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# Free Triiodothyronine (fT3) Assay Development Report

**Theranos, Inc.**

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# 1 ASSAY INFORMATION[ TC "ASSAY INFORMATION" \F C \L "2" ]

## 1.1 Assay Specifications[ TC "Assay Specifications" \f C \l "3" ]

This assay is designed to detect free Triiodothyronine (T3) in human whole blood (automatically processed into plasma by the Theranos system), plasma and serum. The assay has a reportable normal range of 2 pg/ml to 50 pg/ml.

### 1.1.1 Reference Assays [ TC "Reference Assays and Standards" \f C \l "3" ]

The following commercial ELISA kit has been used in house for comparison:
-US Biological (Cat #T8425-06)

### 1.1.2 Materials and methods

[ TC "Materials and Methods" \f C \l "1" ]

In this competitive assay format, the fT3 in the sample competes with T3-AP for binding to the anti-T3 antibody. Briefly, an anti-rabbit antibody serves as the capture surface for the competitive ELISA. Alkaline Phosphatase-labeled triiodothyronine (T3-AP) serves as the tracer. The mixture of sample, Rabbit Anti-T3 Antibody and T3-AP is incubated with the capture surface for 5 minutes followed by six wash steps. Then the alkaline phosphatase substrate is incubated with the capture surface for 5 minutes. The resulting chemiluminescence is read in Relative Light Units (RLU).

The key materials that were used for this assay are listed in Table 1.

**Table [ SEQ Table * ARABIC ]: Materials**

| Name | Supplier | Catalog # |
|---|---|---|
| Triiodothyronine | Sigma | T2877 |
| Goat Anti-Rabbit IgG (Fc) | Pierce | 31216 |
| Triiodothyronine Alkaline Phosphatase Conjugate | Fitzgerald | 65-IT25 |
| Backup product: Triiodothyronine AP conjugate | USBiological | T8425-15 |
| Phospho Glo Substrate | KPL | 55-60-04 |
| Low BSA Blocking Buffer (0.03% BSA (Fraction V, 99% Pure) in TBS, 0.05% Sodium Azide) | Sigma | A3059-500G |
| Carbonate-bicarbonate buffer | Sigma | C3041 |
| Thyroid Hormone Depleted Serum (Calibrator Matrix) | Sunny Lab | SF509-2 |

**Table [ SEQ Table * ARABIC ]: List of Antibodies-Set 1**

| Antibody# | Vendor | Catalog # | Clone # |
|---|---|---|---|
| 1 | US Biological | T8425-01 | 1.B.174 |
| 2 | US Biological | T8425-01B | 3H285 |
| 3 | US Biological | T8425-01C | 3H286 |
| 4 | US Biological | T8425-10A | 9F323 |
| 5 | US Biological | T8425-01G | 9L742 |
| 6 | US Biological | T8425-07D | 10B2497 |
| 7 | US Biological | T8425-07E | 10B2498 |
| 8 | US Biological | T8425-13A | 10g222 |
| 9 | US Biological | T8425-02 | 1.B.175 |
| 10 | My Biosource | MBS310605 | ME.124 |
| 11 | My Biosource | MBS310706 | 3A6 |
| 12 | My Biosource | MBS311698 | 027-10237 |
| 13 | My Biosource | MBS311697 | 291-13121 |
| 14 | Leinco technologies | T105 | 170 |
| 15 | Roche | 10907332103 | N/A |

**Table [ SEQ Table * ARABIC ]: List of Antibodies-Set 2**

| Antibody# | Vendor | Catalog # | Clone# |
|---|---|---|---|
| 16 | US biological | T8425-01E | 4A142 |
| 17 | US biological | T8425-10 | Rabbit Pab |
| 18 | US biological | T8425-12 | Sheep Pab |
| 19 | US biological | T8425-11 | Goat Pab |
| 20 | US biological | T8425-07A | Sheep Pab |
| 21 | Calbioreagents | P139 | Rabbit Pab |
| 22 | Biospacific | A23010067P | MAb |
| 23 | Biospacific | A23070131P | MAb |
| 24 | Biospacific | A23020067P | MAb |
| 25 | Biospacific | D19020131G | Goat PAb |
| 26 | Diasource | 53.163.06 | MO11 |
| 27 | Acris | AM09291PU-N | S-191 |
| 28 | Acris | AM09292PU-N | 218 |
| 29 | Acris | AM05502PU-N | 3T50 (BGN/09/1250) |
| 30 | Immunodiagnostik | A1036.1 | Rabbit PAb |

# 2 ASSAY DEVELOPMENT[ TC "ASSAY OPTIMIZATION" \F C \L "2" ]

## 2.1 Antibody Screening on MTP- Set 1

The initial screen of fT3 antibodies was performed during the Total T3 assay development.

These were performed on Nunc 384 microtiter (MTP) plates. Antibodies were screened to determine ability to bind to T3-AP and to observe any modulation in the presence of the T3 analyte (Table 2, Table 3). MTP plates were directly coated with antibodies in carbonate-bicarbonate buffer at the following concentrations (10 ug/ml, 1 ug/ml and 0.1 ug/ml). A mixture containing T3-AP (1:10K dilution from stock) and T3 analyte (0 ng/mL or 5 ng/mL) in low BSA buffer was added to these wells. This mixture was incubated for 10 minutes followed by three wash steps. Alkaline phosphatase substrate was subsequently added for 10 minutes and the resulting chemiluminescence was read in Relative Light Units (RLU). Most of the antibodies screened showed strong ability to bind T3-AP. Low dose modulation was observed in presence of 5 ng/mL of T3 (comparing RLU data with no T3 analyte added). Modulation to unbound or Free T3 is determined. Antibody #2 and #3 were eliminated for further testing on the Theranos system due to lack of binding to the T3-AP[ TC "Detection Antibody Conjugate Verification" \f C \l "1" ].

**Table [ SEQ Table * ARABIC ]: Antibody Screen (MTP) Set 1**

| Antibody ID | [Ab] ug/mL | Mean RLU 0 ng/mL T3 | Mean RLU 5 ng/mL T3 | Modulation to Free T3 |
|---|---|---|---|---|
| 1 | 10.0 | 36656 | 15427 | 2.4 |
| | 1.0 | 2675 | 1599 | 1.7 |
| | 0.1 | 777 | 280 | 2.8 |
| 2 | 10.0 | 119 | 83 | 1.4 |
| | 1.0 | 83 | 79 | 1.1 |
| | 0.1 | 81 | 79 | 1.0 |
| 3 | 10.0 | 95 | 110 | 0.9 |
| | 1.0 | 87 | 126 | 0.7 |
| | 0.1 | 105 | 81 | 1.3 |
| 4 | 10.0 | 219371 | 185611 | 1.2 |
| | 1.0 | 14153 | 3362 | 4.2 |
| | 0.1 | 1005 | 260 | 3.9 |
| 5 | 10.0 | 253030 | 155303 | 1.6 |
| | 1.0 | 38514 | 11741 | 3.3 |
| | 0.1 | 2948 | 803 | 3.7 |

| Antibody ID | [Ab] ug/mL | Mean RLU 0 ng/mL T3 | Mean RLU 5 ng/mL T3 | Modulation to Free T3 |
|---|---|---|---|---|
| **6** | 10.0 | 3168 | 1035 | 3.1 |
| | 1.0 | 186 | 148 | 1.3 |
| | 0.1 | 91 | 103 | 0.9 |
| 7 | 10.0 | 141215 | 67161 | 2.1 |
| | 1.0 | 12554 | 2832 | 4.4 |
| | 0.1 | 1532 | 325 | 4.7 |
| **8** | 10.0 | 131481 | 79473 | 1.7 |
| | 1.0 | 11228 | 4565 | 2.5 |
| | 0.1 | 1351 | 471 | 2.9 |
| **9** | 10.0 | 186169 | 149284 | 1.2 |
| | 1.0 | 5885 | 1952 | 3.0 |
| | 0.1 | 281 | 201 | 1.4 |
| **10** | 10.0 | 61090 | 34185 | 1.8 |
| | 1.0 | 5130 | 2203 | 2.3 |
| | 0.1 | 539 | 296 | 1.8 |
| **11** | 10.0 | 203908 | 202068 | 1.0 |
| | 1.0 | 18990 | 6500 | 2.9 |
| | 0.1 | 1300 | 440 | 3.0 |
| **12** | 10.0 | 162099 | 112591 | 1.4 |
| | 1.0 | 16988 | 6541 | 2.6 |
| | 0.1 | 1074 | 349 | 3.1 |
| **13** | 10.0 | 148146 | 82210 | 1.8 |
| | 1.0 | 14946 | 4118 | 3.6 |
| | 0.1 | 495 | 225 | 2.2 |

**Antibody Screen (MTP) Set 1 (continued)**

## 2.2 Competitive Assay Screen on the Theranos System

To determine the optimal capture antibody for T3 on the Theranos 3.0 system, screening was performed. Final total T3 conditions after protocol included a 10 fold sample dilution of the serum calibrators in Low BSA assay buffer (0.03% BSA in TBS). A mixture containing 1 ug/ml antibody and 50 ug/ml of 8-ANS was added to the diluted sample. A dilution of 1:100K (from stock) of T3-AP was loaded in the cartridge. Out of all the 15 antibodies screened Antibody #1 was selected as the best antibody based on modulation and was used for further optimization.

**Table [ SEQ Table * ARABIC ]: Competitive Assay Screen on the Theranos System**

| Antibody # | [TT3], ng/mL | Mean RLU | CV% | Modulation |
|---|---|---|---|---|
| 1 | 10.0 | 35000 | 2.6 | 3.3 |
| | 2.5 | 72821 | 8.1 | |
| | 0.5 | 72626 | 10.7 | |
| | 0 | 114398 | 19.0 | |
| 2 | 10.0 | 84204 | 7.8 | 1.1 |
| | 2.5 | 90956 | 9.4 | |
| | 0.5 | 93975 | 2.9 | |
| | 0 | 93979 | 2.8 | |
| 3 | 10.0 | 85276 | 7.5 | 1.0 |
| | 2.5 | 93606 | 4.5 | |
| | 0.5 | 81789 | 13.3 | |
| | 0 | 88121 | 10.8 | |
| 4 | 10.0 | 2166 | 9.6 | 1.0 |
| | 2.5 | 2255 | 6.4 | |
| | 0.5 | 2418 | 2.6 | |
| | 0 | 2219 | 6.1 | |
| 5 | 10.0 | 62072 | 8.0 | 1.3 |
| | 2.5 | 65980 | 14.9 | |
| | 0.5 | 72085 | 11.3 | |
| | 0 | 79911 | 3.6 | |
| 6 | 10.0 | 102731 | 9.4 | 0.8 |
| | 2.5 | 92715 | 4.8 | |
| | 0.5 | 96665 | 3.5 | |
| | 0 | 84362 | 12.9 | |

**Competitive Assay Screen on the Theranos System (continued)**

| Antibody # | [TT3], ng/mL | Mean RLU | CV% | Modulation |
|---|---|---|---|---|
| 7 | 10.0 | 101560 | 7.0 | 1.0 |
| | 2.5 | 98667 | 5.0 | |
| | 0.5 | 101429 | 4.5 | |
| | 0 | 100639 | 3.9 | |
| 8 | 10.0 | 80644 | 9.0 | 1.4 |
| | 2.5 | 109676 | 9.2 | |
| | 0.5 | 124547 | 4.3 | |
| | 0 | 111519 | 9.7 | |
| 9 | 10.0 | 82229 | 12.2 | 1.2 |
| | 2.5 | 87611 | 5.5 | |
| | 0.5 | 95167 | 2.4 | |
| | 0 | 95916 | 7.9 | |
| 10 | 10.0 | 70493 | 2.4 | 1.0 |
| | 2.5 | 72241 | 11.3 | |
| | 0.5 | 81087 | 4.0 | |
| | 0 | 69038 | 4.4 | |
| 11 | 10.0 | 82229 | 12.2 | 1.2 |
| | 2.5 | 87611 | 5.5 | |
| | 0.5 | 95167 | 2.4 | |
| | 0 | 95916 | 7.9 | |
| 12 | 10.0 | 70493 | 2.4 | 1.0 |
| | 2.5 | 72241 | 11.3 | |
| | 0.5 | 81087 | 4.0 | |
| | 0 | 69038 | 4.4 | |
| 13 | 10.0 | 108617 | 2.4 | 0.9 |
| | 2.5 | 100646 | 15.0 | |
| | 0.5 | 83378 | 36.5 | |
| | 0 | 92694 | 4.8 | |
| 14 | 10.0 | 62083 | 5.5 | 1.0 |
| | 2.5 | 59180 | 19.9 | |
| | 0.5 | 65210 | 1.7 | |
| | 0 | 64292 | 13.5 | |
| 15 | 10.0 | 101657 | 4.6 | 1.1 |
| | 2.5 | 120733 | 8.8 | |
| | 0.5 | 89696 | 3.1 | |
| | 0 | 108095 | 2.5 | |

## 2.3 Antibody 1: Titration of Reagents

To improve modulation of the TT3 assay with antibody #1, both antibody and T3-AP levels were titrated systematically on the Theranos system (Table 5). Final optimized loading levels of T3-AP were determined to be 1:10K dilution from stock while the antibody loading concentration was 100ng/ml. Both T3-AP and antibody get diluted an additional 10 fold during the assay protocol run.

**Table [ SEQ Table * ARABIC ]:** Titration of antibody and T3-AP levels

| T3-AP Dilution from Neat In Cartridge | [Ab], ng/ml | [TT3], ng/mL | Mean RLU | CV% | Modulation |
|---|---|---|---|---|---|
| 1:1000 | 1000 | 10 | 222656 | 3.8 | 4.2 |
| | | 1 | 612862 | 2.4 | |
| | | 0 | 929052 | 2.3 | |
| | 100 | 10 | 20093 | 6.9 | 4.5 |
| | | 1 | 81545 | 2.0 | |
| | | 0 | 90664 | 8.7 | |
| | 10 | 10 | 2214 | 7.3 | 4.6 |
| | | 1 | 7586 | 11.7 | |
| | | 0 | 10098 | 17.2 | |
| 1:10K | 1000 | 10 | 29380 | 12.6 | 9.2 |
| | | 1 | 180511 | 9.6 | |
| | | 0 | 271452 | 12.0 | |
| | 100 | 10 | 2908 | 5.1 | 12.9 |
| | | 1 | 22304 | 4.3 | |
| | | 0 | 37401 | 15.7 | |
| | 10 | 10 | 527 | 6.6 | 7.0 |
| | | 1 | 1998 | 3.3 | |
| | | 0 | 3792 | 7.7 | |
| 1:100K | 1000 | 10 | 6659 | 8.7 | 6.0 |
| | | 1 | 26034 | 4.5 | |
| | | 0 | 40386 | 3.7 | |
| | 100 | 10 | 961 | 6.7 | 6.0 |
| | | 1 | 2862 | 13.5 | |
| | | 0 | 5343 | 11.2 | |
| | 10 | 10 | 194 | 5.2 | 3.0 |
| | | 1 | 361 | 1.3 | |
| | | 0 | 585 | 12.5 | |

## 2.4 Antibody 1: Optimizing Tip Coating Buffers

Super block buffer is an albumin free buffer which has shown to be effective by increasing signal to background noise in some cases. However, for this Total T3 assay on the Theranos system, regular blocking buffer (3% BSA) seemed to give the best modulation compared to the Super block buffer when used as a blocking buffer for coating.

**Table [ SEQ Table * ARABIC ]: Tip Coating Buffer**

| Type of Buffer | [TT3], ng/ml | Mean RLU | CV% | Modulation |
|---|---|---|---|---|
| 3% BSA | 10.0 | 2903 | 4.5 | 15.5 |
| | 5.0 | 6482 | 8.7 | |
| | 2.5 | 12753 | 4.2 | |
| | 1.0 | 22822 | 3.0 | |
| | 0.5 | 33770 | 4.5 | |
| | 0.25 | 33587 | 6.9 | |
| | 0.1 | 39458 | 14.1 | |
| | 0 | 44966 | 6.9 | |
| Super Block | 10.0 | 4827 | 7.2 | 8.4 |
| | 5.0 | 8771 | 4.1 | |
| | 2.5 | 15372 | 0.6 | |
| | 1.0 | 25411 | 9.1 | |
| | 0.5 | 28966 | 2.1 | |
| | 0.25 | 36106 | 8.3 | |
| | 0.1 | 36071 | 8.6 | |
| | 0 | 40762 | 18.1 | |

## 2.5 Antibody 1: Cross Reactivity and Interference

To test cross reactivity and interference, 7 different substances that could potentially cross react or interfere with T3 were tested. The test levels chosen for each test substance were three times higher than the highest level present/administered to a patient. There was significant cross reactivity with thyroxine (T4) and some cross reactivity with 3,5-Diiodo-L-thyronine (T2). Some over-recovery in the presence of sodium salicylate is acceptable as the CDC states that anti-inflammatory substances such as this are known to interfere with equilibrium levels of total T3 and free T3 as they play similar roles to displace T3 from its binding proteins. However the level of cross reactivity with T4 and T2 was considered unacceptable and antibody screening was resumed.

**Table [ SEQ Table * ARABIC ]: Antibody 1 Cross Reactivity and Interference**

| Test Substance | [Test Substance], ng/ml | [TT3], ng/ml | Back Calculated Concentration Mean Conc, ng/ml | Conc CV% | %Recovery |
|---|---|---|---|---|---|
| Control | - | 10 | 10.0 | 6.6 | 100 |
| | | 5 | 4.9 | 2.5 | 97 |
| | | 2 | 2.3 | 1.9 | 115 |
| | | 1.5 | 1.3 | 6.2 | 84 |
| | | 1 | 1.1 | 19.7 | 112 |
| | | 0.8 | 0.7 | 7.9 | 91 |
| | | 0.4 | 0.4 | 9.6 | 109 |
| | | 0 | 0.3 | 1.2 | - |
| 3,3',5' Triiodothyronine (Reverse T3) | 1.5 | 10 | 11.1 | 18.6 | 111 |
| | | 5 | 5.4 | 12.6 | 108 |
| | | 1 | 1.1 | 1.4 | 106 |
| | | 0 | 0.3 | 13.3 | - |
| Thyroxine (T4) | 900 | 10 | OORH | OORH | - |
| | | 5 | 12.7 | 2.8 | 254 |
| | | 1 | 7.5 | 6.2 | 750 |
| | | 0 | 7.5 | 5.2 | - |
| 3'-Iodo -L-tyrosine | 30 | 10 | 12.4 | 5.6 | 124 |
| | | 5 | 6.3 | 3.2 | 127 |
| | | 1 | 1.0 | 19.8 | 104 |
| | | 0 | 0.5 | 22.9 | - |
| 3,5-Diiodo-L-thyronine (T2) | 30 | 10 | 10.1 | 1.5 | 101 |
| | | 5 | 6.1 | 17.6 | 122 |
| | | 1 | 1.5 | 8.3 | 148 |
| | | 0 | 0.8 | 23.0 | - |

**Antibody 1 Cross Reactivity and Interference (continued)**

| Test Substance | [Test Substance], ng/ml | [TT3], ng/ml | Back Calculated Concentration | | |
|---|---|---|---|---|---|
| | | | Mean Conc, ng/ml | Conc CV% | %Recovery |
| Sodium salicyate | 400000 | 10 | 11.5 | 2.3 | 115 |
| | | 5 | 7.1 | 6.8 | 141 |
| | | 1 | 1.4 | 25.2 | 138 |
| | | 0 | 0.5 | 21.1 | - |
| Phenylbutazone | 300000 | 10 | 11.4 | 6.1 | 114 |
| | | 5 | 6.2 | 4.5 | 124 |
| | | 1 | 0.9 | 2.5 | 89 |
| | | 0 | 0.3 | 11.1 | - |
| Diphenylhydantoin | 300 | 10 | 12.7 | 8.5 | 127 |
| | | 5 | 6.1 | 0.4 | 122 |
| | | 1 | 1.0 | 15.9 | 100 |
| | | 0 | 0.4 | 16.9 | - |

## 2.6 Competitive Assay Screen on the Theranos System: Set 2

Due to major challenges of finding a T3 antibody such as insufficient modulation and high cross reactivity with T4, new antibodies were purchased and screened for both modulation and cross reactivity with T4. For screening cross reactivity purposes, % RLU difference from the control was calculated, differences of |20%| and below were considered ideal.

Antibody #1 was included as a control. Antibodies #26, #27 and #28 did not bind to the T3-AP (data not shown). Antibody #24 was the best in terms of modulation and low percent difference while Antibody# 30 and #26 are possible back up antibodies for this assay.

**Table [ SEQ Table * ARABIC ]: Competitive Assay Screen on the Theranos System-Set 2**

| | Control | | | | Spiked with 900 ng/mL T4 | | | |
|---|---|---|---|---|---|---|---|---|
| Antibody # | [TT3] ng/mL | Mean RLU | CV% | Modulation | [TT3] ng/mL | Mean RLU | CV% | % Difference from CTL |
| 1 | 10 | 1661 | 7.2 | 12 | 10 | 1251 | 19 | -25 |
| | 5 | 3330 | 2.1 | | 5 | 2105 | 3.6 | -37 |
| | 2 | 5968 | 1.4 | | - | - | - | - |
| | 1 | 9858 | 12.7 | | 1 | 5299 | 12.4 | -46 |
| | 0 | 19864 | 0.7 | | 0 | 7342 | 5.9 | -63 |
| 15 | 10 | 13763 | 2.8 | 6.6 | 10 | 9723 | 8.3 | -29 |
| | 2 | 39855 | 1.2 | | 2 | 17643 | 2.1 | -56 |
| | 0.8 | 73450 | 18.8 | | - | - | - | - |
| | 0.4 | 74578 | 7 | | 0.4 | 29786 | 2.7 | -60 |
| | 0 | 90690 | 8.4 | | 0 | 37616 | 2.5 | -59 |
| 16 | 10 | 13970 | 17.4 | 8 | 10 | 8976 | 9.3 | -36 |
| | 2 | 61121 | 8.5 | | 2 | 27408 | 9.7 | -55 |
| | 0.8 | 81590 | 9.3 | | - | - | - | - |
| | 0.4 | 98914 | 12.9 | | 0.4 | 50499 | 7.3 | -49 |
| | 0 | 111433 | 9.9 | | 0 | 53904 | 2.9 | -52 |
| 17 | 10 | 863 | 4.1 | 7.6 | 10 | 728 | 10.4 | -16 |
| | 2 | 2870 | 4.3 | | 2 | 1284 | 16.5 | -55 |
| | 0.8 | 3843 | 5.2 | | - | - | - | - |
| | 0.4 | 5054 | 2.7 | | 0.4 | 1905 | 7.8 | -62 |
| | 0 | 6553 | 1.8 | | 0 | 2300 | 8.2 | -65 |
| 18 | 10 | 439 | 9.2 | 4 | 10 | 390 | 6.2 | -11 |
| | 2 | 950 | 0.4 | | 2 | 652 | 2.7 | -31 |
| | 0.8 | 1094 | 0.5 | | - | - | - | - |
| | 0.4 | 1451 | 7.4 | | 0.4 | 811 | 10.8 | -44 |
| | 0 | 1740 | 14.5 | | 0 | 928 | 10.2 | -47 |
| 19 | 10 | 464 | 6.6 | 4.6 | 10 | 486.8 | 13.3 | -5 |
| | 2 | 1107 | 3 | | 2 | 839 | 6.5 | -24 |
| | 0.8 | 1522 | 12.9 | | - | - | - | - |
| | 0.4 | 1925 | 11 | | 0.4 | 985 | 5.1 | -49 |
| | 0 | 2137 | 9.9 | | 0 | 1095 | 9.4 | -49 |
| 20 | 10 | 11421 | 12.9 | 9.7 | 10 | 7647 | 1.3 | -33 |
| | 2 | 34111 | 9 | | 2 | 25037 | 8.6 | -27 |
| | 0.8 | 80675 | 5.4 | | - | - | - | - |
| | 0.4 | 77320 | 8.5 | | 0.4 | 33267 | 11.3 | -57 |
| | 0 | 111223 | 3.4 | | 0 | 39265 | 13.2 | -65 |

| | Control | | | | Spiked with 900ng/ml T4 | | | |
|---|---|---|---|---|---|---|---|---|
| Antibody # | [TT3] ng/mL | Mean RLU | CV% | Modulation | [TT3] ng/mL | Mean RLU | CV% | % Difference from CTL |
| **21** | 10 | 18258 | 4.5 | 2.1 | 10 | 15939 | 5.5 | -13 |
| | 2 | 22523 | 7.9 | | 2 | 18387 | 2.7 | -18 |
| | 0.8 | 28293 | 17.6 | | - | - | - | - |
| | 0.4 | 36510 | 11.2 | | 0.4 | 20230 | 13.6 | -45 |
| | 0 | 38849 | 4.6 | | 0 | 27234 | 14.8 | -30 |
| **22** | 10 | 33710 | 9.3 | 6.6 | 10 | 33067 | 14.2 | -2 |
| | 2 | 67576 | 4 | | 2 | 103593 | 10 | -21 |
| | 0.8 | 130915 | 4.1 | | - | - | - | - |
| | 0.4 | 190517 | 20.2 | | 0.4 | 124003 | 8.8 | -45 |
| | 0 | 222975 | 16.7 | | 0 | 121619 | 17.3 | -45 |
| **23** | 10 | 5205 | 9.2 | 5.1 | | | | |
| | 2 | 23002 | 3.5 | | 2 | 15365 | 6.4 | -33 |
| | 0.8 | 28946 | 12.9 | | - | - | - | - |
| | 0.4 | 38697 | 9.2 | | 0.4 | 24539 | 5.0 | -37 |
| | 0 | 37862 | 2.9 | | 0 | 26589 | 20.7 | -30 |
| **24** | 10 | 8427 | 2.6 | 9.4 | 10 | 8729 | 2.9 | 4 |
| | 2 | 33295 | 18.8 | | 2 | 25360 | 3.5 | -24 |
| | 0.8 | 52923 | 8.6 | | - | - | - | - |
| | 0.4 | 68141 | 7.6 | | 0.4 | 46253 | 1.9 | -32 |
| | 0 | 78968 | 5.9 | | 0 | 57046 | 6.0 | -28 |
| **25** | 10 | 388 | 17.6 | 4.9 | 10 | 373 | 11.5 | -4 |
| | 2 | 952 | 9.7 | | 2 | 841 | 4.6 | -12 |
| | 0.8 | 1369 | 14.8 | | - | - | - | - |
| | 0.4 | 1806 | 5.8 | | 0.4 | 1203 | 16.1 | -33 |
| | 0 | 1904 | 8.4 | | 0 | 1237 | 16.8 | -35 |
| **26** | 10 | 16225 | 7.1 | 12.1 | 10 | 11895 | 2.6 | -27 |
| | 2 | 88834 | 1.6 | | 2 | 59382 | 9.9 | -33 |
| | 0.8 | 137688 | 4.5 | | - | - | - | - |
| | 0.4 | 167417 | 13.2 | | 0.4 | 106163 | 2.0 | -44 |
| | 0 | 196894 | 3.1 | | 0 | 130531 | 6.2 | -40 |
| **30** | 10 | 2086 | 2 | 8.2 | 10 | 1665 | 9.0 | -20 |
| | 2 | 8132 | 6.3 | | 2 | 5674 | 3.9 | -30 |
| | 0.8 | 11445 | 14.6 | | - | - | - | - |
| | 0.4 | 12668 | 9.3 | | 0.4 | 9226 | 4.9 | -27 |
| | 0 | 17096 | 16.8 | | 0 | 10882 | 3.4 | -36 |

## 2.7 Alkaline Phosphatase Stabilizer

Antibody #24 was selected as the best antibody in terms of modulation and low cross reactivity with T4. Two commercial stabilizers were tested as the T3-AP conjugate diluents: Stabilzyme AP and Biostab. An in-house AP stabilizer was also tested. This stabilizer consisted of 0.1mM $Zn^{2+}$ and 5mM $Mg^{2+}$ in Low BSA buffer (0.03% BSA). The in-house AP Stabilizer provided the best modulation and was chosen as the final condition.

**Table [ SEQ Table * ARABIC ]: Alkaline Phosphatase Stabilizer**

| | | Signal (RLU) | | | Back-Calculated Conc (ng/mL) | | |
|---|---|---|---|---|---|---|---|
| **AP Stabilizer** | **[TT3], ng/ml** | **Mean RLU** | **CV%** | **Mod.** | **Mean** | **CV %** | **% Recovery** |
| Control (Low BSA Buffer | 10.0 | 12115 | 11.9 | 9.7 | 9.1 | 3.0 | 91 |
| | 5.0 | 22726 | 14.6 | | 6.2 | 14.2 | 125 |
| | 2.0 | 55578 | 11.7 | | 1.7 | 25.3 | 85 |
| | 0.8 | 80271 | 4.5 | | 0.7 | 11.2 | 92 |
| | 0.4 | 95182 | 6.2 | | 0.5 | 16.2 | 120 |
| | 0.0 | 117233 | 5.7 | | 0.3 | 15.5 | |
| Stabilzyme AP | 10.0 | 13454 | 2.6 | 6.6 | 10.3 | 5.1 | 103 |
| | 5.0 | 24423 | 3.1 | | 5.0 | 2.9 | 100 |
| | 2.0 | 52466 | 13.2 | | 2.2 | 7.3 | 111 |
| | 0.8 | 63432 | 7.9 | | 0.7 | 37.8 | 85 |
| | 0.4 | 67319 | 13.2 | | 0.6 | 56.4 | 141 |
| | 0.0 | 89444 | 2.6 | | 0.1 | 17.3 | |
| Biostab | 10.0 | 12285 | 4.4 | 8.0 | 9.4 | 1.7 | 94 |
| | 5.0 | 23296 | 6.1 | | 5.9 | 6.3 | 118 |
| | 2.0 | 55124 | 5.0 | | 1.6 | 9.5 | 81 |
| | 0.8 | 73944 | 10.6 | | 0.9 | 25.5 | 112 |
| | 0.4 | 99833 | 4.2 | | 0.4 | 10.5 | 105 |
| | 0.0 | 98739 | 11.1 | | 0.4 | 28.5 | |
| In House AP Stabilizer | 10.0 | 12110 | 5.6 | 8.2 | 10.5 | 8.8 | 105 |
| | 5.0 | 22983 | 6.0 | | 5.0 | 5.5 | 100 |
| | 2.0 | 47182 | 8.5 | | 2.3 | 13.0 | 113 |
| | 0.8 | 80354 | 14.3 | | 0.9 | 22.2 | 113 |
| | 0.4 | 90280 | 12.7 | | 0.4 | 19.9 | 93 |
| | 0.0 | 107809 | 4.5 | | 0.2 | 18.2 | |

## 2.8 Evaluating Ab24 for the Free T3 Assay Format

Different incubation protocols and incubation times were tested for Antibody 24 in the fT3 assay format. Final conditions after protocol run included a 1:5 sample dilution, 1 ng/ml of antibody #24, and 1:200K dilution(from stock) of T3-AP. Sample mixture-substrate incubation times of 10-10 minutes (control condition) and 5-5 minutes were tested. Modulation was better with the 5 minute reagent incubations and will be used from here after.

| | | Signal (RLU) | | | Concentration (pg/ml) | |
|---|---|---|---|---|---|---|
| Protocol | [fT3], pg/ml | Mean RLU | CV% | Mod | Mean Conc, [fT3],pg/ml | Conc CV% |
| **10,10** | 50.0 | 1888 | 5.7 | 8.5 | 50.2 | 6.0 |
| | 30.0 | 3128 | 14.0 | | 30.9 | 12.8 |
| | 20.0 | 4708 | 8.6 | | 19.7 | 11.4 |
| | 10.0 | 6950 | 10.8 | | 10.8 | 21.3 |
| | 5.0 | 10485 | 4.9 | | 3.9 | 14.5 |
| | 2.0 | 11897 | 4.6 | | 2.6 | 14.6 |
| | 1.0 | 15436 | 2.5 | | 1.0 | 10.9 |
| | 0.0 | 15962 | 1.9 | | 1.2 | |
| **5,5** | 50.0 | 788 | 1.8 | 9.2 | 50.6 | 2.5 |
| | 30.0 | 1232 | 13.7 | | 30.9 | 13.6 |
| | 20.0 | 1928 | 5.8 | | 19.4 | 6.6 |
| | 10.0 | 2933 | 2.6 | | 10.8 | 4.6 |
| | 5.0 | 4115 | 2.8 | | 5.2 | 7.4 |
| | 2.0 | 5884 | 4.9 | | 1.6 | 17.5 |
| | 1.0 | 6275 | 4.1 | | 1.2 | 16.8 |
| | 0.0 | 7239 | 6.0 | | | |

## 2.9 Evaluation of clinical set with the different antibodies

Clinical samples were evaluated with Antibody 24 for the free T3 assay format. There were two outliers and correlation was poor. Backup TT3 antibodies were then tested for modulation and evaluated with clinical samples. Out of these, Antibody # 20 and Antibody #30 showed good modulation and fair sensitivity under these conditions. The clinical correlation was best for Antibody 30 and was selected for further optimizations. The antibodies were screened at the 5-5 minute incubation time. Conditions after protocol run included a 1:5 sample dilution and a 1:200K dilution (from stock) of T3-AP. Loading concentration of antibody 20 was 1:1M dilution from stock while antibody 30 was 1:10K (from a 1:100 fold diluted stock).

**Table [ SEQ Table * ARABIC ]: Standard Curve Data for Antibody 24**

| | Signal (RLU) | | | Back calculated( pg/ml) | |
|---|---|---|---|---|---|
| Nominal [fT3], pg/mL | Mean RLU | CV% | Mod | Mean Conc. [fT3] pg/ml | CV% |
| 50.0 | 1092 | 10.0 | 6.2 | 53.6 | 18.5 |
| 30.0 | 1663 | 2.9 | | 30.6 | 2.7 |
| 20.0 | 2620 | 5.1 | | 19.7 | 6.3 |
| 10.0 | 3533 | 4.7 | | 12.0 | 10.1 |
| 5.0 | 4977 | 6.0 | | 4.3 | 23.5 |
| 2.0 | 5717 | 6.3 | | 2.4 | 29.0 |
| 1.0 | 6592 | 7.5 | | OORL | OORL |
| 0.0 | 6768 | 7.6 | | OORL | OORL |

**Table [ SEQ Table * ARABIC ]: Antibody 24-Clinical test set**

| | | Signal(RLU) | | Back calculated (pg/ml) | | Siemens Immulite(CLIA) |
|---|---|---|---|---|---|---|
| Sample Type | Sample # | Mean RLU | CV% | Mean Conc. [fT3] pg/ml | CV% | Reported [fT3] pg/ml |
| Bioreclamation | 1 | 4736 | 6.1 | 5.2 | 22.0 | 1.96 |
| | 3 | 5077 | 4.0 | 4.0 | 15.2 | 3.8 |
| | 4 | 2741 | 7.9 | 18.6 | 10.7 | 2.86 |
| | 5 | 5565 | 4.9 | 2.7 | 22.7 | 4.52 |
| | 6 | 4825 | 9.3 | 5.0 | 34.4 | 4.2 |
| Sunny Lab | 3 | 3930 | 6.8 | 9.3 | 18.4 | 6.68 |
| | 5 | 3900 | 12.2 | 9.7 | 31.2 | 6.03 |
| | 6 | 2359 | 6.8 | 22.2 | 7.2 | 5.92 |
| | 7 | 4567 | 6.4 | 6.0 | 21.7 | 7.53 |
| | 8 | 3968 | 9.4 | 9.2 | 25.2 | 7.26 |
| | 11 | 3735 | 3.2 | 10.5 | 7.8 | 9.77 |
| | 13 | 3804 | 3.0 | 10.1 | 7.4 | 9.35 |
| | 14 | 3668 | 4.0 | 11.0 | 9.4 | 11.2 |
| | 18 | 2217 | 77.3 | 13.6 | 7.3 | 10.8 |
| | 24 | 3092 | 5.3 | 15.5 | 9.0 | 11.7 |

**Figure [ SEQ Figure * ARABIC ]: Evaluating Antibody 24 with clinical samples**

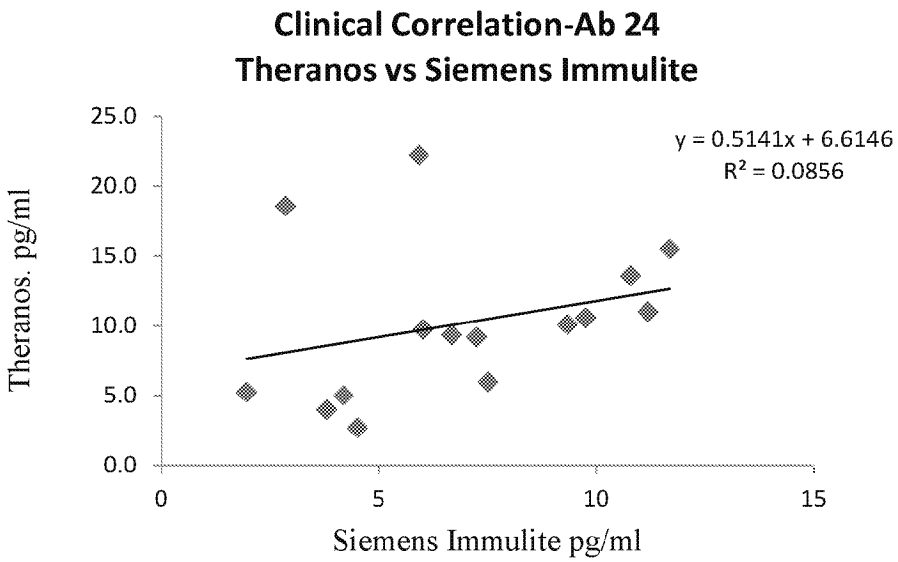



**Table [ SEQ Table * ARABIC ]: Standard Curve Data for Antibody 20**

| Nominal [fT3], pg/ml | Signal (RLU) | | | Back Calculated (pg/ml) | |
|---|---|---|---|---|---|
| | Mean RLU | CV% | Modulation | Mean Conc. [fT3], pg/ml | CV% |
| 50 | 8257 | 8.1 | 18.3 | 52.6 | 10.4 |
| 30 | 14903 | 2.1 | | 29.3 | 1.6 |
| 20 | 24789 | 8.1 | | 20.4 | 5.7 |
| 10 | 46352 | 8.9 | | 11.5 | 11.0 |
| 5 | 79206 | 8.5 | | 4.9 | 16.8 |
| 2 | 121838 | 8.4 | | 1.6 | 26.9 |
| 1 | 128452 | 7.4 | | 1.4 | 23.9 |
| 0 | 151493 | 4.1 | | OORL | OORL |

**Table [ SEQ Table * ARABIC ]: Antibody 20-Clinical test set**

| | | Signal (RLU) | | Back Calculated (pg/ml) | | |
|---|---|---|---|---|---|---|
| Sample Type | Sample # | Mean RLU | CV% | Mean Conc. [fT3], pg/ml | CV% | Reported [fT3] pg/ml |
| Bioreclammation | #4 | 78387 | 8.3 | 5.0 | 15.8 | 2.86 |
| | 18 | 105214 | 3.2 | 2.4 | 9.0 | 3.24 |
| | 19 | 91931 | 10.3 | 3.5 | 26.5 | 2.99 |
| | 20 | 102566 | 7.6 | 2.6 | 19.0 | 3.55 |
| | 22 | 68234 | 5.8 | 6.5 | 10.8 | 3.45 |
| Sunny Lab | 1 | 94725 | 9.9 | 3.3 | 27.0 | 5.45 |
| | 2 | 110736 | 7.2 | 2.1 | 20.6 | 5.85 |
| | 3 | 102260 | 2.9 | 2.6 | 7.9 | 6.68 |
| | 4 | 110073 | 4.3 | 2.2 | 12.5 | 5.15 |
| | 5 | 90149 | 1.5 | 3.6 | 3.6 | 6.03 |
| | 6 | 46803 | 13.2 | 11.4 | 15.7 | 5.92 |
| | 18 | 55122 | 12.0 | 9.2 | 17.7 | 10.8 |
| | 11 | 65903 | 11.7 | 6.9 | 19.4 | 9.77 |
| | 19 | 87462 | 6.8 | 3.9 | 16.0 | 7.43 |
| | 20 | 63683 | 13.5 | 7.4 | 23.7 | 7.78 |
| | 21 | 53867 | 14.7 | 9.5 | 20.5 | 11 |
| | 24 | 50457 | 6.3 | 10.3 | 8.1 | 11.7 |

**Figure [ SEQ Figure * ARABIC ]: Evaluating Antibody 20 with clinical samples**

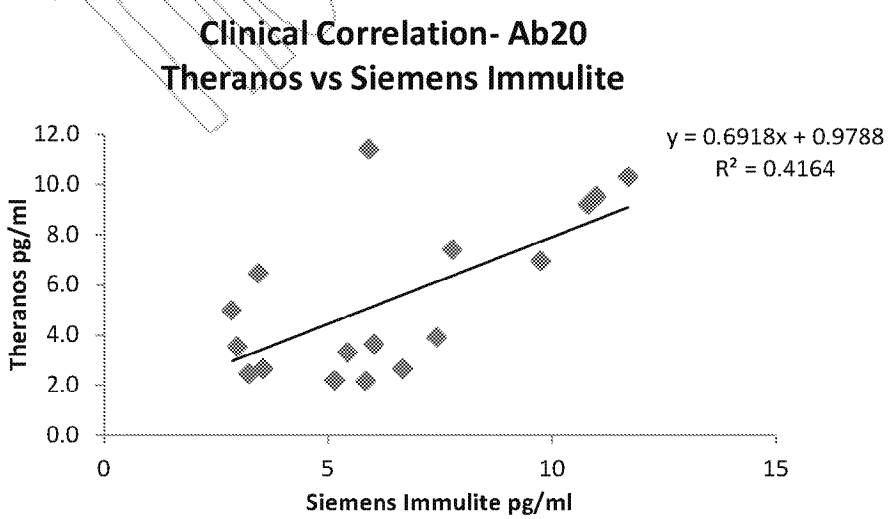


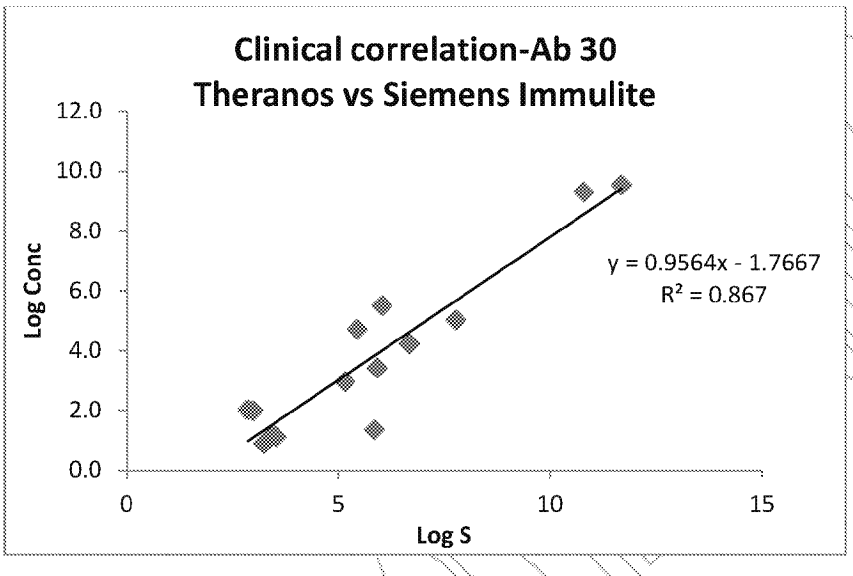
**Table [ SEQ Table * ARABIC ]: Standard Curve Data for Antibody 30**

| Nominal | Signal (RLU ) | | | Back Calculated (pg/ml) | |
|---|---|---|---|---|---|
| [fT3], pg/ml | Mean RLU | CV% | Modulation | Mean Conc [fT3],pg/ml | CV% |
| 50 | 10202 | 4.4 | 9.2 | 52.2 | 6.4 |
| 30 | 17792 | 7.0 | | 28.5 | 6.9 |
| 20 | 23725 | 4.8 | | 21.2 | 5.2 |
| 10 | 37321 | 5.6 | | 11.2 | 10.1 |
| 5 | 53908 | 7.9 | | 4.9 | 21.7 |
| 2 | 72001 | 10.3 | | 1.9 | 40.8 |
| 1 | 80956 | 10.9 | | 1.2 | 50.5 |
| 0 | 94029 | 8.1 | | OORL | OORL |

**Table [ SEQ Table * ARABIC ]: Antibody 30-Clinical test set**

| | | Signal (RLU) | | Back Calculated (pg/ml) | | |
|---|---|---|---|---|---|---|
| **Sample Type** | **Sample #** | **Mean RLU** | **CV%** | **Mean Conc. pg/ml** | **CV%** | **Reported fT3, pg/ml** |
| Biorec | 4 | 70299 | 4.7 | 2.0 | 17.9 | 2.86 |
| | 18 | 86040 | 9.3 | 0.9 | 42.3 | 3.24 |
| | 19 | 70830 | 4.9 | 2.0 | 18.7 | 2.99 |
| | 20 | 81153 | 0.8 | 1.1 | 3.7 | 3.55 |
| | 22 | 80946 | 8.5 | 1.2 | 35.8 | 3.45 |
| Sunny Lab | 1 | 54681 | 7.4 | 4.7 | 20.1 | 5.45 |
| | 2 | 77854 | 4.2 | 1.3 | 18.1 | 5.85 |
| | 3 | 56893 | 8.5 | 4.2 | 25.2 | 6.68 |
| | 4 | 63644 | 8.2 | 3.0 | 27.4 | 5.15 |
| | 5 | 51404 | 2.6 | 5.5 | 7.1 | 6.03 |
| | 6 | 60802 | 8.1 | 3.4 | 23.7 | 5.92 |
| | 18 | 41072 | 1.2 | 9.3 | 2.5 | 10.8 |
| | 20 | 53579 | 8.8 | 5.0 | 25.2 | 7.78 |
| | 24 | 40978 | 12.2 | 9.5 | 24.8 | 11.7 |

**Figure [ SEQ Figure * ARABIC ]: Evaluating Antibody 30 with clinical samples**



## 2.10 Ab30-Titration of reagents

To improve modulation of the fT3 assay with antibody #30, both antibody and T3-AP levels were titrated systematically on the Theranos system. Final optimized loading levels of T3-AP was determined to be 1:50K dilution from stock while the antibody loading concentration was 1:10K dilution (from 1:100K diluted stock). These conditions have good modulation between each level especially at the lower fT3 levels. Conditions after protocol run included a 1:5 sample dilution at 5-5 incubation time.

**Table [ SEQ Table * ARABIC ]: Antibody 30-Titration of Reagents**

| Antibody 30 | T3-AP | Assigned [fT3], pg/ml | Mean RLU | CV% | Modulation |
|---|---|---|---|---|---|
| 1:100K | 1:10K | 50 | 1320 | 5.9 | 8.8 |
| | | 30 | 2173 | 10.4 | |
| | | 20 | 2769 | 8.7 | |
| | | 10 | 4779 | 10.7 | |
| | | 5 | 7337 | 4.2 | |
| | | 2 | 9449 | 7.0 | |
| | | 1 | 10855 | 8.1 | |
| | | 0 | 11589 | 11.0 | |
| 1:10K | 1:50K | 50 | 2481 | 10.0 | 10.4 |
| | | 30 | 3999 | 2.5 | |
| | | 20 | 6772 | 6.5 | |
| | | 10 | 9242 | 7.9 | |
| | | 5 | 13917 | 10.8 | |
| | | 2 | 20012 | 3.9 | |
| | | 1 | 20488 | 9.8 | |
| | | 0 | 25828 | 12.1 | |
| 1:10K | 1:100K | 50 | 1074 | 9.6 | 8.6 |
| | | 30 | 1608 | 4.1 | |
| | | 20 | 2412 | 3.0 | |
| | | 10 | 3669 | 7.2 | |
| | | 5 | 4850 | 6.5 | |
| | | 2 | 7318 | 18.6 | |
| | | 1 | 7903 | 12.1 | |
| | | 0 | 9192 | 12.9 | |

## 2.11 Antibody #30 –Optimizing Tip coating buffer

Super block buffer is an albumin free buffer which has shown to be effective by increasing signal to background noise in some cases. However, for this Free T3 assay on the Theranos system, regular blocking buffer (3% BSA) seemed to give the best modulation and sensitivity compared

to the Super block buffer when used as a blocking buffer for coating. Final optimized loading levels of T3-AP was determined to be 1:50K dilution from stock while the antibody loading concentration was 1:10K dilution (from 1:100K diluted stock). Conditions after protocol run included a 1:5 sample dilution at 5-5 incubation time.

**Table [ SEQ Table * ARABIC ]: Antibody 30-Optimizing Tip Coating Buffer**

| Tip Coat Buffer | Assigned | Signal (RLU) | | | Concentration(pg/ml) | |
|---|---|---|---|---|---|---|
| | [fT3] pg/ml | Mean RLU | CV% | Modulation | Mean Conc [ft3] pg/ml | CV% |
| Regular 3% BSA | 50 | 2481 | 10.0 | 10.4 | 49.8 | 12.3 |
| | 30 | 3999 | 2.5 | | 29.8 | 2.3 |
| | 20 | 6772 | 6.5 | | 17.5 | 7.7 |
| | 10 | 9242 | 7.9 | | 11.3 | 13.0 |
| | 5 | 13917 | 10.8 | | 4.9 | 27.6 |
| | 2 | 20012 | 3.9 | | 1.6 | 13.6 |
| | 1 | 21582 | 6.4 | | 1.2 | 24.9 |
| | 0 | 25828 | 12.1 | | OORL | OORL |
| Super Block Buffer | 50 | 2599 | 6.5 | 9.3 | 52.1 | 9.8 |
| | 30 | 4347 | 9.7 | | 29.5 | 8.2 |
| | 20 | 6365 | 2.7 | | 21.2 | 2.6 |
| | 10 | 10857 | 7.0 | | 10.4 | 13.6 |
| | 5 | 14502 | 3.8 | | 5.3 | 10.8 |
| | 2 | 20192 | 12.2 | | 1.8 | 54.4 |
| | 1 | 21361 | 7.3 | | 1.3 | 26.0 |
| | 0 | 24247 | 10.7 | | OORL | OORL |

## 2.12 Antibody #30-Cross Reactivity

To test cross reactivity for antibody 30, substances that could potentially cross react with T3 were tested in Low BSA buffer instead of serum. No cross reactivity was observed with any of the substances tested. CDC states that anti-inflammatory substances such as phenylbutazone are known to interfere with equilibrium levels of total T3 and free T3 as they play similar roles to displace T3 from its binding proteins. Final optimized loading levels of T3-AP were determined to be 1:50K dilution from stock while the antibody loading concentration was 1:10K dilution (from 1:100K diluted stock). Conditions after protocol run included a 1:5 sample dilution at 5-5 incubation time.

**Table [ SEQ Table * ARABIC ]: Antibody 30- Cross Reactivity Data**

| Analyte | ng/ml | Mean RLU | CV% | |
|---|---|---|---|---|
| T3 | 50 | 407 | 19.1 | |
| | 30 | 485 | 4.7 | |
| | 20 | 513 | 24.7 | |
| | 10 | 968 | 10.8 | |
| | 5 | 1503 | 12.1 | |
| | 2 | 3003 | 14.9 | |
| | 1 | 4156 | 23.9 | |
| | 0 | 18380 | 9.4 | |
| 3,3',5' Triiodothyronine (rT3) | 1.5 | 18026 | 4.3 | OORL |
| | 1.0 | 15364 | 7.2 | OORL |
| | 0.5 | 18047 | 2.7 | OORL |
| | 0.3 | 14226 | 14.5 | OORL |
| | 0.1 | 17851 | 17.6 | OORL |
| | 0.0 | 20853 | 10.0 | OORL |
| T2 | 30 | 13559 | 10.4 | OORL |
| | 20 | 11665 | 16.7 | OORL |
| | 10 | 15659 | 14.8 | OORL |
| | 5 | 14186 | 14.1 | OORL |
| | 2 | 18507 | 4.6 | OORL |
| | 0 | 19949 | 10.0 | OORL |
| 3 iodo tyrosine | 30 | 15599 | 7.5 | OORL |
| | 20 | 18729 | 13.6 | OORL |
| | 10 | 18405 | 14.5 | OORL |
| | 5 | 16296 | 7.7 | OORL |
| | 2 | 17668 | 3.4 | OORL |
| | 0 | 19949 | 10.0 | OORL |

## 2.13 Calibration Verification

The Biorad Liquichek controls were tested for the Free T3 Assay. Here, three level serum controls from BioRad (Liquichek Immunoassay Plus) were measured on the Theranos System. These results were compared to those reported by the clinical analyzers. The controls correlate very well with the Siemens Dimension clinical analyzer. Final optimized loading levels of T3-AP were determined to be 1:50K dilution from stock while the antibody loading concentration was 1:10K dilution (from 1:100K diluted stock).

**Table [ SEQ Table * ARABIC ]: Antibody 30-Standard Curve Data with Dexter**

| | Signal (RLU) | | | Concentration(pg/ml) | | |
|---|---|---|---|---|---|---|
| Assigned [fT3], pg/ml | Mean RLU | CV% | Mod | Mean Conc [fT3], pg/ml | CV% | %Recovery |
| 50 | 2037 | 5.5 | 10.8 | 50.2 | 6.6 | 100 |
| 30 | 3236 | 2.7 | | 30.1 | 2.9 | 100 |
| 20 | 4777 | 8.4 | | 19.6 | 9.4 | 98 |
| 10 | 7792 | 5.3 | | 10.7 | 6.7 | 107 |
| 5 | 12154 | 6.4 | | 5.0 | 12.2 | 101 |
| 2 | 16519 | 3.1 | | 1.9 | 13.3 | 97 |
| 1 | 18189 | 5.2 | | OORL | OORL | OORL |
| 0 | 22022 | 4.6 | | OORL | OORL | OORL |

**Figure [ SEQ Figure * ARABIC ]: Standard curve fit with Dexter**

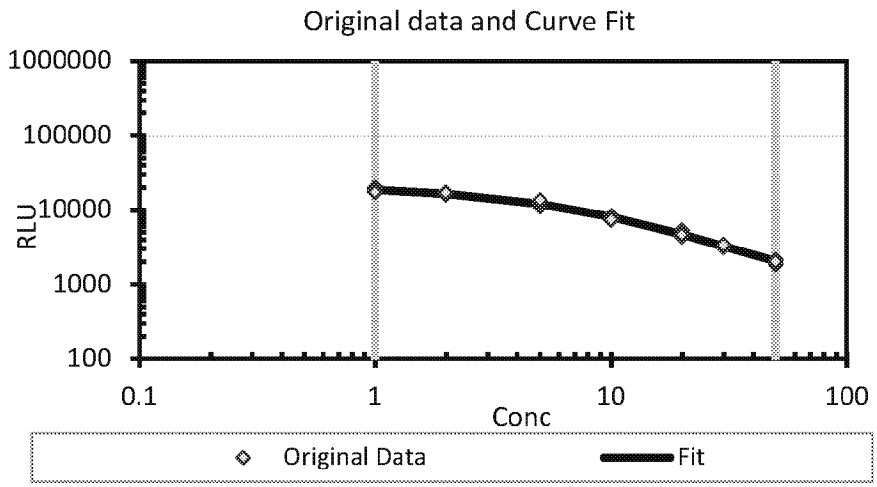
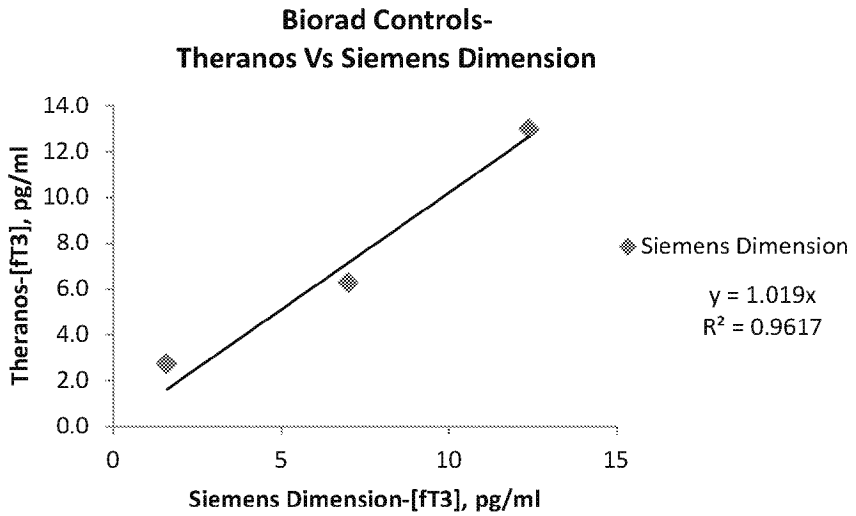


**Table [ SEQ Table * ARABIC ]: Biorad Liquichek Controls**

| | Reported [fT3] in pg/ml | | | | |
|---|---|---|---|---|---|
| Biorad Control | Theranos | Abott Architect | Roche Elecsys (pg/ml) | Siemens Dimension Vista systems | Siemens Immulite |
| Level 1 | 2.7 | 2.0 | 2.05 | 1.59 | 2.43 |
| Level 2 | 6.3 | 5.75 | 6.49 | 7 | 5.15 |
| Level 3 | 13.0 | 10.1 | 11.2 | 12.4 | 7.86 |

**Figure [ SEQ Figure * ARABIC ]: Biorad Controls-Theranos Vs Siemens Dimension**




## 2.14 Clinical Correlation

Serum samples from patients were obtained from both Sunny Labs and Bioreclammation. These samples were run on the Siemens Immulite (In House). These results were compared to those obtained on the Theranos system. A set of clinical samples across the fT3 range were selected to run on the Theranos system for the clinical correlation analysis. Final optimized loading levels of T3-AP were determined to be 1:50K dilution from stock while the antibody loading concentration was 1:10K dilution (from 1:100K diluted stock). This was done at the 5-5 incubation time.

The fT3 values obtained from Siemens Immulite correlated very well to the values obtained from the Theranos System.

**Table [ SEQ Table * ARABIC ]: Standard Curve Data-Dexter Analyses**

| | Signal (RLU) | | | Concentration(pg/ml) | | |
|---|---|---|---|---|---|---|
| Assigned [fT3], pg/ml | Mean RLU | CV% | Mod | Mean Conc. [fT3], pg/ml | CV% | %Recovery |
| 50 | 2037 | 5.5 | 10.8 | 50.2 | 6.6 | 100 |
| 30 | 3236 | 2.7 | | 30.1 | 2.9 | 100 |
| 20 | 4777 | 8.4 | | 19.6 | 9.4 | 98 |
| 10 | 7792 | 5.3 | | 10.7 | 6.7 | 107 |
| 5 | 12154 | 6.4 | | 5.0 | 12.2 | 101 |
| 2 | 16519 | 3.1 | | 1.9 | 13.3 | 97 |
| 1 | 18189 | 5.2 | | OORL | OORL | OORL |
| 0 | 22022 | 4.6 | | OORL | OORL | OORL |

**Figure [ SEQ Figure * ARABIC ]: Standard Curve-Dexter Analyses**

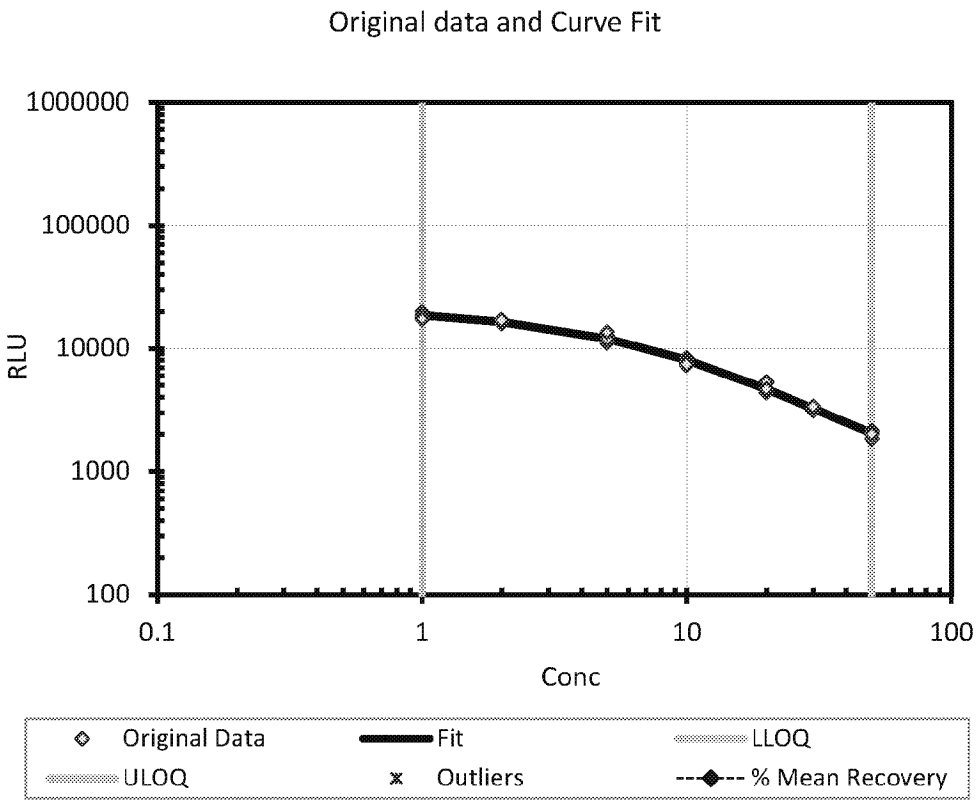
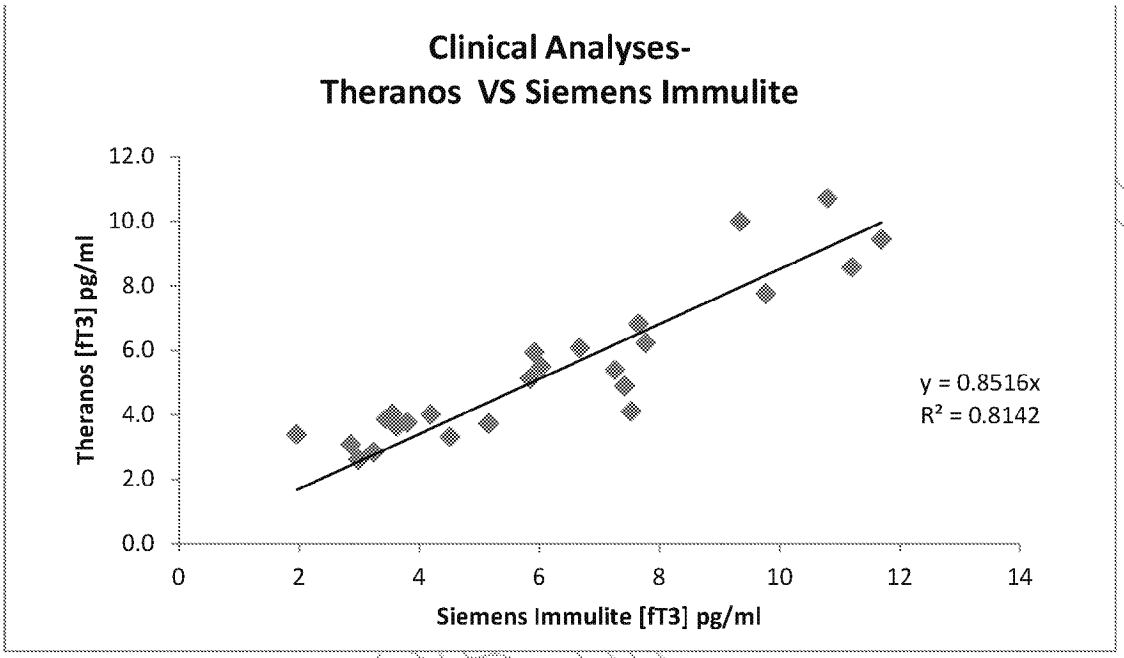


**Table [ SEQ Table * ARABIC ]: Clinical Sample Analyses**

| Sample Type | Sample# | Theranos,[fT3]-pg/ml | Siemens Immulite, [fT3]-pg/ml |
|---|---|---|---|
| Bioreclammation | 1 | 3.4 | 1.96 |
| | 2 | 3.6 | 3.63 |
| | 3 | 3.7 | 3.8 |
| | 4 | 3.1 | 2.86 |
| | 5 | 3.3 | 4.52 |
| | 6 | 4.0 | 4.2 |
| | 18 | 2.8 | 3.24 |
| | 19 | 2.6 | 2.99 |
| | 20 | 4.0 | 3.55 |
| | 22 | 3.9 | 3.45 |
| Sunny Labs | 2 | 5.1 | 5.85 |
| | 3 | 6.1 | 6.68 |
| | 4 | 3.7 | 5.15 |
| | 5 | 5.5 | 6.03 |
| | 6 | 5.9 | 5.92 |
| | 7 | 4.1 | 7.53 |
| | 8 | 5.4 | 7.26 |
| | 11 | 7.7 | 9.77 |
| | 13 | 10.0 | 9.35 |
| | 14 | 8.6 | 11.2 |
| | 17 | 6.8 | 7.66 |
| | 18 | 10.7 | 10.8 |
| | 19 | 4.9 | 7.43 |
| | 20 | 6.2 | 7.78 |
| | 24 | 9.4 | 11.7 |

**Figure [ SEQ Figure * ARABIC ]: Clinical Correlation-25 Sample Set**



## 2.15 Interfering Matrix Effects

Spike recovery was tested in grossly lipemic, icteric and hemolyzed serum samples to ascertain whether there may be interference in the assay results when measuring these types of samples. ARUP states that hemolyzed samples are completely unacceptable; however, the recovery for these samples on the Theranos system is good. The signal RLU was calculated to the concentration based on the standard curve and equation listed on Table 22. Final optimized loading levels of T3-AP were determined to be 1:50K dilution from stock while the antibody loading concentration was 1:10K dilution (from 1:100K diluted stock). This was done at the 5-5 incubation time.

**Table [ SEQ Table * ARABIC ]: Spike Recovery in Interefering Matrix**

| | Nominal spike | Signal (RLU) | | Recovered [fT3] | | Total Nominal plus endogenous | %Recovery |
|---|---|---|---|---|---|---|---|
| Matrix | [fT3], pg/ml | Mean RLU | CV% | Mean Conc pg/ml | CV% | [fT3], pg/ml | |
| Icteric | 0 | 13885 | 3.8 | 3.5 | 10.9 | 3.5 | 100 |
| | 2 | 11409 | 9.0 | 5.6 | 18.3 | 5.5 | 102 |
| | 5 | 10738 | 4.8 | 6.2 | 9.3 | 8.5 | 73 |
| | 15 | 6171 | 19.2 | 14.8 | 24.1 | 18.5 | 80 |
| Hemolyzed | 0 | 15531 | 6.5 | 2.5 | 23.3 | 2.5 | 99 |
| | 2 | 11367 | 7.8 | 5.6 | 16.0 | 4.5 | 125 |
| | 5 | 10937 | 2.0 | 6.0 | 3.8 | 7.5 | 80 |
| | 15 | 6429 | 8.7 | 13.7 | 11.1 | 17.5 | 78 |
| Lipemic | 0 | 14507 | 8.3 | 3.1 | 24.2 | 3.1 | 101 |
| | 2 | 12229 | 4.1 | 4.8 | 9.2 | 5.1 | 94 |
| | 5 | 9895 | 5.0 | 7.2 | 8.7 | 8.1 | 89 |
| | 15 | 5699 | 8.3 | 15.9 | 10.2 | 18.1 | 88 |

**Figure [ SEQ Figure * ARABIC ]: Spike Recovery in Icteric Sera**

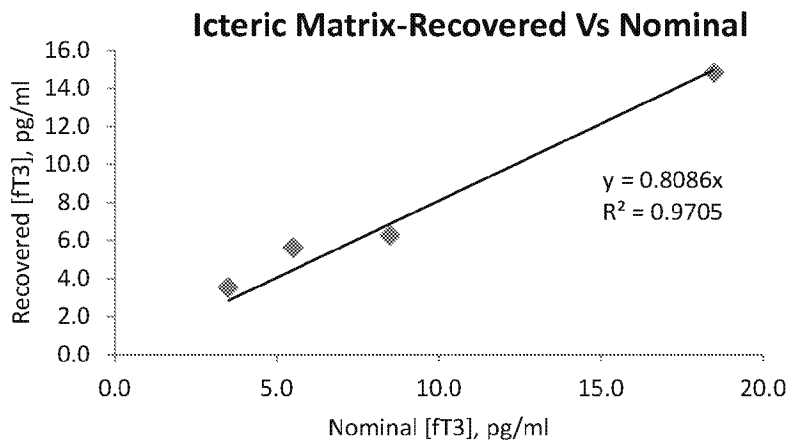


**Figure [ SEQ Figure * ARABIC ]: Spike Recovery in Hemolyzed Sera**

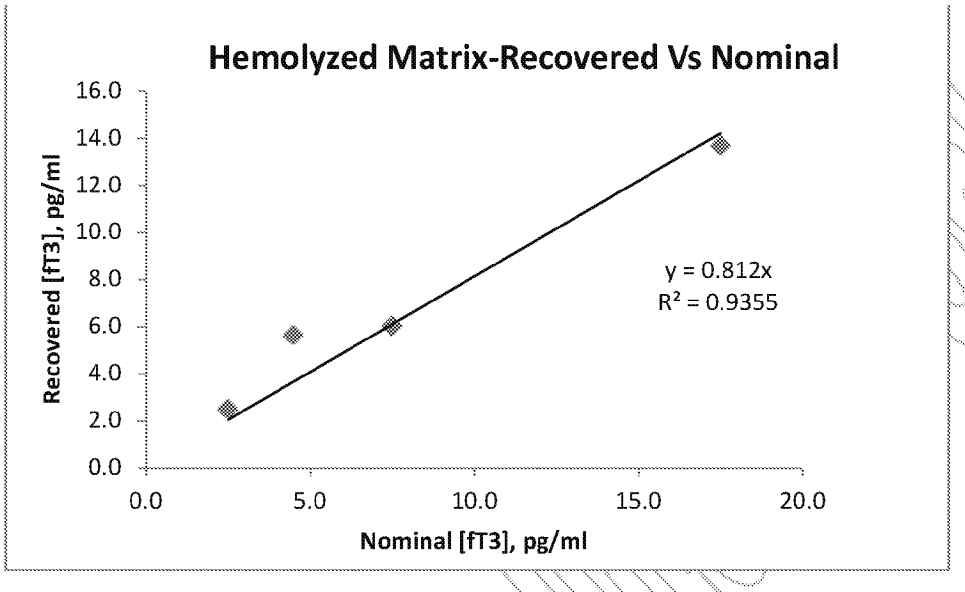
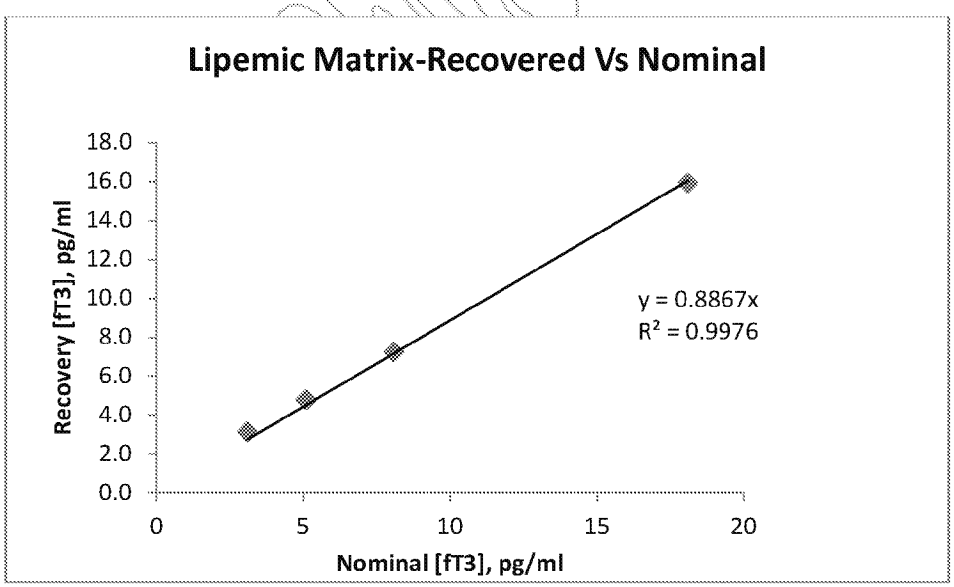



**Figure [ SEQ Figure * ARABIC ]: Spike Recovery in Lipemic Sera**



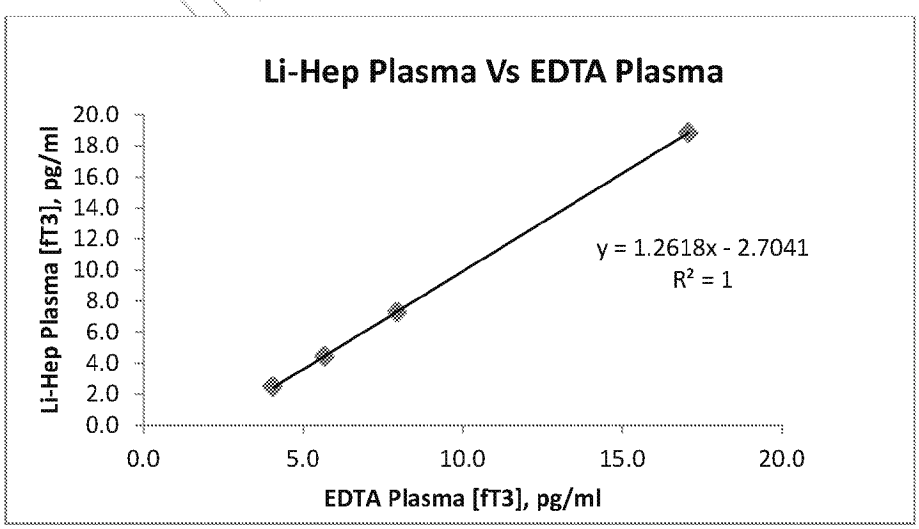
## 2.16 Effects of Anticoagulant

The Theranos System will be able to prepare plasma from both EDTA and lithium-heparin-treated blood. Matched plasma samples were prepared from both EDTA tubes and from Li-Hep tubes. The correlation was good indicating either type of anticoagulant would work well for this assay in this system. Final optimized loading levels of T3-AP were determined to be 1:50K dilution from stock while the antibody loading concentration was 1:10K dilution (from 1:100K diluted stock). This was done at the 5-5 incubation time.

**Table [ SEQ Table * ARABIC ]: Effect of Anticoagulant**

| | Nominal Spike Level | Signal (RLU) | | Recovered [fT3], pg/ml | | Total Nominal Spike plus Endogenous | |
|---|---|---|---|---|---|---|---|
| Anticoagulant | [fT3], pg/ml | Mean RLU | CV% | Mean Conc. pg/ml | CV% | [fT3], pg/ml | % Recovery |
| Li-Hep Plasma | 0 | 15549 | 4.4 | 2.5 | 15.7 | 2.5 | 100 |
| | 2 | 12635 | 1.9 | 4.4 | 4.3 | 4.5 | 99 |
| | 5 | 9851 | 4.1 | 7.3 | 7.1 | 7.5 | 98 |
| | 15 | 4921 | 4.9 | 18.9 | 5.8 | 17.5 | 108 |
| EDTA Plasma | 0 | 13189 | 9.5 | 4.1 | 23.6 | 4.1 | 100 |
| | 2 | 11297 | 5.8 | 5.7 | 12.0 | 6.1 | 94 |
| | 5 | 9358 | 6.2 | 8.0 | 10.1 | 9.1 | 88 |
| | 15 | 5358 | 6.3 | 17.1 | 7.4 | 19.1 | 90 |

**Figure [ SEQ Figure * ARABIC ]: Effect of Anticoagulant**



## 2.17 Matrix Effects: Serum vs Plasma

Theranos System will be capable of testing either serum prepared in the lab or plasma prepared on board from a whole blood sample. Matched Serum and Lithium Heparin Plasma from a 9 normal donors were analyzed. The max, min and average is comparable between both matrices. Spike recovery was also performed with matched serum and plasma samples from one of the donors. Correlation data was good indicating both plasma and serum samples can be used for the assay. Final optimized loading levels of T3-AP were determined to be 1:50K dilution from stock while the antibody loading concentration was 1:10K dilution (from 1:100K diluted stock). This was done at the 5-5 incubation time.

**Table [ SEQ Table * ARABIC ]: Standard Curve Data**

| | Signal (RLU) | | | Concentration (pg/ml) | | |
|---|---|---|---|---|---|---|
| [fT3], pg/ml | Mean RLU | CV% | Mod | Mean Conc [fT3] pg/ml | CV% | %Recovery |
| 50 | 2037 | 5.5 | 10.8 | 51.2 | 10.6 | 102 |
| 30 | 3236 | 2.7 | | 27.9 | 2.7 | 93 |
| 20 | 4777 | 8.4 | | 19.6 | 7.5 | 98 |
| 10 | 7792 | 5.3 | | 11.2 | 7.9 | 112 |
| 5 | 12154 | 6.4 | | 4.6 | 16.9 | 92 |
| 2 | 16519 | 3.1 | | 1.7 | 11.9 | 85 |
| 1 | 18189 | 5.2 | | OORL | OORL | OORL |
| 0 | 22031 | 5.9 | | OORL | OORL | OORL |

**Figure [ SEQ Figure * ARABIC ]: Standard Curve**

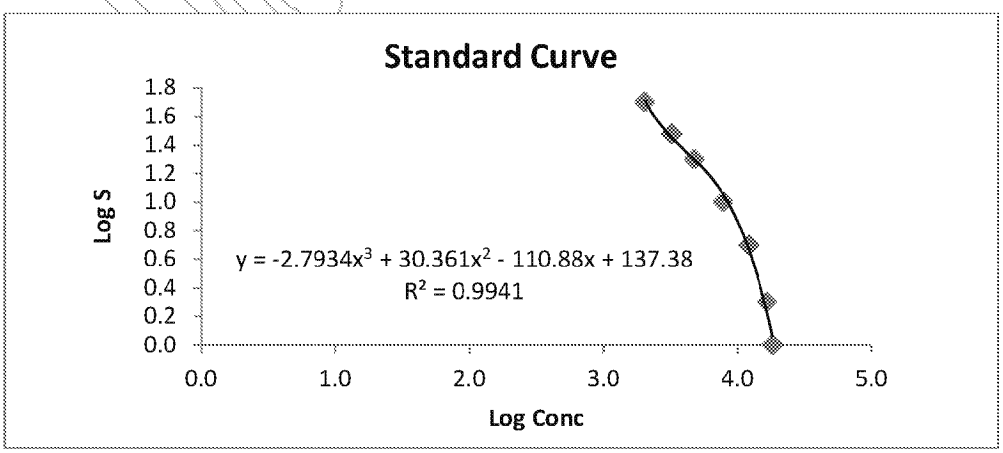


**Table [ SEQ Table * ARABIC ]: Matrix Effects: Lithium-Heparin Plasma vs Serum Samples**

| Donor # | Serum[fT3], pg/ml | Plasma [fT3], pg/ml |
|---|---|---|
| 1 | 2.9 | 2.7 |
| 2 | 4 | 4.7 |
| 3 | 2.3 | 3.0 |
| 4 | 2.7 | 2.6 |
| 5 | 3.3 | 3.4 |
| 6 | 4.1 | 3.7 |
| 7 | 2.2 | 2.5 |
| 8 | 2.6 | 2.1 |
| 9 | 4.4 | 4.0 |
| Min | 2.2 | 2.1 |
| Max | 4.4 | 4.7 |
| Average | 3.2 | 3.2 |



**Figure [ SEQ Figure * ARABIC ]: Matched Li-Hep Plasma Samples Vs Serum Samples**

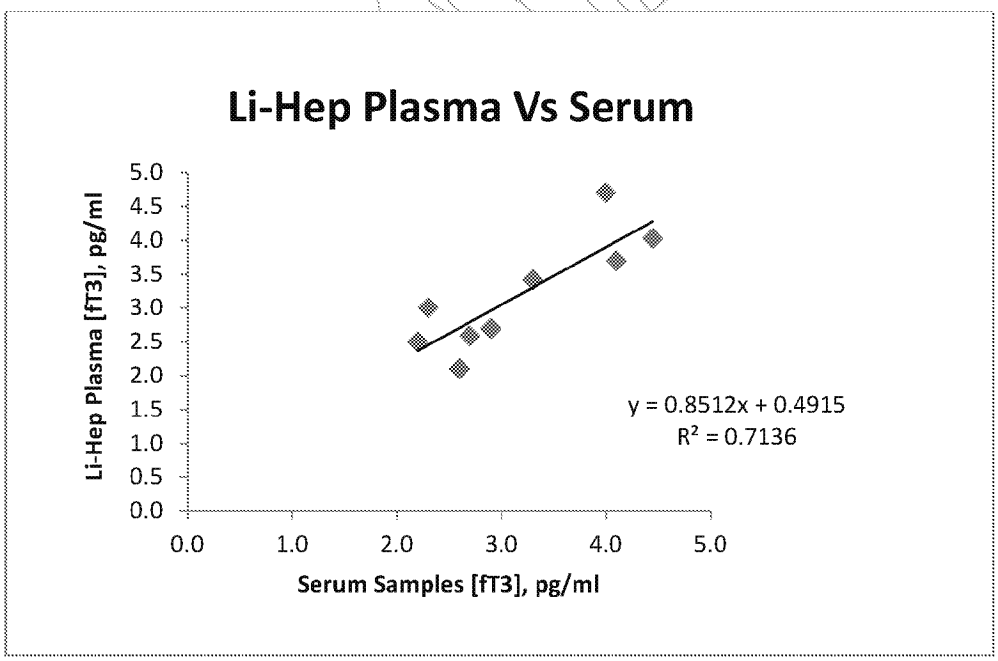
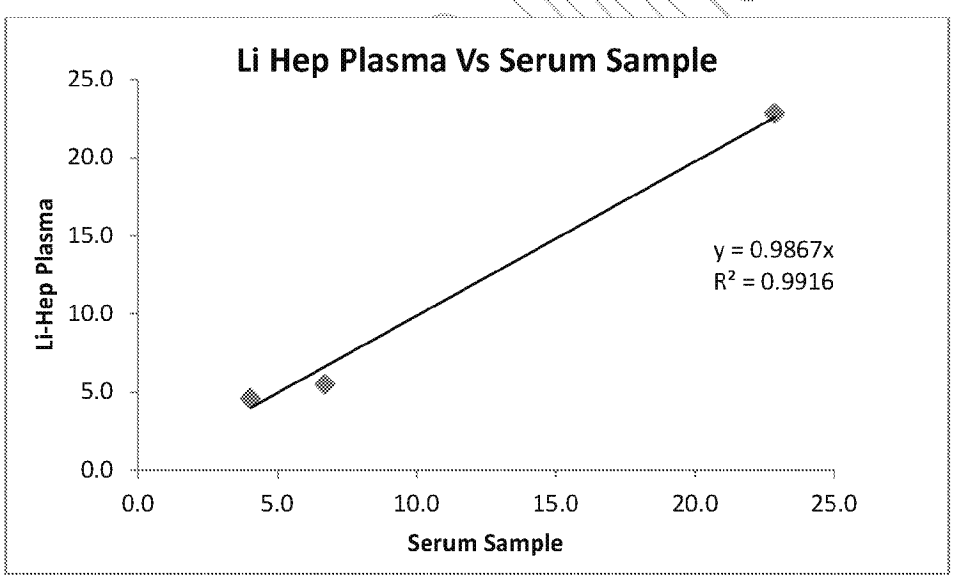


**Table [ SEQ Table * ARABIC ]: Spike Recovery-Li Hep Plasma Vs Serum Sample**

| | Nominal Spike Level | Signal RLU | | Recovered [fT3], pg/ml | | Nominal Spike plus Endogenous | %Recovery |
|---|---|---|---|---|---|---|---|
| Matrix | [fT3] pg/ml | Mean RLU | CV% | Mean Conc | CV% | [fT3], pg/ml | |
| Serum | 0 | 12761 | 7.3 | 4.0 | 21.8 | 4.0 | 100 |
| | 2 | 10376 | 6.1 | 6.7 | 13.4 | 6.0 | 111 |
| | 15 | 4020 | 6.5 | 22.9 | 5.7 | 19.0 | 120 |
| Li-Hep Plasma | 0 | 12191 | 9.8 | 4.6 | 24.1 | 4.6 | 100 |
| | 2 | 11377 | 8.2 | 5.5 | 19.7 | 6.6 | 83 |
| | 15 | 4154 | 20.4 | 22.8 | 18.7 | 19.6 | 116 |

**Figure [ SEQ Figure * ARABIC ]: Spike Recovery- Li-Hep Plasma Vs Serum**



## 2.18 Stability

Stability monitoring is ongoing for the the assay reagents stored at 4°C and protected from light. All reagents used for the standard curve will be evaluated at each time point.