ThyroSpot© Point-of-Care TSH Assay System

Name:
i-calQ ThyroSpot Smartphone Point-of-Care Quantitative TSH Immunoassay System

Intended Use:
The i-calQ ThyroSpot TSH assay is an in vitro diagnostic device for the quantitative determination of TSH in human serum. The assay is validated for use as a laboratory method to screen for congenital and acquired hypothyroidism, aid in the clinical diagnosis of thyroid disease, monitor iodine nutrition at the population level, and assist in the clinical management of thyroid disorders.

Test System Summary and Explanation:

1. TSH Physiology and Pathology

Human thyroid stimulating hormone (TSH) is a glycoprotein secreted by the anterior pituitary gland. TSH travels from the pituitary gland via the blood stream to the thyroid gland where TSH activates cells in the thyroid gland that produce thyroxine, T4, the principle thyroid hormone.

Many factors, including hormones and neurotransmitters, regulate the pituitary secretion of TSH. Thyroxine, through the process of negative feedback, is the major regulator of thyroid hormone production. When T4 levels fall below a set point, TSH levels increase. This results in a rise in circulating T4 levels. When T4 levels exceed a set point, TSH secretion from the pituitary decreases and this, in turn, results in a decrease in T4. The pituitary gland responds to very small T4 deviations from the set point and, as a result, TSH levels outside the normal range are a very sensitive indicator of thyroid disease. Elevations in TSH are the earliest abnormality seen when the thyroid gland is underactive (primary hypothyroidism). Suppressed TSH levels occur when excess amounts of thyroid hormones are present in the blood (primary hyperthyroidism). TSH levels below 0.4 mIU/L serum are often due to excess thyroid hormones, while TSH levels above 10 mIU/L are usually the result of thyroid hormone deficiency. TSH levels above 20 mIU/L are diagnostic of hypothyroidism.(http://www.thyroidmanager.org/).

In iodine sufficient regions, congenital primary hypothyroidism affects approximately 1 in 2800 newborns.(1) In populations with insufficient iodine intake, the incidence of congenital primary hypothyroidism is substantially higher. (2). TSH measurement is routinely used to screen for congenital primary hypothyroidism. TSH levels > 50 mIU/L serum in newborns older than 48 hours require immediate confirmation and treatment to avoid a progressive and permanent loss of brain development. TSH levels > 16 mIU/L serum in newborns older than 48 hours are consistent with primary hypothyroidism and should be repeated in 7 to 10 days. If TSH exceeds 20 mIU/L serum on repeat testing, the infant should be treated for primary hypothyroidism. (3) TSH measurement can also be used to track the treatment of primary hypothyroidism, as TSH levels should be approximately 1.0 mIU/L serum in patients on appropriate treatment.(4) Additionally, newborn TSH measurement can also be used to monitor a population's iodine nutrition status. Because the percentage of newborn TSH values > 5 TSH mIU/L serum is an indicator of a population's iodine intake, routine measurement of TSH in newborns older than 48 hours is an index of the degree of iodine deficiency in a population.(5)

2. Biological Principles of the Procedure

A. TSH Immunochromatographic Assay

The i-calQ smartphone point-of-care TSH assay uses a single-use, disposable immunoassay that is read with a smartphone-based image analysis algorithm. The assay measures serum TSH in the range 1.0 to 100.0 mIU/L serum. The i-calQ smartphone point-of-care TSH assay can be used to screen for congenital and acquired primary hypothyroidism, monitor the treatment of hypothyroidism, and assess a population's iodine intake.

Each single-use TSH assay consists of a multi-layer membrane held inside a plastic cassette. The membrane assembly consists of three layers at the proximal end of a nitrocellulose chromatographic strip. The sample pad top layer, upon which a 15 microliter sample of serum is deposited, lies on the conjugate pad. The conjugate pad contains dehydrated mobile phase murine monoclonal antibodies covalently linked to a colloidal nanoparticle. The sample pad overlaps with a buffer pad which extends to the proximal end of the strip. When solubilized by serum and buffer, the colloidal nanoparticles in solution bind to the beta TSH subunit present in the serum sample. The nitrocellulose membrane is beneath and in contact with the conjugate pad. Serum in buffer flows through the conjugate pad onto the nitrocellulose membrane. Capillary forces drive the bulk flow of in buffer the length of the nitrocellulose membrane. Serum in buffer that has reached the terminal end of the nitrocellulose membrane is absorbed onto a sump.

Goat polyclonal anti-beta TSH antibodies that recognize a second epitope distinct from that recognized by the mobile phase murine monoclonal antibody are deposited on and bound to the nitrocellulose membrane at a defined distance from the edge of the conjugate pad. These “capture antibodies” represent the test line. A “control line” consisting of goat anti-mouse IgG antibodies is deposited on the nitrocellulose membrane downstream from the test line. TSH in serum is sequestered at the test line to form a “sandwich”. The “sandwich” consists of TSH positioned between antibodies affixed to nitrocellulose and antibodies to which colloidal nanoparticles have been attached. Bulk flow of serum in buffer continues through the control line region of the nitrocellulose membrane where the remaining murine IgG conjugated to nanoparticles are captured.

The captured TSH labeled with colloidal nanoparticles generates a signal in the visible range at the test line. The intensity of the visual signal correlates with the amount of TSH present in the serum sample. The presence of the control line indicates that sufficient volume and time have elapsed for the serum and buffer to travel the length of the nitrocellulose membrane and that the conditions are appropriate for antigen antibody binding.

The immunochromatographic membrane assembly is held in position inside a plastic cassette housing. Each cassette holds a TSH immunoassay strip and a calibration strip. The cassette contains a port for specimen addition, a port for buffer addition, and a window for imaging the test and control lines. There is a curved indexing feature on the top of each cassette that is used for correctly positioning the cassette in the reader. See Figure 1.

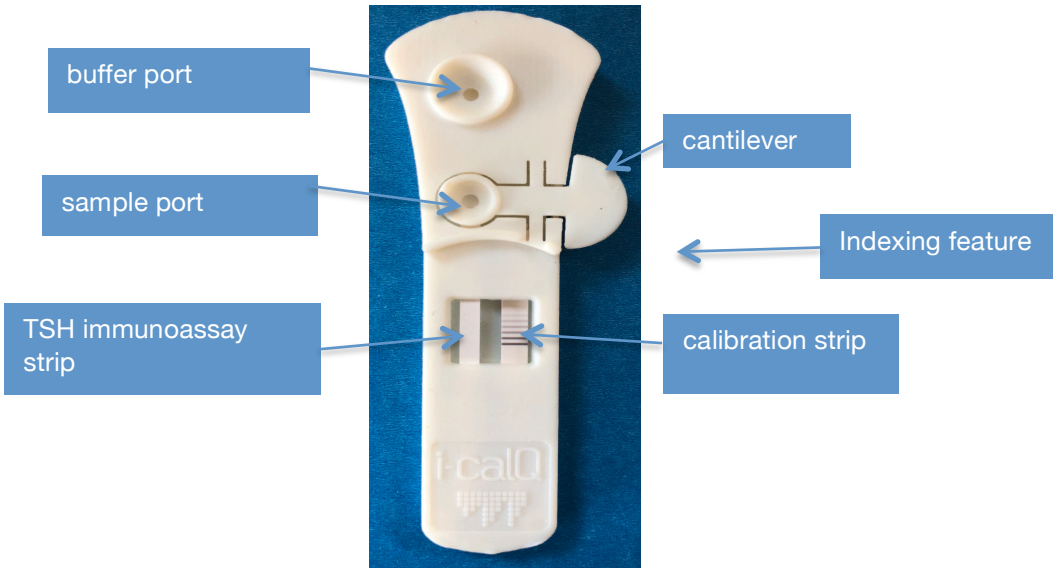

Figure 1
TSH cassette

Each assembled cassette is packaged in a sealed foil pouch. Each pouch is labeled with the product name, lot number, and expiration date. The i-calQ TSH assay, consisting of a membrane assembly, cassette, and foil pouch is a single-use device.

B. Immunochromatographic Assay Reader and Software

The TSH concentration, indicated by the amount of light reflected from the test line, is quantified using a smartphone camera with a cassette reader attachment and an image analysis app. To control for variations in illumination, test image acquisition is performed inside of a light-box. The light-box provides controlled and defined illumination.

The i-calQ TSH image analysis app is pre-installed on each smartphone. The i-calQ software runs in kiosk-mode and automatically loads when the phone is powered on. An internet connection is not needed. After the software launches, select the TSH image analysis icon on the home page or press Start on the app and then the image analysis icon to bring up the test imaging screen. See Figure 2.

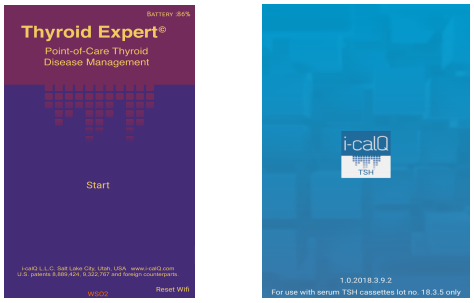

Figure 2
App Landing Page and Image Analysis Icon

The app images and interprets the test seen on the phone screen. All results are stored on the handset and can be accessed via the file browser icon on the Preview Test screen. Each test's acquired and image and image analysis results are stored. These results can be transferred to a computer using the mini-USB charging cable and an Android file transfer application.

C. Software Verification and Calibration

The cassette is inserted into the i-calQ colorimetric smartphone reader attachment 20 minutes after the specimen has been added to the cassette. The phone, reader, and cassette are then inserted into the light-box. The smartphone reader uses the smartphone's camera and controlled illumination to image the control and test lines visible through the cassette window. An algorithm loaded on the smartphone (the i-calQ TSH app) identifies the control and test lines and quantifies the intensity of the reflected light generated by plasmon surface resonance and Raleigh Mie light scattering from the fold nanoparticles. (6) A direct relationship exists between the amount of TSH in the sample and the intensity of the reflected light detected by a smartphone camera.

Each cassette contains a calibration strip. Calibration is performed in parallel with each TSH measurement. The calibration strip lines are imaged in parallel with each TSH test. Each line corresponds to a TSH concentration. The i-calQ TSH app measures the intensity of each calibration line to verify software performance and lighting conditions and compensate for any variation in ambient illumination present at the time that each test image is acquired.

3. Results Interpretation

The American Thyroid Association recommends the use of functional, not analytic, TSH assay sensitivity (7). Analytically sensitive "third / fourth generation" TSH assays can reliably identify TSH concentrations below reference interval values, making these assays appropriate for use in populations with a low risk of thyroid disease. Because TSH assay results must be interpreted in a clinical context, "risk of thyroid disease" represents both the incidence of thyroid disease and the economic, resource, and psychosocial consequences of detecting TSH values below the reference interval that are not due to thyroid disease. The i-calQ ThyroSpot point-of-care TSH Assay System has the functional sensitivity to

perform newborn thyroid screening, detect elevations in TSH consistent with the diagnosis of primary hypothyroidism, monitor a population's iodine nutrition status, and assess the adequacy of thyroid hormone replacement.

i-calQ Smartphone Point-of-Care TSH Assay System Components

Each box of i-calQ point-of-care TSH assays contains supplies for 50 tests:

50 individual wrapped TSH cassettes
2 chase buffer dropper bottles

Storage and Stability

All i-calQ smartphone point-of-care TSH assay supplies should be stored indoors at between 4°C – 30°C ambient temperature and 10 – 95% relative humidity in unopened foil pouches. Materials should not be exposed to direct heat, light or water. When stored and handled as directed, i-calQ smartphone point-of-care TSH assay supplies are stable until the expiration date printed on each foil cassette and dropper bottle.

Instrument Procedure

Remove the phone and reader from the light-box by sliding the phone towards the rear of the light-box and then lifting the phone up. See Figure 3A. The phone and reader are inserted into the light-box by lowering the phone and reader into the light-box through the circular cutout that corresponds to the reader. See Figure 3B.

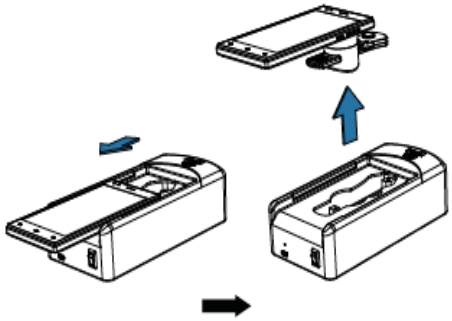

Figure 3A
Removing reader from light-box

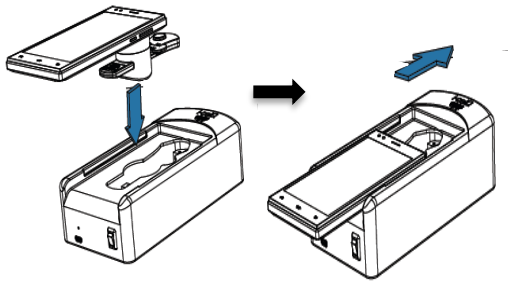

Figure 3B
Inserting reader into light-box

The toggle switch on the back face of the light-box controls the power to the LED's inside the light box. Prior to use, check that the LED on each side of the light-box is emitting light when the toggle switch is in the ON position. The light-box can remain on while testing is performed and showed be switched to the OFF position when testing is completed. Illumination within the light-box is maintained at a constant level independent of battery strength. A mini-USB connector is used to charge the rechargeable battery in the light box. A red LED above the micro-UB connector indicates that charging is taking place when the light-box is connected via USB to a power source. The absence of an illuminated red light when the light-box is connected to a power source indicates that the battery is fully charged. The light-box should be stored at 2-30° C. out of direct heat and light and away from moisture. The light box can be cleaned with a damp cloth. Contact i-calQ (contact@i-calQ.com) for any questions about light-box use.

The cassette reader is attached to the back of the smartphone via a bayonet mount. The reader can be removed and dissembled for cleaning. Contact i-calQ (contact@i-calQ.com) for detailed instructions on removing, cleaning, and reinstalling the reader. Prior to use confirm that the reader is correctly aligned with the phone's camera. See Figure 4.

Figure 4
Correct Alignment of i-calQ Reader with Handset.
Note that the i-calQ logo is parallel to the phone.

. The phone and reader should be stored in the light-box when not in use.

Specimen Collection and Sample Preparation

- Human serum should be used.
- Pre-analytic factors, especially ambient temperature > 30° C., can affect specimen integrity. (8)
- Samples may be stored at 2 – 8° C. for up to 5 days prior to testing. If specimens will be tested more than 5 days after collection, they should be frozen and stored at < -10° C. Specimens, tests, and buffer must be at room temperature when used for testing. Frozen specimens must be completely thawed, thoroughly mixed, and brought to room temperature prior to testing. Specimens that have undergone repeated freezing and thawing cycles should not be used.
- Serum samples that are cloudy, viscous, or contain particulate matter should not be used.
- Protective clothing including laboratory coats and disposable gloves must be worn while assaying samples. There should be no eating, drinking, or smoking in the area where specimens and kit reagents are handled.
- Standard laboratory practices for the handling and disposal of potentially infectious biological material should be used while performing a point-of-care TSH assay.

Assay Procedure / Test Instructions
See Figure 5 for step by step instructions.

- Read the instructions carefully and completely before using this test.
- Allow samples and i-calQ TSH test devices and buffer to come to room temperature prior to testing.
- Remove the cassette from its protective.
- Place cassette on a flat surface.
- Add 15 microliters of serum to the sample well on the cassette.
- Wait 60 seconds for the serum sample to be completely absorbed.
- Push the cantilever down to snap into place.
- Add 65 microliters (2 drops) of buffer into the buffer well.
- Keep cassette flat after adding sample and buffer.

- Use the i-calQ reader and app to read the test result 20 minutes after adding the buffer..
- Test results obtained after 30 minutes are not accurate.
- Test results are displayed as an image of the test with the regions of the test and control lines that were analyzed indicated by red boxes and a numerical TSH result. Inspect the image to confirm that the box locations are superimposed on control and test lines.
    - If no test line is present, a red box will be displayed at a predetermined distance from the control line. The absence of a test line beneath this box indicates that the TSH concentration present in the sample is below the detection threshold of the test.
    - The image analysis should be verified to confirm that control and test boxes were correctly identified. If boxes do not correspond to test and control line positions, remove and reinsert cassette into reader and then reimage.
    - If removing and repositioning the cassette and repeating the imaging does not result in correctly identifying the test and control lines, inspect the cassette membrane for the presence of dirt or debris. Clean off debris by gently blowing on the cassette.

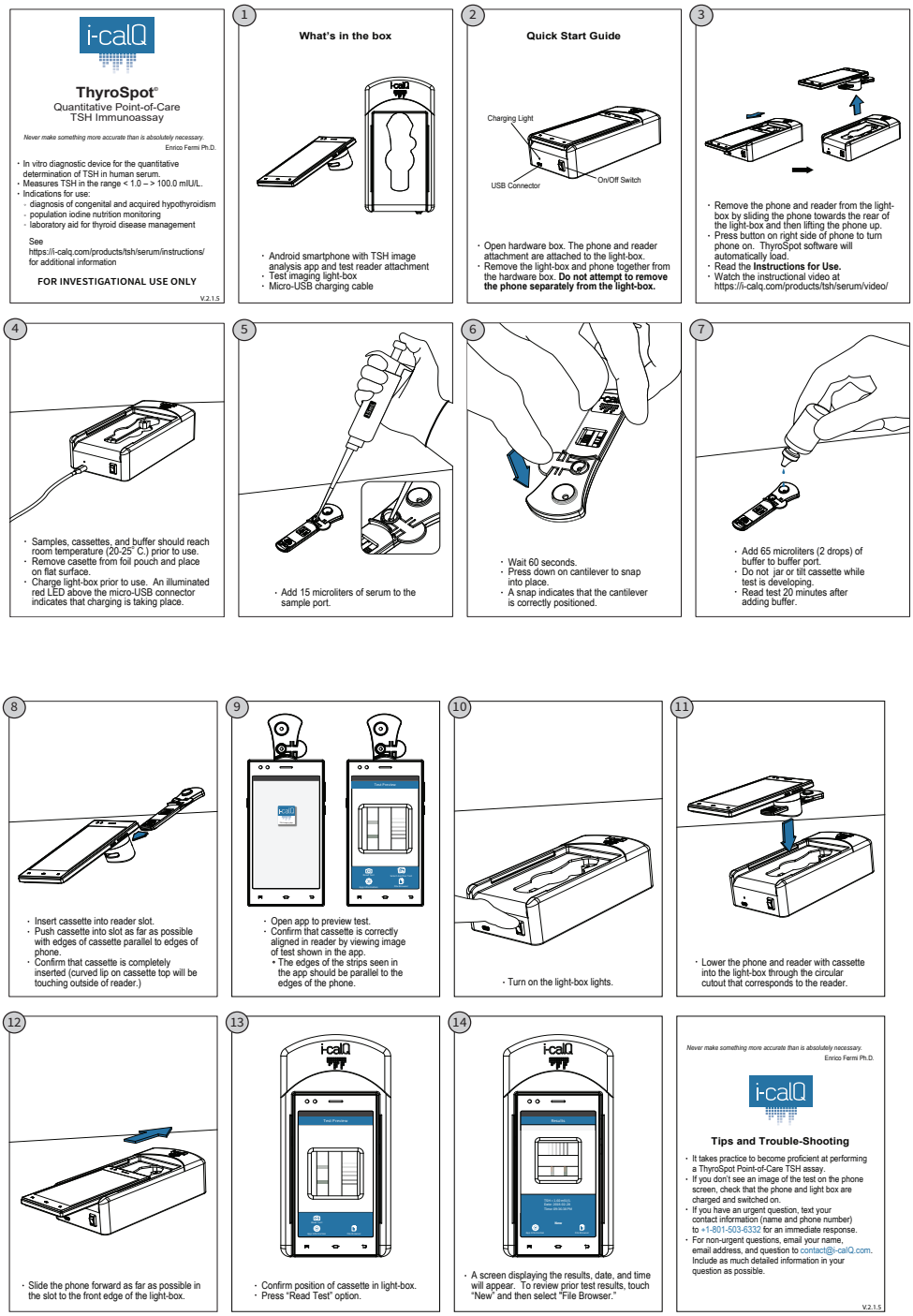

Figure 5
Step by Step Instructions

Limitations of the Assay

The diagnosis of thyroid disease should not be based upon a single TSH determination.

- The i-calQ smartphone point-of-care TSH assay should not be used to diagnose hyperthyroidism or monitor the treatment of thyrotoxicosis or thyroid cancer.
- Patient samples may contain heterophile antibodies or antibodies that recognize mouse monoclonal antibodies. These antibodies can affect assay performance and produce anomalous results.
- TSH variants may result in artefactually low or elevated TSH results.
- Patients with central hypothyroidism may have TSH levels that are within the reference interval. TSH should not be used for the diagnosis or monitoring of patients with pituitary or hypothalamic disease.
- Intercurrent illness can affect pituitary secretion of TSH and result in deviations in circulating TSH concentrations outside the reference interval.

Expected Values

Shown in Table 1, the reference interval for serum / plasma TSH, based upon the analysis of 465,593 TSH values from individuals ages 1 -104 years with no thyroid disease (9), is:

Age (years)	TSH (mIU/L)
1 – 20	0.53 – 6.45
21- 40	0.52 – 6.25
41 – 61	0.53 – 6.55
61 – 80	0.54 – 6.80
80 +	0.57 – 7.55

Table 1
Reference Interval for TSH in Individuals with No Thyroid Disease

Assay Validation and Performance Characteristics

The following studies were performed at a single site using a single lot of product, and two smartphones with reader attachments. With the exception of precision/repeatability experiments, all validation studies used human specimens that had been stored for at $\leq 5^{\circ}$ C.

1. Quantitative Recovery

Known gravimetric amounts of International Reference Preparations of pituitary (WHO International Standard Thyroid Stimulating Hormone, Human, for Immunoassay NIBSC code: 81/565) and recombinant (WHO Reference Reagent Thyroid-Stimulating Hormone (TSH), Recombinant, Human NIBSC code 94/674) human TSH in the range 0.5 – 100 mIU/L were added to patient plasma samples with TSH < 0.01 mIU/L as determined by Abbott Architect immunoassay. 20 microliter aliquots of each sample were assayed using the procedure described above. Results are displayed in Tables 2 and 3.

TSH added mIU/L	Instrument 1 % recovered	Instrument 2 % recovered
100	73.1	68.6
50	77.5	76.7
20	70.2	89.1
10	49.7	52.7
5	62.4	58.6
0.5	163.7	153.5

Table 2
Quantitative Recovery of Human Pituitary IRP TSH

TSH added mIU/L	Instrument 1 % recovered	Instrument 2 % recovered
100	62.4	69
50	68.02	70.3
20	55.8	63.04
10	71.5	80.7
5	48.5	51.9
0.5	123.3	118.47

Table 3
Quantitative Recovery of Human Recombinant IRP TSH

The variability in quantitative recovery between two types of TSH International Reference Preparations demonstrates the intrinsic biological variation in TSH immunoreactivity. Recombinant TSH lacks sialic acid, as CHO cells used to manufacture recombinant TSH do not have express sialytransferase. Additionally, pituitary TSH undergoes post-translational modification in sialic content based upon negative feedback of thyroxine on TSH secretion. Consequently, neither recombinant nor pituitary TSH International Reference Preparations are identical to TSH present in human blood. (10, 11, 12) Both recombinant and pituitary TSH International Reference Preparations in fact represent surrogate markers of circulating TSH in humans with and without primary thyroid disease. TSH's intrinsic immunovariability constrains the analytic precision of any TSH immunoassay. TSH immunoassays, including the predicate and similar "third-generation" TSH immunoassays that claim a functional sensitivity of <0.01 mIU/L, do not take into account intra- and inter-individuality variability in TSH immunopotency.

2. Serial Dilution

A known gravimetric amount of International Reference Preparations of pituitary (WHO International Standard Thyroid Stimulating Hormone, Human, for Immunoassay NIBSC code: 81/565) and recombinant (WHO Reference Reagent Thyroid-Stimulating Hormone (TSH), Recombinant, Human NIBSC code 94/674) human was added to patient plasma samples with TSH < 0.01 mIU/L as determined by Abbott Architect immunoassay to

obtain an initial TSH concentration of 150 mIU/L. This sample was serially diluted using 1:1 dilution with plasma that contained TSH < 0.01 mIU/L. 20 microliter aliquots of each sample were assayed using the procedure described above. The results are displayed in Table 4.

International Reference Preparation	**Instrument**	**Results X = Abbott Architect TSH Y = i-calQ TSH**	**Range TSH mIU/L**
Pituitary TSH	1	y = 0.96x – 3.41 r = .98	0.59 – 150.
	2	y = 0.65 - 1.57 r= .98	"
Recombinant TSH	1	y = 0.96x - 3.41 r = .98	"
	2	x = 0.57x - .60 r = .99	"

Table 4
Serial Dilution: x = Abbott Architect TSH, y = point-of-care TSH

3. Limit of Detection

The limit of detection represents the lowest amount of analyte in a patient sample that can be detected but not necessarily quantified as an exact value. The limit of detection was calculated using NCCLS EP17-A protocol in which LOD is defined as:

LOD = blank (mean) + 1.645*(blank standard deviation) + 1.645* standard deviation of mean of low sample measurements

The results are shown in Table 5.

Specimen	**TSH (mIU/L) mean + standard deviation**	
	Instrument 1	Instrument 2
TSH < 0.01	.07 + .36	.26 + .34
.3 ≤ TSH ≤ .6	.67 + .05	.79 + .03
Limit of detection	**0.74 mIU/L**	**0.90 mIU/L**

Table 5
Limit of Detection

These data demonstrate that the i-calQ smartphone point-of-care TSH assay's lower limit of detection corresponds to the Abbott Architect TSH assay values of .74 and .9 for instruments 1 and 2 respectively.

4. Linearity / Range / Limit of Quantification

Linearity is defined as the range of results that are directly proportional the concentration of TSH in patient specimens. Range is the interval between the lower and upper TSH concentrations that can be measured with a CV < 20%. The lowest amount of TSH that can be quantified with a C.V. < 20% represents the limit of quantification. The reportable range for the i-calQ smartphone point-of-care TSH assay is 1.0 – 100.0 mIU/L. The coefficient of variation for TSH measurements in this range is 3 – 17% (Figure 3). Serum and plasma TSH

concentrations below 1.0 mIU/L are displayed a < 1 mIU/L. Serum and plasma TSH concentrations over 100 mIU/L are displayed as > 100 mIU/L.

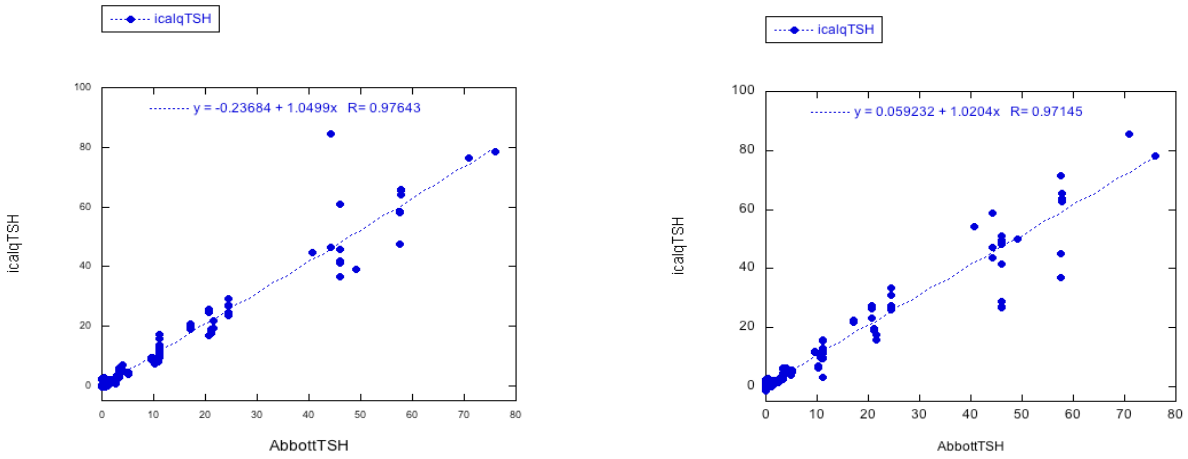

Figure 3
Assay Range

5. Precision / Repeatability

Precision represents the closeness of agreement / degree of scatter between a series of measurements obtained from multiple sampling of the same homogeneous sample under defined conditions. Repeatability expresses the precision when operating conditions are held constant and the testing interval is measured in days.

The precision / repeatability of the i-calQ smartphone point-of-care TSH assay was determined by repeated measurement of the TSH concentration in Bio-Rad Liquicheck controls. The results are displayed in Table 6.

BioRad Standard	Instrument	N	Mean + standard deviation mIU/L	Coefficient of variation
Level 1	1	9	0.85 ± .13	17%
	2	9	0.71 ± .11	15%
Level 2	1	10	2.89 ± .27	9.3%
	2	8	2.88 ± .16	5.5%
Level 3	1	10	12.42 ± .82	6.6%
	2	8	12.12 ± .67	5.5%

Table 6
Precision / Repeatability

6. Hook (Prozone) Effect

The hook effect describes artefactually low reported TSH values due to the presence of TSH in excess of the amount of reagents present in the assay. TSH was measured in patient samples with TSH concentrations between 132.8 and 530.17 mIU/ml. These results were reported as TSH > 1000 mIU/L., demonstrating that the i-calQ smartphone point-of-care TSH has no hook effect with maximal levels of patient sample TSH.

7. Assay Specificity

a. Analysis of variance was used to determine the effect of other glycoprotein hormones on TSH measurement with the smartphone point-of-care assay. These, displayed in Table 7, show that hCG, LH, and FSH in clinically relevant concentrations have no effect on TSH measurement by the i-calQ smartphone point-of-care TSH assay.

glycoprotein hormone	Instrument	a	b	p value
hCG (range 170 – 230,409 mIU/ml, n = 11)	Instrument 1 Instrument 2	1.794 1.34	8.98 e-7 6.4 e-7	0.962 0.627
LH (range 12.2 – 45.3 mIU/ml, n = 13)	Instrument 1 Instrument 2	0.822 0.968	1.33 e-2 3.53 e-3	0.0055 0.0187
FSH (range 51.6 – 74.5 mIU/ml, n = 9)	Instrument 1 Instrument 2	0.62 0.72	1.12 e-2 8.13 e-3	0.004 0.004

Table 7 Effect on Glycoprotein Hormones on TSH Assay Performance

b. Effect of interfering substances

Bilirubin (concentrations up to 34.5 mg/dL), triglycerides (concentrations up to 34013 mg/dL), and hemoglobin (500 mg/dL) do not interfere with the i-calQ smartphone point-of-care TSH immunoassay.

c. Matrix Effects

Effect of specimen matrix on assay performance was studied in 32 simultaneous drawn EDTA / serum specimen pairs, 7 simultaneous drawn EDTA plasma / lithium specimen pairs / and 2 simultaneous drawn lithium heparin / serum specimen pairs.
Specimen matrix does not have a significant effect on smartphone TSH immunoassay and that the assay is valid for use with serum, EDTA plasma, and lithium heparin plasma samples. The lack of matrix effect in the smartphone point-of-care TSH assay is equivalent to the lack of matrix effect in the predicate TSH assay.

8. Accuracy by Correlation

TSH concentration was determined in patient specimens with TSH in the range <0.01 – 530.17 mIU/L as measured by predicate assay. Two lots of product were used. Results are shown in Table 6.

Statistical Analysis	Instrument	Number of Specimens	Intercept	Slope	Correlation Coefficient
Least squares	1	253	.41	.99	r = .91
	2	134	1.64	1.01	r = .89

Table 6
Smartphone TSH Correspondence with Predicate TSH Assay

These results demonstrate that the i-calQ point-of-care smartphone TSH immunoassay generates serum and plasma TSH results that are equivalent to results provided by the predicate device, the Abbott Architect TSH immunoassay.

9. Duration of Substantial Equivalence / Reagent Stability Studies

Individually foiled wrapped i-calQ TSH immunoassays, stored at temperatures between 4° C. and 40° C. and relative humidity between 10 – 90%, showed no loss of component functionality over 36 months. When stored as directed, cassette stability is 24 months from date of manufacture. Test expiration date is indicated on each foil pouch.

Buffer Label

i-calQ Smartphone Point-of-Care TSH Buffer
Contains anti-microbial preservative
Lot number: 01096-01
Expiration date: 2018-11

Individual Foil Cassette Label

ThyroSpot Point-of-Care TSH Assay
Lot number: 18.3.
Expiration date: 2020-03

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