ***g*** at 4°C for 4 min. ^{125}I counted in 360 μL SN	300 μL MeOH, vtx 2 min, 13,800 ***g*** for 5 min, 400 μL SN + 200 μL buffer (0.02 M NH_4OAc, 4% MeOH, 4% PE)
Column	-	Acquity Premier BEH C18, 130 Å, 11.7 μm, 2.1 × 100 mm)		-	Acquity Premier BEH C18, 130 Å, 1.7 μm, 2.1 × 100 mm
HPLC system	-	Acquity Premier UPLC System (Waters)	Paper chromatography (3001-614, Whatman, GE Healthcare, Chicago)	-	Acquity UPLC System (Waters)
Detector	Wizard2 2,470 Automatic Gamma Counter (Revvity, USA)	Ramona star detector (Elysia-Raytest, Belgium)	Autoradiographed chromatograms, bands in γ counter (Titertek, Germany)	Wizard2 2,470 Gamma Counter (Revvity, USA)	Radiomatic 150 TR Flow Scintillation Analyzer (Revvity, USA)
Calculation	Tissue blank-adjusted activity with 1 nM T4	Assay blank-adjusted activity with 1 nM T4	Assay blank-adjusted percentage ^{125}I-T3 produced from ^{125}I-T4	Tissue blank-adjusted activity with 1 nM T4	Assay blank-adjusted activity with 1 nM T4
Formula	(2x ^{125}I)/(total ^{125}I-T4)* 100	(^{125}I + ^{125}I-T3)/((^{125}I + ^{125}I-T3) + ^{125}I-T4)* 100	200* (^{125}I-T3)/((^{125}I-T3) + ^{125}I-T4))	Sample cpm-background cpm/total cpm/0.6* substrate (300 fmol)/min/mg)	(^{125}I + ^{125}I-T3)/((^{125}I + ^{125}I-T3) + ^{125}I-T4))* 100
Unit	^{125}I-fmol/min/per mg protein	T3 fmol/min/mg protein	^{125}I + T3 fmol/min/mg protein	^{125}I-fmol/min/mg protein	^{125}I + [^{125}I]-T3 fmol/min/mg/protein

PE buffer, 0.1 M sodium phosphate, 2 mM EDTA pH 7.2; PED10, 0.1 M sodium phosphate, 2 mM EDTA pH 7.2, 10 mM dithiothreitol (DTT); PED20 buffer, 0.1 M sodium phosphate, 2 mM EDTA pH 7.2, 20 mM DTT; PED50 buffer, 0.1 M sodium phosphate, 2 mM EDTA pH 7.2, 50 mM DTT; T/S 25 buffer, 20 mM Tris–base, 0.25 M sucrose, DTT 25 mM, pH 7.0–7.4; BAT, brown adipose tissue; vtx, vortexing; NHS, normal horse serum; SN, supernatant.

*In-house single-labeled tracer according to Wiersinga *et al.* (5). †The D2 assay in Amsterdam is based on the protocol previously described by Werneck-de-Castro *et al.* (6). ‡Single ^{125}I-labeled T3, T4 and rT3 were produced as described (9). §The assay is based on the protocols previously described (7, 8).

Table 4 Original methods used by the laboratories participating in the study and used for measuring samples in round 1.

D3	Amsterdam UMC	Erasmus MC	Maine	Budapest	Chicago[$]
Tissue	Cells (C + D) and liver 2.3	Cells (C + D) and liver 2.3	Cells (C + D) and liver 2.3	-	Cells (C + D)
Duplo	Yes	Yes	Yes	-	Yes
Protein concentration					
C	0.002 and 0.0002 mg/mL	0.000071 mg/mL	0.0027 mg/mL		0.24 mg/mL
D	0.01 and 0.001 mg/mL	0.00031 mg/mL	0.018 and 0.0018 mg/mL		1.58 mg/mL
Liver	6.45 mg/mL	1.53 mg/mL	3.95 mg/mL		
Buffer	PED20	PED20	T/S 25	-	PE
Substrate	1 nM T3	1 nM T3	2 nM T3	-	1 nM T3
Tracer	1 × 10^5 cpm [^{125}I]-T3 (NEX110X, Revvity)	2 × 10^5 cpm [^{125}I]-T3***	1–2 × 10^5 cpm [^{125}I]-T3 (NEX110X, Revvity)	-	2.5 × 10^5 cpm [^{125}I]-T4 (Revvity, USA)
Buffer tracer	PE	PED20	T/S 25	-	PE
Final volume (uL)	150	100	50	-	300
Assay blank (background)	PED20 + 1 nM T3 + 1 × 10^5 cpm [^{125}I]-T3	PED20 + 1 nM T3 + 1 × 10^5 cpm [^{125}I]-T3	T/S 25 + 2 nM T3 + 1–2 × 10^5 cpm [^{125}I]-T3	-	PED20 + 1 nM T3 + 2.5 × 10^5 cpm ^{125}I-T3
Tissue blank	Assay blank + 500 nM T3	Assay blank + 500 nM T3	Assay blank + 1 μM T3	-	Assay blank + 500 nM T3
Positive control	Mouse liver	None	Neonatal mouse liver	-	None
Incubation time (min)	120 at 37°C	120 at 37°C	10–60 at 37°C	-	300 at 37°C
Reaction stop (on ice)	150 μL ethanol, vtx, 5 min incubation, 2,370 ***g*** at 4°C for 20 min, 100 μL SN + 100 μL 0.02 M NH_4OAc (pH 4)	125 μL 0.1% HOAc in acetonitrile, vtx 5 s, 1 h on ice, 2,862 ***g*** at 4°C, 100 μL SN with 125 μL 0.02 M NH_4OAc (pH 4)	50 μL EtOH	-	300 μL MeOH, vtx 2 min, 13.800 ***g*** for 5 min, 400 μL SN + 200 μL buffer (0.02 M NH_4OAc, 4% MeOH, 4% PE)
Column	4.6 × 250 mm symmetry C18	Acquity Premier BEH C18 (130 Å, 11.7 μm, 2.1 × 100 mm)		-	Acquity Premier BEH C18 (130 Å, 11.7 μm, 2.1 × 100 mm)
HPLC system	Shimadzu HPLC system	Acquity Premier UPLC System (Waters)	Paper chromatography (3001-614, Whatman, GE Healthcare, Chicago)	-	Acquity UPLC System (Waters)
Detector	Radiomatic 150 TR Flow Scintillation Analyzer (Revvity, USA)	Ramona star detector (Elysia-Raytest, Belgium)	Autoradiographed chromatograms, bands counted in γ counter (Titertek)	-	Radiomatic 150 TR Flow Scintillation Analyzer (Revvity, USA)
Calculation	Tissue blank-adjusted activity with 1 nM T3	Tissue blank-adjusted activity with 1 nM T3	Assay blank-adjusted percentage ^{125}I-T2 produced from ^{125}I-T3	-	Tissue blank-adjusted activity with 1 nM T3
Formula	(^{125}I-T2)/(^{125}I-T2 + ^{125}I-T3)×100	(^{125}I-T2)/(^{125}I-T2 + ^{125}I-T3)×100	100* (^{125}I-T2)/((^{125}I-T2) + ^{125}I-T3))		(^{125}I-T2)/(^{125}I-T2 + ^{125}I-T3)×100
Unit	T2 fmol/min/mg protein	T2 fmol/min/mg protein	T2 fmol/min/mg protein	-	T2 fmol/min/mg protein

PE buffer, 0.1 M sodium phosphate, 2 mM EDTA pH 7.2; PED10, 0.1 M sodium phosphate, 2 mM EDTA pH 7.2, 10 mM dithiothreitol (DTT); PED20 buffer, 0.1 M sodium phosphate, 2 mM EDTA pH 7.2, 20 mM DTT; PED50 buffer, 0.1 M sodium phosphate, 2 mM EDTA pH 7.2, 50 mM DTT; T/S 25 buffer, 20 mM Tris–base, 0.25 M sucrose, DTT 25 mM, pH 7.0–7.4; vtx, vortexing; SN, supernatant. The Budapest laboratory did not measure D3 activity due to unavailability of the HPLC system.

[$]The assay is based on the protocols previously described (7, 8). ***Single ^{125}I-labeled T3, T4 and rT3 were produced as described (9).

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Table 5 Consensus protocol used for quality control round 2. It is recommended that the conversion should be below 30–50% and that the protein concentration of a sample should be adjusted accordingly. PTU is added to suppress expected D1 activity.

Deiodinase	Protein concentration in reaction	Stability additives	Substrate additives	Incubation time
D1	±0.10 mg/mL liver and kidney	10 mM DTT	100 nM rT3	30 min
D2	Pituitary homogenized in 300 µL buffer; ±2 mg/mL BAT	25 mM DTT, 0.25 M sucrose	1 nM T4, 0.5 mM PTU for pituitary	120 min
D3	±2.0 mg/mL liver; ±0.3 mg/mL high D3 liver; ±0.06 mg/mL placenta	10 mM DTT	1 nM T3; 0.5 mM PTU for liver	120 min (liver); 60 min (placenta)

Results

Type 1 deiodinase (Fig. 1)

D1 was measured in cells overexpressing D1 (round 1), mouse liver (rounds 1 and 2) and mouse kidney (round 2) by HPLC using T4 or rT3 as a tracer. In the first round of exchange, laboratories used their own protocol to measure the activity. D1 activity in the cells overexpressing D1 (cell line A) greatly varied between 18 and 275 pmol/min/mg with a median of 78 pmol/min/mg (Fig. 1A). The substantial variance cannot be explained by using T4 instead of rT3 as a tracer as the highest value was found in the laboratory using a T4 tracer.

Endogenous D1 activity was measured in liver and kidney in two separate rounds (Fig. 1B); in the second round, laboratories used a harmonized protocol in an attempt to decrease the variance. The median D1 activity in mouse liver (round 1) varied between 6.5 and 15.7 with a median of 7.5 pmol/min/mg. The variation cannot be explained by using T4 instead of rT3 as a tracer. D1 activity in mouse liver measured using a harmonized protocol (round 2) showed a median value around 26 pmol/min/mg with a maximal 1.5-fold deviation. The activity measured using T4 instead of rT3 was around threefold lower. D1 activity in mouse kidney homogenates was comparable across three different laboratories.

Type 2 deiodinase (Fig. 2)

D2 was measured in cells overexpressing D2 or both D2 and D3 (round 1), mouse pituitary (round 1), rat pituitary (round 2) and mouse BAT (round 2). Three different techniques were used to quantify the radioactive products: HPLC, paper chromatography or measurement of released radioactive iodine after protein precipitation. First, very high levels were obtained in D2-overexpressing cells in all laboratories within a similar range with a maximum 1.4-fold deviation from the median and with paper chromatography consistently measuring the highest activity.

Endogenous activity of D2 is often low and difficult to measure using HPLC as the detection limit of a HPLC system is around 1% conversion. This is supported by the fact that the two laboratories that measure D2 using HPLC were unable to measure a signal in the small amount of mouse pituitary tissue provided in round 1. In this round, only the other two methods were used for the pituitary D2 activity, resulting in a median of 1.6 fmol/min/mg and paper chromatography measuring 2.5-fold higher.

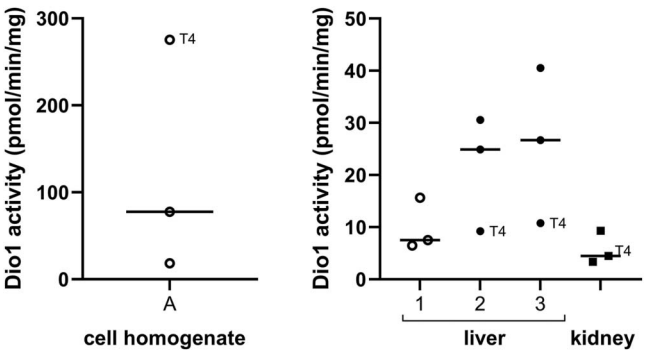

Figure 1

Measured deiodinase activity and median value for the recombinant cell line homogenate with D1 overexpression (cell line A, panel 1A), liver (samples 1–3) and kidney (panel 1B). D1 activity measured in round 1 is represented by open symbols and using the harmonized protocol (round 2) by solid symbols. T4, laboratory uses a T4 tracer instead of rT3.

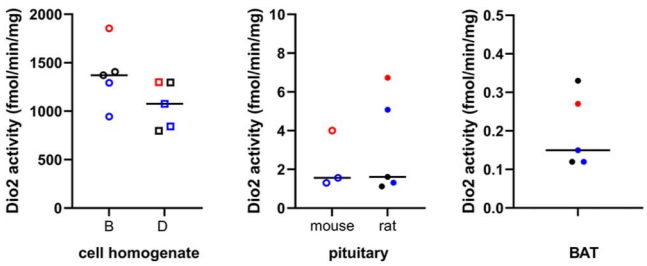

Figure 2

Measured deiodinase activity and median value for the recombinant cell line homogenates with D2 overexpression (B) and D2 + D3 overexpression (D), pituitary and BAT. D2 activity measured in round 1 is represented by open symbols and using the harmonized protocol (round 2) by solid symbols. Samples measured by HPLC are given in black, measured with paper chromatography in red and measured with protein precipitation in blue.

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All three methods were used in round 2 for the measurement of the larger rat pituitary. This resulted in a median of 1.6 with a range of 1.1–6.7 fmol/min/mg. Using paper chromatography resulted in the highest activity, while the measurements with HPLC were in a similar range. BAT D2 activity was low but in similar range with a median of 0.15 fmol/min/mg in all laboratories.

Type 3 deiodinase (Fig. 3)

D3 was measured in cells overexpressing D3 or both D2 and D3 (round 1), mouse liver (rounds 1 and 2) and human placenta pituitary (round 2). D3 activity was measured by HPLC or paper chromatography technique using T3 as a substrate. Very high D3 activity was measured in D3-overexpressing cells by all laboratories with a median of 5,256 fmol/min/mg and a high deviation of fourfold difference above and below the median. For the cells overexpressing both D2 and D3, the median was also high with 1,155 fmol/min/mg and an even larger maximum deviation of almost sevenfold lower than the median. Paper chromatography consistently measured the highest values; if this technique was excluded, the maximum deviation was threefold. In the same round, low endogenous D3 activity was measured in mouse liver sample 1 by HPLC or paper chromatography with a median of 0.25 fmol/min/mg and ranging between 0.13 and 0.33.

In round 2, using the harmonized protocol, endogenous activity was measured in mouse liver (samples 2 and 3) and human placenta. Activity levels in mouse liver were again in a similar range with on average 1.5-fold difference between the laboratories, and no obvious differences were seen between the used techniques. In contrast, D3 activity in placenta differed greatly between laboratories with a fivefold difference; the difference could not be explained by the technique as one of the HPLC labs measured the same low activity as detected by paper chromatography. Despite the overall differences in absolute activity levels, all laboratories that measured endogenous hepatic D3 activity in round 2 were able to pick up the anticipated higher activity in liver sample 3 versus liver sample 2. All results are also given in Supplemental Table 1 (see section on Supplementary materials given at the end of the article).

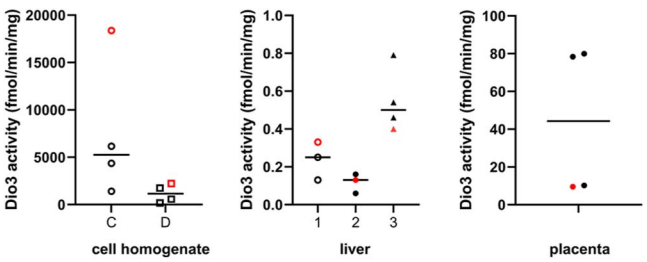

Figure 3

Measured deiodinase activity and median value for the recombinant cell line homogenates with D3 overexpression (C) and D2 + D3 overexpression (D), liver (samples 1–3) and placenta. D3 activity measured in round 1 is represented by open symbols and using the harmonized protocol (round 2) by solid symbols. Samples measured by HPLC are represented in black and measured with paper chromatography are represented in red.

Discussion

The present study utilized standardized samples in in-house and harmonized protocols with different detection methods to assess the activity of deiodinases across different research laboratories, ultimately aiming to improve the comparability of the existing assays. The study involved shipping samples prepared in the Endocrine Laboratory in Amsterdam UMC (one of the participating laboratories) to five leading laboratories that measure deiodinase activities, in a variety of tissue materials using different techniques. In round 1 of the exchange, laboratories were allowed to use their own protocol. This resulted in substantial differences in results for all analyzed materials and all three deiodinases. The recombinant cell models overexpressing D1, D2 and/or D3 (3) displayed the largest variation. This variation was likely due to the very high activity levels, which required multiple sample dilutions and resulted in samples with a very low protein concentration, as well as multiple rounds of thawing and refreezing to optimize assay conditions. The observed variation therefore does not necessarily reflect the actual differences between techniques and laboratories, but rather a suboptimal protocol for a sample with very high activities, which are rarely found in mammalian tissues. This is supported by the results from the liver and pituitary homogenates in the same round that showed a smaller variance in the activity levels. In an attempt to reduce the variance in activity, in round 2, a harmonized protocol (similar protein concentration in reaction, similar stability additives, similar substrate additives and similar incubation time) was employed along with a standardized tissue homogenate. However, using a harmonized protocol and the same tissue homogenate did not result in less variation in deiodinase activity levels between the participating laboratories. This indicates that protein concentrations, additives and incubation time were not responsible for the differences in results.

Possibly, differences in quantification techniques might underlie the differences in enzyme activity. The most comparable results were seen for the

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radioactive techniques using HPLC and/or protein precipitation. Although it is challenging to implement these techniques across all laboratories because of the lack of available, affordable and suitable radiolabeled substrates (especially rT3), employing similar techniques could have the potential to reduce intra-laboratory variability. The unavailability of commercial rT3 as a tracer forces laboratories to choose T4 as a substrate for D1, which has a lower affinity for D1 compared to rT3 (10). This is likely an important reason for some of the differences observed between the only laboratory using T4 and the other laboratories using rT3. An alternative solution would be in-house radiolabeling of the substrates. Other factors, such as equipment, reagents or sample handling, could play an important role, too. For better inter-laboratory comparison, the advice would be that all laboratories use the same technique, substrates and protocol. Even with the protocol given in the Basic Guideline of the American Thyroid Association (2), this is still difficult to achieve.

Despite the differences in results between participating investigators, it is reassuring that deiodinase activities measured in samples from animal tissues were within ranges consistent with the extensive literature in the past decades generated by multiple laboratories, many of them not participating in the present study. It was equally reassuring that all participating laboratories consistently measured very comparable fold changes in D3 activity in the liver samples of round 2, indicating lower inter-laboratory variability in the measurement of relative deiodinase activity values. As deiodinase assays involve three main steps, i.e., sample preparation, incubation with the substrate, followed by the separation and quantification of the relevant metabolites, it will be insightful to identify which of those steps accounts for the most variability in the results obtained. For example, different laboratories could be asked to quantify relevant metabolites and hormone conversion rate in a deiodinase reaction sample originated in a given laboratory. Still, other factors that are difficult to reproduce across laboratories may significantly influence deiodinase assay results, even if the assay reaction is theoretically performed in the same standardized conditions. These factors may include assay pH, total amount of protein assayed, inaccuracies in the specific activity of the radioactive tracer obtained from commercial providers and concentration of dithiothreitol to support enzyme activity.

An important approach toward more comparable results could be the use of tissue blanks in the assays. Not all laboratories include a tissue blank as a control where the measurable conversion is suppressed by saturating the enzyme with an excess non-radioactive substrate. This control provides information on background levels as well as possible not suppressed activity of other deiodinase enzymes. Another recommendation would be to include a positive control for each series. For D1 and D3, this can be made by homogenizing a suitable large tissue (e.g. liver for D1 and brain or placenta for D3), measuring the protein concentrations of the homogenate and storing sufficient aliquots at −80°C to avoid thawing/freezing cycles. For D2, a homogenate as an internal control sample is not possible, because the D2 enzyme is much more unstable and sensitive to thawing/freezing cycles. In that case, a series of tissues, such as rat pituitaries or mouse neonatal BAT, from the same experimental conditions can be used as a control. Analyzing this internal control sample will show if the conversion percentage is within an acceptable range, i.e. below 50%, and keeps track of the activity rates over time, which is useful for trend analysis. An upward or downward trend in measured activity in these controls over time could indicate problems with sensitivity, standards or equipment. The inclusion of these types of internal control samples is accepted as an alternative approach to external quality control as required for the ISO 15189 accreditation due to the lack of independent reference standards and external quality control programs.

It can be concluded that the exchange of quality control materials as performed in this study is most useful if the laboratories use the same technique, the same substrate and the same protocol optimized for the specific matrix and equipment. Without further standardization and optimization, it is important to note that absolute activity levels can only be compared within and between experiments from the same laboratory. However, in many experimental settings, the primary goal of the deiodinase assays is to detect relative differences between experimental groups. In this context, adjusting assay conditions, such as protein concentration or DTT levels, to optimize the sensitivity of the assay may sometimes be more necessary than strictly maintaining uniform conditions. Nevertheless, with proper analysis that accounts for matrix differences, careful calculation and the inclusion of sufficient controls as recommended here, the differences found between experimental groups – and therefore the conclusions of different studies – should be reproducible and comparable between laboratories and techniques. A limitation of the study is the limited number of samples, which significantly constrains the statistical robustness of the study. It is likely that an increase in the number of samples analyzed would have ameliorated the observed differences in activities across laboratories. Future studies aimed at identifying sources of variability and establishing universal assay protocols should prioritize larger cohorts and standardized sample preparation procedures and consistent reagent concentrations.

Main conclusions

(i) There are substantial inter-laboratory differences in the measurement of deiodinase activity, even among groups employing similar techniques. This observation underscores the sensitivity of these assays to subtle variations in methodology.
(ii) Absolute deiodinase activity cannot be necessarily compared across laboratories at present as they may be inherently tied to local assay conditions, equipment and methodology.
(iii) Despite variability in absolute deiodinase activity, the participating laboratories using the radioactive tracers and the HPLC technique for D1 and all three techniques for D3 detected the expected differences between samples. All three methods are suitable to measure overexpressed D2 activity, while chromatography or protein precipitation methods appear more sensitive than the HPLC approach to measure lower levels of D2 activity, as those in tissues.
(iv) The use of external quality control materials is only effective when laboratories use the same method with identical protocols. In this study, the harmonization of assay conditions did not reduce variability between laboratories.

Recommendations

(i) Reporting normalized activity values would facilitate a meaningful comparison between laboratories.
(ii) Regular external quality checks are essential to harmonize deiodinase assays across different laboratories.
(iii) Internal controls are essential for assessing assay reliability over time.
(iv) When inter-laboratory comparison is a research priority, D1 and D3 activities can be compared between laboratories using the same detection method (e.g. HPLC).
(v) Paper chromatography or protein precipitation methods are preferable to measure endogenous, lower levels, of D2 activity.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/ETJ-26-0041.

Declaration of interest

ACB is a consultant for AbbVie, Acella, Synthonics and Xeris. JW, PM, BG, AH, SL, MM, WEV, SR, FS-L and AB state that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

The shipment of materials was funded by the European Thyroid Association. The work carried out at The University of Chicago was funded in part by grant DK15070 and DK58538, and DK095908 from the National Institutes of Health (USA), and the work by the HUN-REN IEM Budapest was funded by RRF-2.3.1-21-2022-00011 of NRDIO Hungary.

Author contribution statement

JW performed the measurements and wrote the manuscript. PM, SL, AH and FS-L performed the measurements, commented on the manuscript draft and approved the final manuscript. BG, MM. WEV, SR and ACB commented on the manuscript draft and approved the final manuscript. AB supervised the project, reviewed and commented on the manuscript draft and approved the final manuscript.

Acknowledgments

We would like to thank Marja van Veen (Endocrine Laboratory, Department of Laboratory Medicine, Amsterdam UMC) and Andrea Juhász (Lab. of Molecular Cell Metabolism, HUN-REN Institute of Experimental Medicine, Budapest, Hungary) for technical assistance and Kostja Renko and Lutz Schomburg (Institut fur Experimentelle Endokrinologie, Charite – Universitatsmedizin Berlin, Germany) for providing us with the cell lines overexpressing D1, D2 and D3.

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