Validation of a Multiplex Assay to Detect Thyroid Hormones in Rat Serum

Abstract # 1892

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Abstract

Endocrine disruptors are substances that can cause harmful effects to the endocrine system. Thyroid hormones, triiodothyronine (T3), thyroxine (T4), and thyroid stimulating hormone (TSH) are involved in development of central nervous and skeletal systems. Test guidelines on the screening and testing of chemicals published by the Organisation for Economic Co-operation and Development (OECD) have been updated to include thyroid hormone measurements for assessing endocrine disrupter potency. It's therefore important that non-clinical toxicology studies are able to provide data assessing potential adverse effects on endocrine endpoints.

Several guidelines require analysis of circulating levels of thyroid hormones in juvenile rats. Sample volume and assay sensitivity can become limiting factors. Traditional enzyme-linked immunosorbent assays (ELISA) require relatively large sample volumes and suitability can be limited by poor sensitivity, inaccuracy, and biologically irrelevant assay ranges.

The Rat Thyroid Hormone Panel (Merck), enables multiplexing of thyroid hormones in a single analysis, minimising required sample volumes. The panel operates using internally colour coded microspheres, coated with a specific capture antibody. Sample analyte is captured by the bead and biotinylated detection antibody is introduced. The reaction mixture is finally incubated with Streptavidin-Phycoerythrin conjugate. Each microsphere is identified, and the result quantified based on fluorescent reporter signals.

A validation was performed evaluating kit performance in both multiplex and singleplex protocols for the relative quantification of T3, T4 and TSH in rat serum. Data capture was performed using a Luminex MAGPIX and xPONENT software. Method limitations for standard parameters were investigated.

The panel was acceptable with respect to parallelism, intra and inter run precision, accuracy, recovery, carry-over and calibration standards. The functional range determined was: 194.0 to 10,000.0 pg/mL (T3), 751.6 to 100,000.0 pg/mL (T4), 15.8 to 4,000.0 pg/mL (TSH) and were suitable for the analysis of changes in circulating levels of endogenous analyte.

The validation facilitated sample volume reduction to 12 µl for measurement of 3 analytes simultaneously, reducing sample volume requirements by 90%. The method is fit-for-purpose for measuring relative serum concentrations of T3, T4 and TSH in support of toxicological studies with acceptable precision and accuracy.

Introduction

Triiodothyronine (T3) and thyroxine (T4) are key hormones secreted from the thyroid gland and play a vital role in the development of the central nervous and skeletal systems, whose syntheses and release are regulated by Thyroid-Stimulating Hormone (TSH). These hormones play critical roles in the regulation of energy and both mental and physical development. Exogenous substances that can alter endocrine system functions are referred to as Endocrine disruptors (ED). Such substances can cause hormonal interference and exert adverse effects on numerous systems such as developmental, reproductive, neurological and immunological (1, 2).

Guidelines published by the Organisation for Economic Co-operation and Development (OECD) require the evaluation of circulating serum T3, T4 and TSH in order to evaluate thyroid function in both adult (Dams, Parental males) and juvenile rats at Postnatal Day 4 and 13 (PND4, PND13).

An alternative platform was required to previously validated traditional enzyme-linked immunosorbent assays (ELISA), in order to reduce the sample volume required, while also providing a suitable dynamic range for measurement in rats of varying life stages. Therefore, the performance was evaluated for a multiplex platform that was able to simultaneously measure T3, T4 and TSH from a single serum sample.

The MILLIPLEX MAP kit - Rat Thyroid Hormone Magnetic Bead Panel (MERCK), is a multiplex kit used for the simultaneous quantification of T3, T4 and TSH on the Luminex MAGPIX analyser in rat serum. The kits operate by using internally colour-coded microspheres with two fluorescent dyes (green and red), coated with a specific capture antibody. After analyte capture, a biotinylated detection antibody is introduced. The reaction mixture is incubated with Streptavidin-Phycoerythrin conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere. Each individual microsphere is identified, and the result of its bioassay is quantified based on fluorescent reporter signals (3).

Validation Method

The method provided by the manufacturer was validated, with steps such as incubation times and shaking settings optimised where required. Figure 1 provides a brief workflow of the assay procedure. Raw data was captured and processed using xPONENT 4.2 Software.

Calibration Standards and Quality Control (QCs) samples were prepared in buffer and analysed in duplicate. The Calibrators and QCs were also matrix matched via the addition of analyte free matrix (serum) as part of the method procedure, to compensate for any non-specific matrix effects. Control rat serum was analysed as part of parallelism investigations to confirm endogenous levels. Validation samples (VQC) consisted of serum matrix spiked with T3, T4 and TSH at High, Mid and Low concentrations, in order to evaluate accuracy and precision, and were analysed in replicates of 6.

Both intra-batch (single analytical run) and inter-batch (>3 analytical runs) precision and accuracy was evaluated. Parallelism was assessed to determine if dilution of test samples resulted in a biased measurement of the analyte concentration. Where possible 4 - 5 Control serum samples were diluted in analyte-free matrix and the analyte concentrations determined. Measured concentrations (not corrected for dilution) were plotted against the resulting net median fluorescence (MFI) value, along with the Calibration curve.

Assay Limits for all 3 analytes were determined as shown below (4):

- The Limit of Blank (LoB) = 10 replicates of blank. LOB= $\bar{x}$ + 1.645s, where $\bar{x}$ is the mean of the blank results and s is their Standard Deviation (SD).
- The Limit of Detection (LoD) = LOB +1.645s (low conc. Sample).
- The Lower Limit of Quantitation (LLoQ) = lowest concentration at which the analyte could be reliably detected. Acceptance criteria of bias ±20% and precision CV <20%.
- The Upper limit of Quantitation (ULoQ) was the highest used calibrator that returned acceptable results (Accuracy ±25%, and Precision ≤20%).

(FIGURE 1)

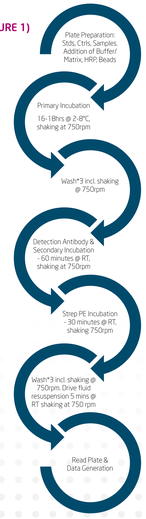

Figure 1: Simplified Assay Workflow for MILLIPLEX MAP kit - Rat Thyroid Hormone Magnetic Bead Panel

Results

For calculated concentrations of samples, the Mean, SD and CV(%) were calculated for each analyte/parameter. Percent change (Relative Error (RE%)) from the nominal value was calculated for each sample mean.

Results are presented below for the following parameters for all 3 analytes: VQC (pooled) Precision and Accuracy (Tables 1-3), Calibration Standards (pooled) Precision and Accuracy (Tables 4-6) and Parallelism (Figures 2-6). Method statistics, including assay limits are also presented (Tables 7-9). Stability assessments (short-term, processed, freeze-thaw and long-term (28 day)), were investigated separate to this study. Extended stability investigations (>3 months) are currently ongoing.

Precision and Accuracy

A CV ≤20 % (≤25 % at LLoQ) and a mean percent change (RE%) of ±20 % (±25 % at ULoQ/LLoQ) was considered acceptable. Intra- and inter-assay precision and accuracy of both VQC and Calibration Standards were within this acceptance criteria.

Pooled values for VQC (n=4) and Calibration Standards (n=8) are shown below.

T3 Parameter	VQC1 5356 pg/mL	VQC2 2645 pg/mL	VQC3 1186 pg/mL	VQC4 630 pg/mL
Mean	5815	3127	1434	677
SD	250	146	167	153
CV (%)	4.3	4.7	11.6	22.7
RE (%)	8.6	18.2	20.9	7.5

Table 1 : T3 Pooled Precision (CV%) and Accuracy (RE%)

T4 Parameter	VQC1 80746 pg/mL	VQC2 58271 pg/mL	VQC3 24938 pg/mL	VQC4 12243 pg/mL	VQC5 6272 pg/mL
Mean	83141	66857	29792	13057	6474
SD	6022	2384	1196	1648	1331
CV (%)	7.2	3.6	4.0	12.6	20.6
RE (%)	3.0	14.7	19.5	6.7	3.2

Table 2 : T4 Pooled Precision (CV%) and Accuracy (RE%)

TSH Parameter	VQC1 1929 pg/mL	VQC2 1015 pg/mL	VQC3 509 pg/mL	VQC4 248 pg/mL	VQC5 124 pg/mL	VQC6 63 pg/mL
Mean	1952	999	509	262	134	67
SD	69	40	38	20	11	5
CV (%)	3.6	4.0	7.5	7.7	8.4	7.4
RE (%)	1.2	-1.6	0.0	5.8	7.7	7.4

Table 3 : Pooled TSH Precision (CV%) and Accuracy (RE%)

T3 Parameter	Cal_1 156.3 pg/mL	Cal_2 312.5 pg/mL	Cal_3 625 pg/mL	Cal_4 1250 pg/mL	Cal_5 2500 pg/mL	Cal_6 5000 pg/mL	Cal_7 10000 pg/mL
Mean	146	327	644	1175	2660	5405	8834
SD	15	39	51	55	159	285	492
CV (%)	10.0	11.9	8.0	4.7	6.0	5.3	5.6
RE (%)	-6.4	4.6	3.0	-6.0	6.4	8.1	-11.7

Table 4 : Pooled T3 Calibration Standard Precision (CV%) and Accuracy (RE%)

T4 Parameter	Cal_1 3125 pg/mL	Cal_2 6250 pg/mL	Cal_3 12500 pg/mL	Cal_4 25000 pg/mL	Cal_5 50000 pg/mL	Cal_6 100000 pg/mL
Mean	3065	6254	12272	24836	56268	92102
SD	84	246	228	856	3995	22269
CV (%)	2.7	3.9	1.9	3.4	7.1	24.2
RE (%)	-1.9	0.1	-1.8	-0.7	12.5	-7.9

Table 5 : Pooled T4 Calibration Standard Precision (CV%) and Accuracy (RE%)

TSH Parameter	Cal_1 62.5 pg/mL	Cal_2 125 pg/mL	Cal_3 250 pg/mL	Cal_4 500 pg/mL	Cal_5 1000 pg/mL	Cal_6 2000 pg/mL	Cal_7 4000 pg/mL
Mean	63	123	251	509	1008	1932	4087
SD	1	3	9	18	13	56	72
CV (%)	1.0	2.7	3.5	3.5	1.2	2.9	1.8
RE (%)	0.5	-1.2	0.5	1.8	0.8	-3.4	2.2

Table 6 : Pooled TSH Calibration Standard Precision (CV%) and Accuracy (RE%)

Parallelism

Parallelism was demonstrated where the sample dilutions were parallel to the Calibration curve, with no apparent trend for increasing or decreasing estimates of analyte concentrations (Figures 2 & 5). Results were also back calculated based on dilution factor and the precision of the diluted samples was determined (Figures 3, 4 & 6). The CV% of the back calculated concentration was acceptable where the CV% was ≤30%, in >50% of the samples in a dilution series .

Parallelism results indicated that native T3, T4 and TSH were detected in the assay with a similar sensitivity to the exogenous thyroid hormone Calibration Standard. Diluted samples were parallel to the Calibration curve indicating a comparable selectivity between analyte and antibody from endogenous sample and Calibration analyte. Parallelism was not achieved in samples diluted greater than 1:18, with a positive shift in relative results observed and high CV (>30 %). A minimum required dilution (MRD) of 1:6 to prevent bead aggregation and matrix effects will be used as recommended by the manufacturer and was confirmed as suitable for use as part of these investigations.

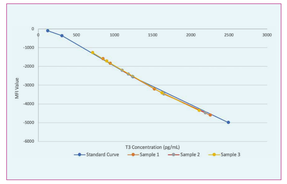

Figure 2: Parallellism T3: Representative Samples & Calibration Curve.

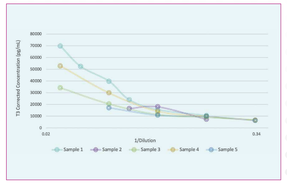

Figure 3: T3 Parallelism: Dilution Corrected (1:3 to 1:48) Control Rat Serum Samples.

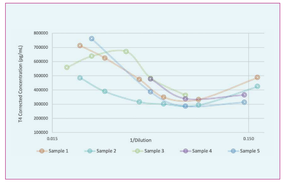

Figure 4: T4 Parallelism: Dilution Corrected (1:6 to 1:56) Control Rat Serum Samples.

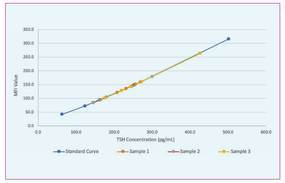

Figure 5: Paralellism TSH: Representative Samples & Calibration Curve

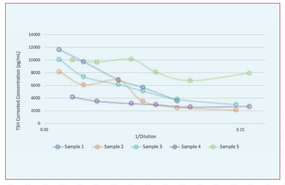

Figure 6: TSH Parallelism: Dilution Corrected (1:6 to 1:56) Control Rat Serum Samples.

Method Statistics and Comparison

The determined limits (LoB, LoD, LLoQ, ULoQ) that met the acceptance criteria are detailed below, along with validated manual ELISA kits previously in use (Tables 8-10). For LLoQ determination all analytes met the precision and bias criteria (Table 7). As a minimum required dilution of 1:6 was determined for serum samples, LLoQ and ULoQ values have been corrected to reflect this, and in order to apply the dilution corrected nominal value to any test results <LLoQ or >ULoQ.

Parameter	T3 (625 pg/mL)	T4 (3125 pg/mL)	TSH (62.5 pg/mL)
Mean (pg/mL)	644	2982	58
SD	81	430	5
CV (%)	12.5	14.4	8.4
Bias (%)	3.0	-4.6	-7.2

Table 7 : LLoQ Precision (CV%) and Bias (%)

T3 Parameter	Manual ELISA[1]	MILLIPLEX
LoD	480 pg/mL	194 pg/mL
LLoQ	1050 pg/mL	3750 pg/mL
ULoQ	7500 pg/mL	60000 pg/mL
Sample Volume	50 µL	12 µL

Table 8 : Assay Limits and Sample Volumes for T3 manual ELISA and MILLIPLEX

[1] Manual ELISA unacceptable due to insufficient levels of accuracy / precision.

T4 Parameter	Manual ELISA	MILLIPLEX
LoD	0.23 pg/mL	752 pg/mL
LLoQ	6100 pg/mL	18750 pg/mL
ULoQ	250000 pg/mL	600000 pg/mL
Sample Volume	20 µL	12 µL

Table 9 : Assay Limits and Sample Volumes for T4 manual ELISA and MILLIPLEX

TSH Parameter	Manual ELISA	MILLIPLEX
LoD	100 pg/mL	16 pg/mL
LLoQ	1000 pg/mL	375 pg/mL
ULoQ	80000 pg/mL	24000 pg/mL
Sample Volume	50 µL	12 µL

Table 10 : Assay Limits and Sample Volumes for TSH manual ELISA and MILLIPLEX

Conclusion

The methodology was able to determine concentrations of T3, T4 and TSH within the working range of the assay and was deemed suitable for the measurement of serum samples from both parental and juvenile rats. Precision and accuracy were deemed acceptable for all analytes for both multiplex and singleplex formats and capable of identifying group trends and changes in analyte concentration.

As a result, OECD developmental and reproductive toxicology guidelines 421, 422, 414 and 443 which include an evaluation of thyroid function can be directly supported. The low sample volume (12 µL) improves over traditional ELISA methodology (120 µL). This has allowed for the implementation of the 3Rs (replace, reduce, refine) by reducing the volume of serum required by 90%.

Future method optimisation will focus on additional limit testing and MRD using native samples with high concentrations of analyte as part of extended parallelism assessment.

References

(1) Kacheyaverko O, et al. Report from the BfR expert hearing on practicability of hormonal measurements: recommendations for experimental design of toxicology studies and integrated hormonal endpoints. Archives of Toxicology 2019; 93:1157-1167.
(2) Yang D, et al. Endocrine-disrupting chemicals: Review of Toxicological Mechanisms using Molecule Pathway Analysis. Journal of Cancer Prevention 2017; vol. 20, No.1: 12-24.
(3) MILLIPLEX MAP Kit - Rat Thyroid Hormone Magnetic Bead Panel, kit insert, MERCK (MILLIPORE), Rev 02-Jun-2017.
(4) Armbruster DA, and Pry T. Limit of Blank, Limit of Detection and Limit of Quantitation. Clin Biochem Rev. 2008;29:549-552.

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