ARBOR ASSAYS™

DetectX® Thyroxine (T_4)

ELISA Kit

1 Plate Kit – Catalog No. K050-H1
5 Plate Kit – Catalog No. K050-H5

Species Independent

Sample Types Tested:
Serum, EDTA Plasma, Heparin Plasma, Tissue Culture Media, and Fecal Extracts

Please read this insert completely prior to using the product. For research use only.
Not for use in diagnostic procedures.
www.ArborAssays.com

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SUPPLIED COMPONENTS & STORAGE

		K050-H1	K050-H5	Description
Goat anti-Mouse Clear Coated 96-well Plate	Quantity	1	5	Strip well plates coated with goat anti-mouse IgG
	Catalog No.	X012-1EA	X012-1EA	
Thyroxine (T_4) Standard	Volume	40 µL	200 µL	Thyroxine at 1,000 ng/mL in stabilizing solution
	Catalog No.	C177-40UL	C177-200UL	
DetectX® Thyroxine (T_4) Antibody	Volume	3 mL	13 mL	Mouse monoclonal antibody specific for Thyroxine
	Catalog No.	C175-3ML	C175-13ML	
DetectX® Thyroxine (T_4) Conjugate	Volume	3 mL	13 mL	Thyroxine-peroxidase conjugate in stabilizing solution
	Catalog No.	C176-3ML	C176-13ML	
Assay Buffer Concentrate	Volume	28 mL	55 mL	5X concentrate that must be diluted
	Catalog No.	X065-28ML	X065-55ML	
Dissociation Reagent	Volume	1 mL	5 mL	ONLY to be used with Serum and Plasma samples
	Catalog No.	X058-1ML	X058-5ML	
Wash Buffer Concentrate	Volume	30 mL	125 mL	20X concentrate that must be diluted
	Catalog No.	X007-30ML	X007-125ML	
TMB Substrate	Volume	11 mL	55 mL	3,3',5,5'-Tetramethylbenzidine, a peroxidase substrate
	Catalog No.	X019-11ML	X019-55ML	
Stop Solution	Volume	5 mL	25 mL	1M solution of hydrochloric acid CAUSTIC
	Catalog No.	X020-5ML	X020-25ML	
Plate Sealer	Quantity	1	5	-
	Catalog No.	X002-1EA	X002-1EA	

Once opened, the kit can be stored at 4ºC up to the expiration date on the kit label.

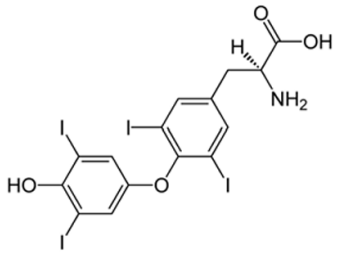
OTHER MATERIALS REQUIRED

- Distilled or deionized water
- Adjustable pipettes with disposable tips. Repeater pipette or multichannel pipettes with corresponding tips are also recommended.
- Glass or high-quality polypropylene test tubes for standard and sample preparation
- An orbital microplate shaker
- A plate reader capable of measuring absorbance at 450 nm
- Software for converting optical density (OD) readings from the plate reader and carrying out four parameter logistic curve (4PL) fitting. Contact your plate reader manufacturer for details.
- Optional: automated plate washer. Refer to Plate Washing Instructions for more details.
    - https://bit.ly/3tBT7N4

PRECAUTIONS

- Read this insert completely prior to using the product.
- This kit may not perform as described if any reagent or procedure is replaced or modified. Do not interchange reagents from different kit lots.
- Take appropriate safety precautions, such as: avoid breathing fumes, wear personal protective equipment (gloves, clothing, eye and face protection), and familiarize yourself with SDS documents.
    - https://www.ArborAssays.com/documentation/msds/K050-H_MSDS.pdf
- Ensure all buffers used for samples are azide free and that any plate washing system is rinsed well with deionized water prior to using the supplied Wash Buffer. Buffers, including other manufacturers' wash buffers, that contain sodium azide will inhibit color production from the peroxidase.
- **Take appropriate precautions when handling the Stop Solution, which is a caustic acid.**

BACKGROUND



Thyroxine is the main hormone produced by the thyroid gland. The thyroid hormones, triiodothyronine (T_3) and thyroxine (T_4), are tyrosine-based hormones produced by the thyroid gland that are primarily responsible for regulation of metabolism.

Iodine is necessary for the production of T_3 and T_4.[1] Iodine deficiency leads to decreased production of T_3 and T_4, enlarged thyroid tissue, and causes the disease known as goiter. The major form of thyroid hormone in the blood is thyroxine (T_4), which has a longer half-life than T_3.[2] The ratio of T_4 to T_3 secreted from the thyroid gland is roughly 14 to 1.[3] T_4 is converted to the active T_3 (three to four times more potent than T_4) within cells by deiodinases (5'-iodinase).[4] These are further processed by decarboxylation and deiodination to produce iodothyronamine (T_{1a}) and thyronamine (T_{0a}).[5] All three isoforms of the deiodinases are selenium-containing enzymes, thus selenium is essential for T_3 production.

ASSAY PRINCIPLE

The DetectX® Thyroxine (T_4) ELISA kit is designed to quantitatively measure T_4 present in serum, plasma, extracted dried fecal samples, and tissue culture media samples. Please read the complete kit insert before performing this assay.

A T_4 stock solution is provided to generate standard curves for the assay and all samples should be read off the standard curve. Standards or diluted samples are pipetted into a clear microtiter plate coated with an antibody to capture mouse antibodies. A T_4-peroxidase conjugate is added to the standards and samples in the wells. The binding reaction is initiated by the addition of a monoclonal antibody to T_4 to each well. After an hour incubation the plate is washed, and substrate is added. The substrate reacts with the bound T_4-peroxidase conjugate. After a short incubation, the reaction is stopped, and the intensity of the generated color is detected in a microtiter plate reader capable of measuring 450 nm wavelength. The concentration of the T_4 in the sample is calculated, after making suitable correction for the dilution of the sample, using software available with most plate readers.

REAGENT PREPARATION

Except for the reagents listed below, all kit components are ready for use.

Reagent	Preparation	Stability
1X Assay Buffer	Warm 5X Assay Buffer Concentrate to room temperature and mix thoroughly by inversion. Mix 1 volume 5X Assay Buffer Concentrate with 4 volumes deionized water.	1X Assay Buffer is stable for 3 months at 4°C
1X Wash Buffer	Warm 20X Wash Buffer Concentrate to room temperature and mix thoroughly by inversion. Mix 1 volume 20X Wash Buffer Concentrate with 19 volumes deionized water.	1X Wash Buffer is stable for 3 months at room temperature

SAMPLE PREPARATION

For samples containing particulates, centrifuge prior to use. Upon collection, all samples should be frozen rapidly and stored at -80°C until testing.

Sample Type	Procedure

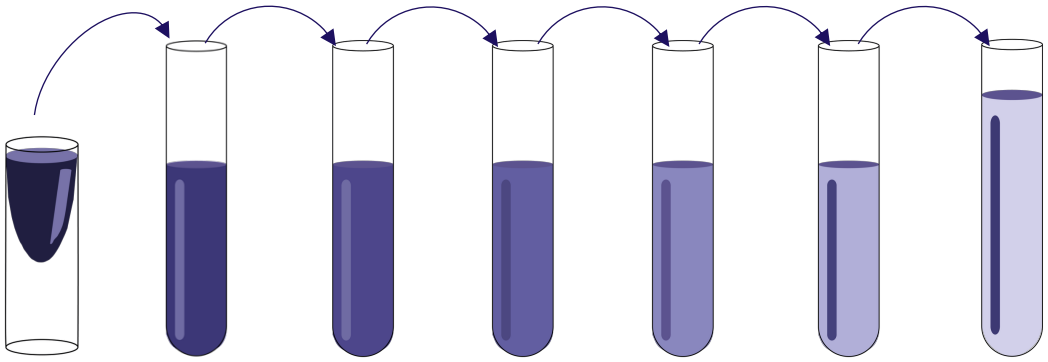
Serum and Plasma	• Serum and Plasma samples must be treated with Dissociation Reagent.
	  ○ Addition of this reagent yields the total thyroxine concentration in the sample.
	  ○ ONLY use Dissociation Reagent with serum and plasma samples.
	  ○ Allow the Dissociation Reagent to warm completely to room temperature before use.
	• In a microcentrifuge tube:
	  ○ Add 5 µL Dissociation Reagent
	  ○ Add 5 µL of Serum or Plasma
	  ○ Vortex gently to mix
	  ○ Incubate at room temperature for 5 minutes
	  ○ Add 90 µL 1X Assay Buffer to yield a 1:20 dilution
	  ▪ A minimum 1:20 dilution of sample is necessary to eliminate sample matrix interference.
	• Samples may require further dilution with 1X Assay Buffer to fall within the standard curve range.
	• **Mouse serum samples need to be diluted ≥ 1:30.**
	• **Mouse plasma samples are not recommended due to over-recovery.**

Dried Fecal Samples	• A detailed Extraction Protocol available on our website at: www.arborassays.com/resources/#protocols. • The ethanol concentration in the final sample added to the well should be ≤ 5%.
Tissue Culture Media (TCM)	• This assay has been validated using RPMI-1640. Other types of TCM should be validated before use. • Samples should be diluted in TCM and read off a standard curve generated in the same TCM. • Samples may require further dilution with TCM or 1X Assay Buffer to fall within the standard curve range.

⚠ **Use all samples within 2 hours of dilution.**

STANDARD PREPARATION

1. Label tubes Standard 1 through Standard 6.
2. Add 490 µL 1X Assay Buffer to Standard 1 tube.
3. Add 100 µL 1X Assay Buffer to Standard 2 – 6 tubes.
4. Add 10 µL of the Thyroxine (T_4) Standard stock solution to Standard 1 tube. Vortex thoroughly.
   ⚠ **The Thyroxine (T_4) Standard stock solution contains an organic solvent. Prerinse the pipet tip several times to ensure accurate delivery.**
5. Transfer 100 µL of Standard 1 into Standard 2 tube to make a 2-fold dilution. Vortex thoroughly.
6. Transfer 100 µL of the mixed solution from Standard 2 into Standard 3 tube to make a 2-fold dilution. Vortex thoroughly.
7. Continue serially diluting into the remaining tubes. This process and the final concentrations are summarized in the table below.



	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5	Standard 6
1X Assay Buffer (µL)	490	100	100	100	100	100
Addition	Stock	Std 1	Std 2	Std 3	Std 4	Std 5
Volume of Addition (µL)	10	100	100	100	100	100
Final Concentration (ng/mL)	20	10	5	2.5	1.25	0.625

⚠ **Use all Standards within 2 hours of dilution.**

ASSAY PROTOCOL

Before You Begin:

- **Room Temperature for this assay is defined as 22ºC – 24ºC.**
- **Ensure all reagents have been warmed to room temperature.**
- **Dilute Samples as described in Sample Preparation.**
- **Run all Standards and Samples in duplicate.**
- Use the blank plate template on the back page of this booklet to design your plate layout and aid in proper sample and standard identification.
- Be sure to shake the plate as directed. Failing to shake the plate or altering the shaking speed during incubations will result in decreased signal.
- Set plate parameters on the plate reader for a 96-well Corning Costar 2592 plate. See ArborAssays.com for plate dimension data.
- Determine the number of well strips to be used and return unused well strips to foil pouch with desiccant. Seal the foil pouch and store at 4°C. Desiccant color will change from blue to pink if the foil pouch is not properly sealed.
- If you are using only part of a strip well plate, at the end of the assay discard the used wells and retain the plate frame for use with the remaining unused wells.

1. Add 10 µL Samples or Standards into duplicate wells.
2. Add 35 µL 1X Assay Buffer into duplicate NSB (non-specific binding) wells.
3. Add 10 µL 1X Assay Buffer into Zero Standard (maximum binding or B0) wells.
4. Add 25 µL DetectX® Thyroxine (T_4) Conjugate to each well.
5. Add 25 µL DetectX® Thyroxine (T_4) Antibody to each well, **except the NSB wells**.
6. Cover the plate with a plate sealer and shake at room temperature at 700-900 rpm for **1 hour.**
7. Remove the plate sealer, aspirate the plate, and wash each well 4 times with 300 µL 1X Wash Buffer. Tap the plate dry on clean absorbent towels.
8. Add 100 µL TMB Substrate to each well.
    - The substrate solution will begin to turn blue.
9. Incubate at room temperature for **30 minutes** without shaking.
10. Add 50 µL Stop Solution to each well.
    - The substrate solution will begin to turn yellow.
11. Read the optical density at 450 nm within 10 minutes.

CALCULATION OF RESULTS

Follow the instructions below, or use this online tool: https://myassays.com/assay.aspx?id=1304

1. Use four-parameter logistic curve (4PL) software to calculate the T_4 concentration for each sample. Gather all raw data OD readings from each Sample and Standard, including the Zero (B0) Standard and NSB.
2. Average the duplicate OD readings for each Sample, Standard, B0, and NSB (Mean OD).

EXAMPLE:

Sample	Replicate 1 OD	Replicate 2 OD	Mean OD
NSB	0.064	0.044	0.054
B0	1.562	1.572	1.567
Sample 1	0.438	0.498	0.468

3. Subtract the NSB from the Mean OD for each Sample, Standard, and the B0 (Net OD).

EXAMPLE:

Sample	Mean OD	NSB Mean OD	Net OD
B0	1.567	0.054	1.513
Sample 1	0.468	0.054	0.414

4. Divide the Net OD for each Sample and Standard by the Net OD for the B0 and multiply by 100% (%B/B0).

EXAMPLE:

Sample	Net OD	B0 Net OD	%B/B0
Sample 1	0.414	1.513	27.4

5. Plot the standard curve with %B/B0 for the Standards on the y-axis and T_4 concentration (ng/mL) on the x-axis. Perform a 4PL fit.

   Use the sample %B/B0 readings and the 4PL fit to calculate T_4 concentrations in diluted samples. If diluted sample concentrations are outside of the range of the standards, the sample should be prepared again at a more appropriate dilution.

EXAMPLE:

Sample	Net OD	%B/B0	Sample T_4 Concentration (ng/mL)
Sample 1	0.414	27.4	6.67

6. If the original sample was diluted, multiply the sample T_4 concentration by the sample dilution factor to determine the concentration of T_4 in the original sample.

EXAMPLE:

Sample	Sample T_4 Concentration (ng/mL)	Sample Dilution Factor	Original Sample T_4 Concentration (ng/mL)
Sample 1	6.67	20	133.4

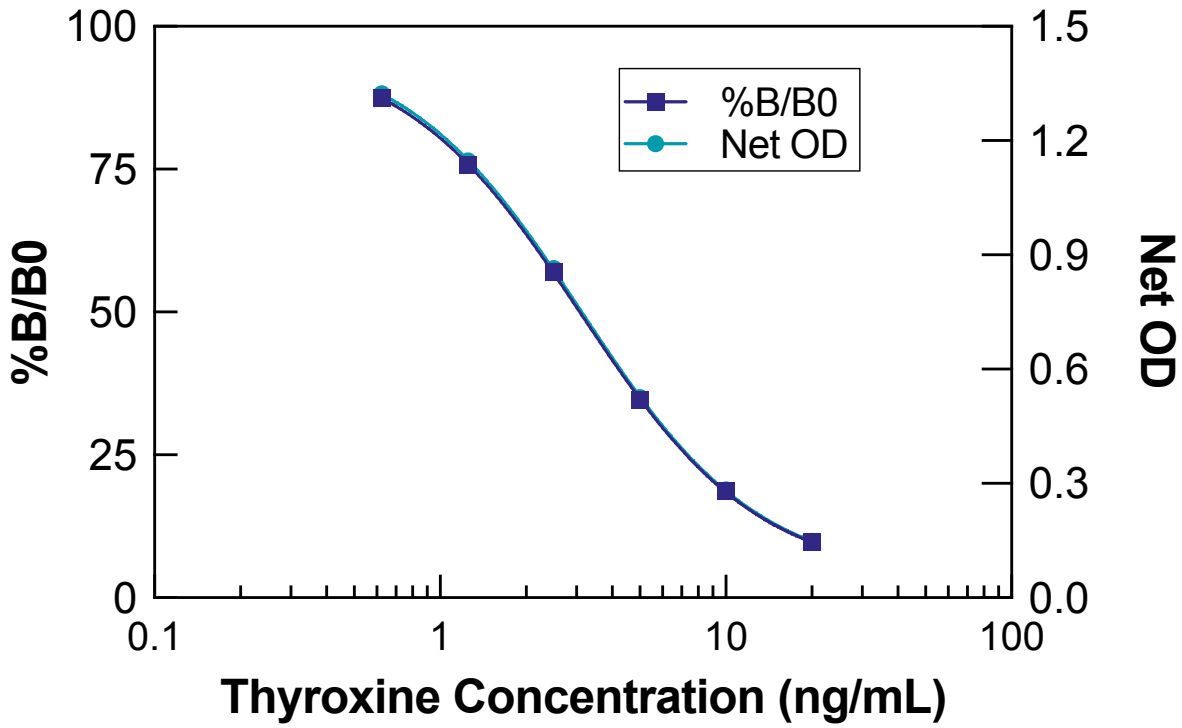
TYPICAL DATA

⚠ **Always run your own standard curve. This data should NOT be used to interpret results.**

Sample	Mean OD	Net OD	%B/B0	Thyroxine Concentration (ng/mL)
NSB	0.054	-	-	-
Standard 1	0.202	0.148	9.7	20
Standard 2	0.337	0.283	18.6	10
Standard 3	0.580	0.526	34.6	5
Standard 4	0.918	0.864	57.0	2.5
Standard 5	1.201	1.147	75.7	1.25
Standard 6	1.377	1.323	87.5	0.63
B0	1.567	1.513	100.0	0
Sample 1	0.468	0.414	27.4	6.67
Sample 2	0.965	0.911	60.2	2.27

Conversion factor: 77.7 ng/mL of Thyroxine is equivalent to 100 nM.

Typical Standard Curve



VALIDATION DATA

Sensitivity and Limit of Detection

Sensitivity was calculated by comparing the ODs for twenty wells run for each of the B0 and Standard 6. The detection limit was determined at two standard deviations from the B0 along the standard curve.

Sensitivity was determined as 0.33 ng/mL

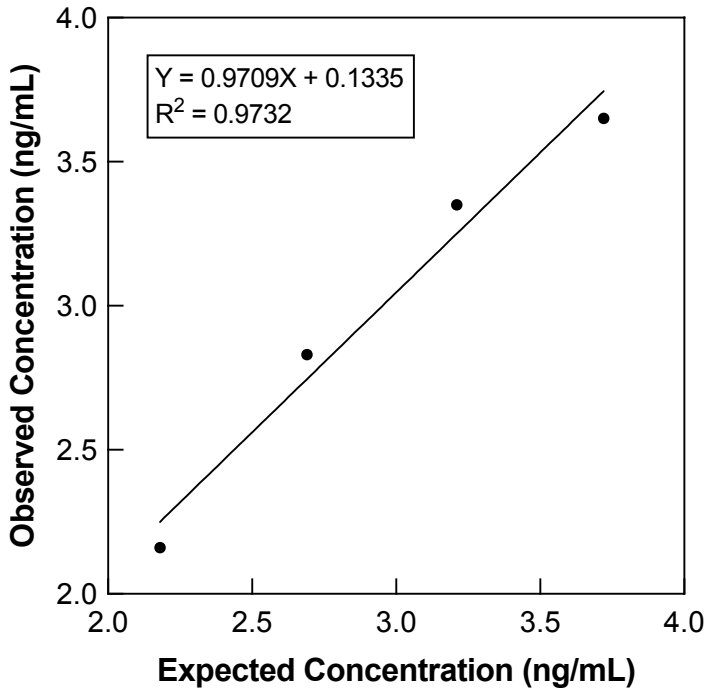
The Limit of Detection was determined in a similar manner by comparing the ODs for twenty wells run for each of the B0 and a low concentration human sample.

The Limit of Detection was determined as 0.30 ng/mL

Linearity

Linearity was determined in human plasma by diluting two samples with known thyroxine concentration with 1X Assay buffer. One sample had a T_4 concentration of 1.66 ng/mL (Low Sample); a second sample had a T_4 concentration of 4.24 ng/mL (High Sample). The two samples were mixed in the ratios given below and the measured concentrations were compared to the expected values for each given ratio.

Low Sample	High Sample	Expected Concentration (ng/mL)	Observed Concentration (ng/mL)	% Recovery
80%	20%	2.18	2.16	99.3
60%	40%	2.69	2.83	105.1
40%	60%	3.21	3.35	104.4
20%	80%	3.72	3.65	98.0
			Mean Recovery	**101.7%**



Intra Assay and Inter Assay Precision

For intra assay precision, two human serum samples and one human plasma sample were diluted in 1X Assay Buffer, and 44 replicates were run in one assay. For inter assay precision, two human serum samples and one human plasma sample were diluted in 1X Assay Buffer and duplicates of each sample were run in 27 assays over multiple days by 5 operators. %CV represents the variation in concentration (not optical density) as determined using a standard curve.

	Intra Assay Precision		Inter Assay Precision	
Sample	Thyroxine Concentration (ng/mL)	%CV	Thyroxine Concentration (ng/mL)	%CV
1 (serum)	6.6	5.0	6.7	11.2
2 (serum)	2.4	8.0	2.3	18.3
3 (plasma)	1.3	11.0	1.2	20.8

SAMPLE VALUES

5 human plasma samples were diluted in 1X Assay Buffer and tested in the assay. 5 human serum samples were diluted in 1X Assay Buffer and tested in the assay. 8 fecal samples from a variety of mammalian species were extracted, diluted in 1X Assay Buffer, and tested in the assay. The adjusted average concentration and sample range are shown below.

The normal reference range for serum thyroxine (total T_4) is 50-125 ng/mL (Mayo Medical Labs).

Sample Type	Recommended Minimum Dilution	Average Adjusted Concentration (ng/mL)	Adjusted Concentration Range (ng/mL)
Serum	1:20	62.1	40.6 – 71.0
Plasma	1:20	47.9	37.1 – 61.6
Dried Feces	Extracted	150.9	22.6 – 361.8

INTERFERENCE

Potentially interfering substances were evaluated in the assay and change in signal was calculated.

Interferent	Effect at High Thyroxine Concentration		Effect at Low Thyroxine Concentration	
	% Added	Effect	% Added	Effect
DMSO	1.25%	7.6% increase	2.5%	9.4% increase
Ethanol	5%	9.2% decrease	5%	10.7% increase
Methanol	2.5%	9.0% decrease	2.5%	6.0% increase
DMF	10%	8.8% increase	2.5%	2.2% decrease
Hemoglobin	0.4 mg/dL	11.6% decrease	0.4 mg/dL	3.4% decrease
Bilirubin	0.5 mg/dL	7.6% decrease	0.05 mg/dL	5.3% decrease
Lipemia	1.0 mg/dL	3.1% decrease	1.0 mg/dL	8.9% decrease

CROSS REACTIVITY

The following cross reactants were tested in the assay and calculated at the 50% binding point.

Steroid	Cross Reactivity (%)
Thyroxine	100%
Reverse T3 (3,3',5'-Triiodo-L-thyronine)	89.0%
T_3 (3,3',5-Triiodo-L-thyronine)	5.2%

TROUBLESHOOTING

Issue	Possible Cause & Solution
Reagent Shortage	• Check under the cap for additional reagent. Pulse spin reagent containers to collect contents prior to opening when possible. • When using a multichannel pipette, return unused reagent to container for later use.
Erratic Values	• Ensure the assay plate has been properly blotted after assay washes to remove residual wash buffer. • Prerinse pipet tips with desired reagent prior to aspirating the required volume. • Deliver volume with care to prevent splashing into adjacent wells. • Ensure all samples have been run at or below the minimum dilution to eliminate sample matrix interference.
High Background	• Ensure assay plate has been properly washed with the number of washes indicated in the protocol. • Reagent contamination during assay setup. • Verify antibody was not added to the NSB wells.
Low Signal	• Confirm tools, equipment, reagents, and containers used do not contain any trace of sodium azide. • Altering shaking speeds or excluding shaking during incubation steps. • Verify the plate reader wavelength is 450 nm. • Confirm reagents were at room temperature prior to use.

CITATIONS

1. Dilas, L. T., Bajkin, I., Icin, T., Paro, J. N., & Zavisić, B. K. (2012). *Medicinski pregled*, *65*(11-12), 489–495.
2. Zutinic, A., Blauw, G. J., Pijl, H., Ballieux, B. E., Westendorp, R. G. J., Roelfsema, F., & van Heemst, D. (2020). Circulating Thyroid Hormone Profile in Response to a Triiodothyronine Challenge in Familial Longevity. *Journal of the Endocrine Society*, *4*(10), bvaa117.
3. Gomes-Lima, C., Wartofsky, L., & Burman, K. (2019). Can reverse T3 assay be employed to guide T4 vs. T4/T3 therapy in hypothyroidism? *Frontiers in Endocrinology*, *10*.
4. Idrose AM. Acute and emergency care for thyrotoxicosis and thyroid storm. *Acute Med Surg*. 2015 May 12;2(3):147-157.
5. Bellusci, L., Laurino, A., Sabatini, M., Sestito, S., Lenzi, P., Raimondi, L., Rapposelli, S., Biagioni, F., Fornai, F., Salvetti, A., Rossi, L., Zucchi, R., & Chiellini, G. (2017). New insights into the potential roles of 3-iodothyronamine (T1AM) and newly developed thyronamine-like TAAR1 agonists in neuroprotection. *Frontiers in Pharmacology*, *8*.

RELATED PRODUCTS

Kits	Catalog No.
Urinary Creatinine Detection Kits	K002-H1/H5
Cortisol Enzyme Immunoassay Kits	K003-H1/H5
Glucose Colorimetric Detection Kit	K039-H1
Glucose Fluorescent Detection Kit	K039-F1
Triiodothyronine (T3) Enzyme Immunoassay Kits	K056-H1/H5

LIMITED WARRANTY

Arbor Assays warrants that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.

We must be notified of any breach of this warranty within 48 hours of receipt of the product. No claim shall be honored if we are not notified within this time period, or if the product has been stored in any way other than outlined in this publication. The sole and exclusive remedy of the customer for any liability based upon this warranty is limited to the replacement of the product, or refund of the invoice price of the goods.

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CONTACT INFORMATION

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OFFICIAL SUPPLIER TO ISWE

Arbor Assays and the International Society of Wildlife Endocrinology (ISWE) signed an exclusive agreement for Arbor Assays to supply ISWE members with assay kits and reagents for wildlife conservation research.

PLATE LAYOUT