Development of an Early Stage Biomarker within Discovery: Considerations for Assay Requirements

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Contents

Part 1

+ Biology of Thyroid Hormones
+ Reasons for Measuring Thyroid hormones

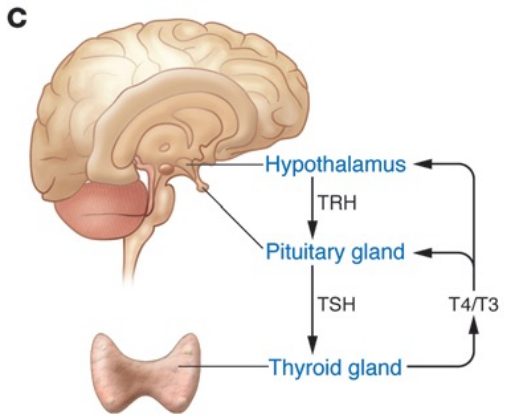
Part 2

+ History of Assay Development
+ Issues during Assay Development
+ Future considerations for the Assay going ‘Back to the Future’.

Preparing For the Analysis

Biology of Thyroid Hormones

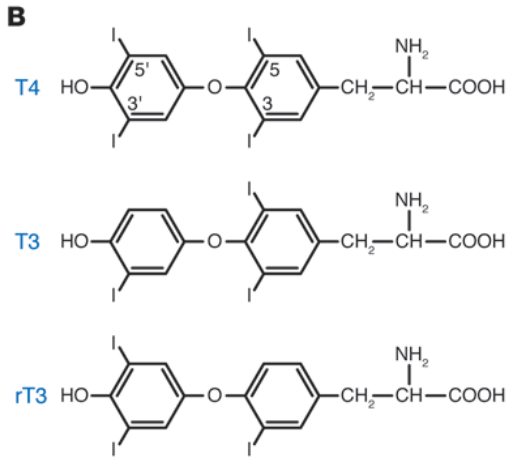
- Thyroid hormones regulate the whole body metabolism having an effect on every cell in the body.
- For example regulating
  - Breathing
  - Heart rate
  - Nervous system
  - Muscle strength
  - Much more

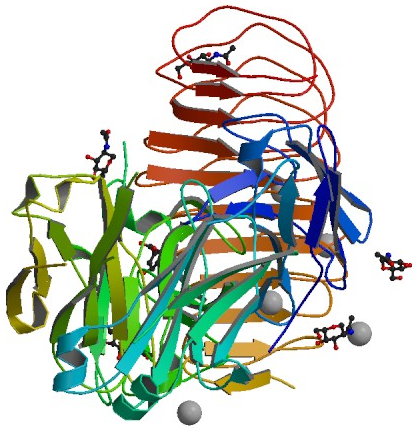


- Levels of Thyroid hormones are tightly regulated by the hypothalamic-pituitary – thyroid axis
  - TSH being secreted in response to TRH with Somatostatin inhibiting the release of TSH

Thyroid Hormones

- Three key hormones: T3, T4 and TSH
- T3 is more active than T4, although T4 is more abundant
- 99% of T3 and T4 is bound to plasma protein
- T4 is converted to T3 by deiodinase
- TSH is released by the Pituitary gland in response to TRH

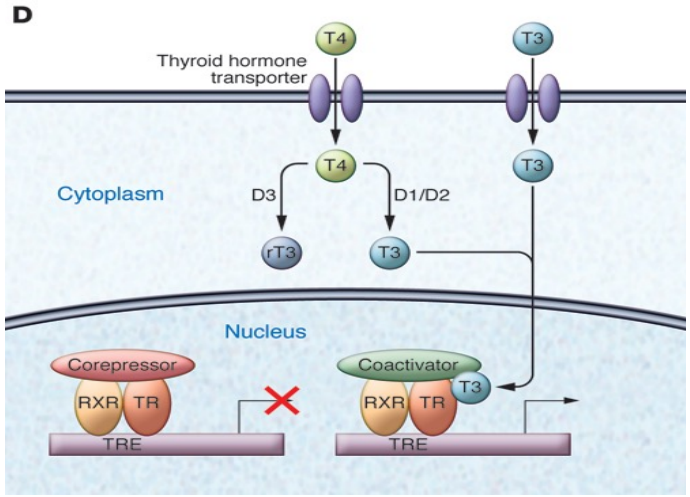




TSH

How Thyroid Hormones Regulate Metabolism

- T3 and T4 are transported into the cell.
- Soluble Thyroid hormone receptors bind T3 and T4 and transport to the nucleus.
- Coactivators bind to the complex and initiate gene transcription.



Reasons for Measuring

- Thyroid hormones used for PD and Safety Biomarkers
- Majority of the projects we are involved in are for safety endpoints
- Used for both In-vitro and in-vivo models
- Data used for internal Discovery assessment, but in addition thinking ahead beyond Discovery
  - Part of Pre-Clinical regulations which are ever changing
  - OECD 441 and 442
  - Initially adult assessment but now measuring levels in pups.

PD and Toxicological effects

- Changes in Thyroid regulation have a serious impact on development and metabolic regulation.
- Mainly due to two classes of effect
  - Thyroid hormone synthesis and regulation
  - Thyroid hormone mechanism e.g. transport, cellular uptake.
- When asked to develop a generic screening assay many factors still need to be considered and questions asked
- Initially we were asked for screening of the hormones in normal adult Rats

Assay Options

- RIA
  - Large sample volume
  - limitations on kits available
- In house development using Gyrolab or MSD
  - Large time investment
  - Gyrolab ideal for pre-clinical samples
  - MSD and Gyrolab generally have good sensitivity
- Luminex Multiplex
  - Low sample volume and multiplex therefore less processing time.
- Individual ELISA's for T3, T4 and TSH
  - High cost and large sample volume required

What was requested by the projects

- Initially screening of Thyroid hormones in adult Rats using an Immunoassay technique.
  - Reason for the request of an Immunoassay was historic data using RIA
    - RIA not sensitive enough and used large volumes therefore Rat samples had to be pooled.
- However as the project progressed they then considered effects in young animals and pups.
- No discussion on levels or consideration for animal maturity and Thyroid hormone development.
  - What was really required of the assay?

Systematic Analytical Validation

Table 4. Method-performance characteristics to be validated for categories of validation.

Performance characteristic	Manufacturer's validation	Manufacturer's validation with in-house kit verification*	Exploratory method validation	Advanced method validation	CLIA validation‡
Dynamic range (LLOQ, ULOQ)	No	No	Yes	Yes	Yes
Sensitivity	No	No	Yes	Yes	Yes
Curve fitting	No	Yes	Yes	Yes	Yes
Selectivity and specificity	No	No	Yes	Yes	Yes
Parallelism	No	No	Yes	Yes	No
Dilutional linearity	No	No	Yes	Yes	Yes
Precision and accuracy (analytical)	No	Yes	Yes	Yes	Yes
Relative accuracy/recovery (biological)	No	No	Yes	Yes	No
Robustness	No	No	No	Yes	No
Sample handling and collection, processing, storage and analyte stability	No	No	Yes	Yes	Yes
Reportable range	No	No	Dynamic range	Dynamic range	Yes
Reference interval	No	No	Initiate	Yes	Yes

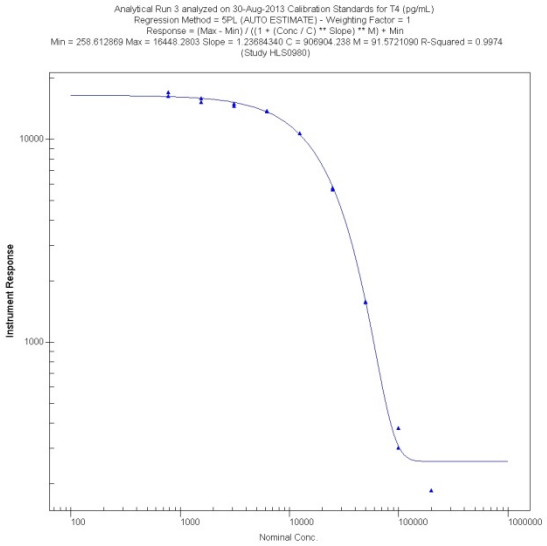
**Manufacturer validations are not standardized. The degree of verification is guided by the package insert and the intended use of the study data.*
‡Some performance characteristics terms may vary for CLIA validations.
CLIA: Clinical Laboratory Improvement Amendments; LLOQ: Lower limit of quantitation; ULOQ: Upper limit of quantitation.

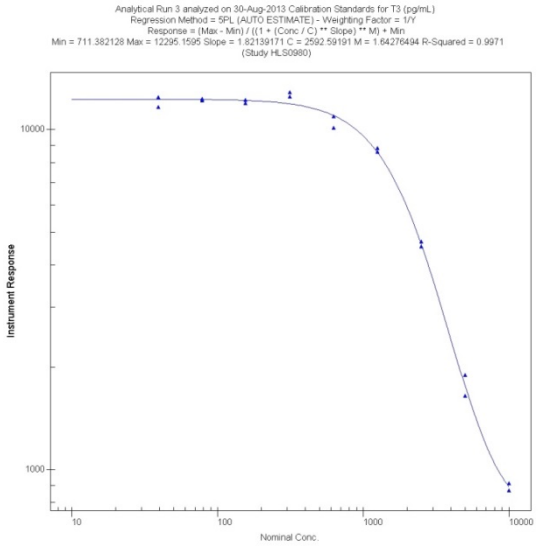
Notwatzke, cole & Bowsher, Bioanalysis 2010 2 (2) 237 - 243

Analytical Challenges

Initial Phase

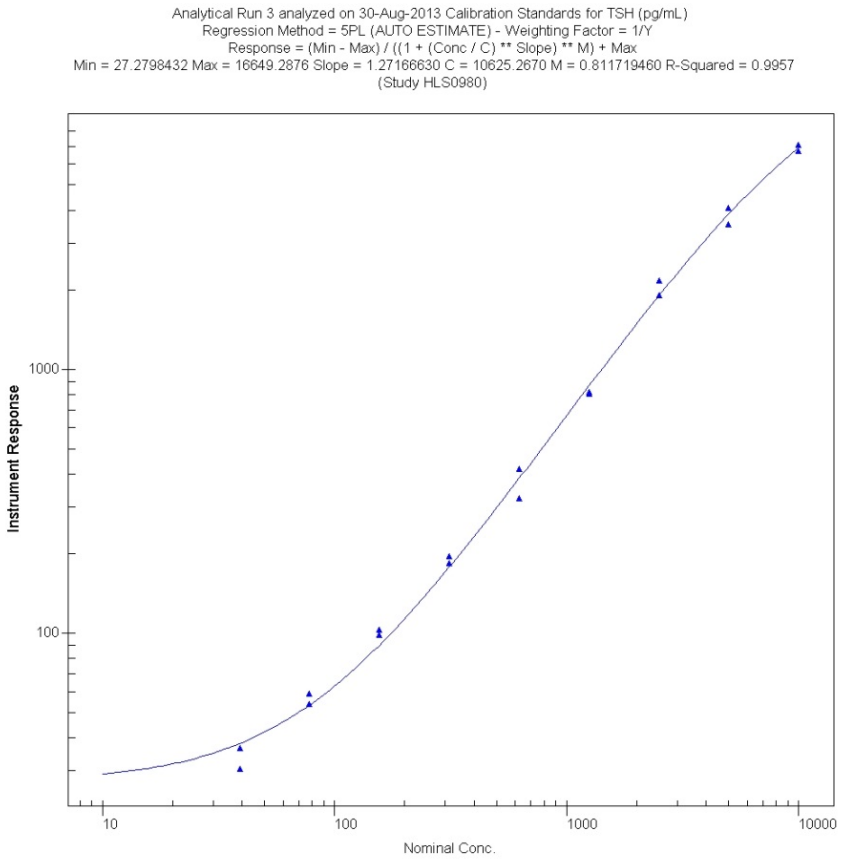
- Request to measure T3, T4 and TSH in Rat Plasma and Serum?
- Filter based bead assay
- Ordered Milliplex Luminex kit which had all 3 analytes
  - Only TSH worked to acceptable levels
  - CV and RE issues for T3 and T4
  - T3 and T4 competitive assays





Initial Phase – TSH Data

+ More defined curve covering the dynamic range required



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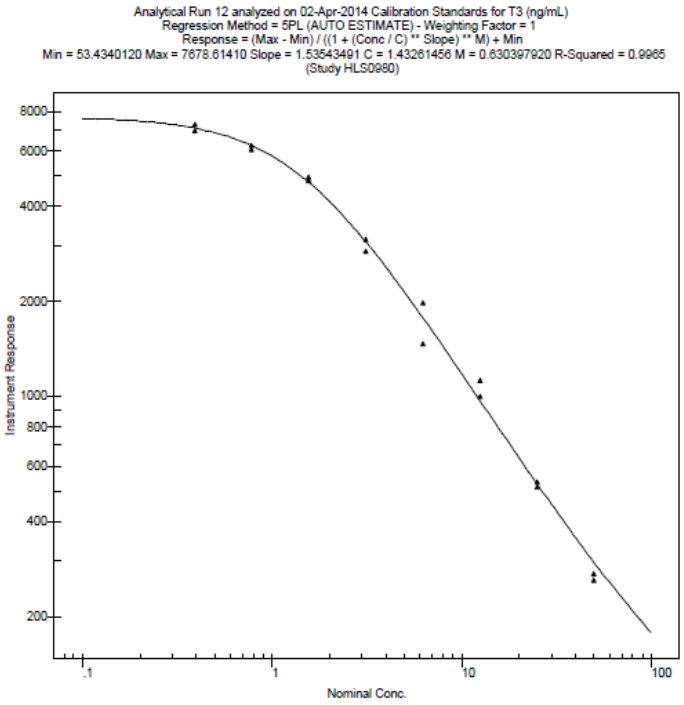
T3 and T4 Assay – Further Development

- Used a new magnetic kit for T3 and T4
  - Performed well
  - Sensitivity issues
  - Neat samples
  - Endogenous levels were at the bottom of the calibration curve
  - Looked at extraction
  - Can measure levels in adult animals but not in Pups.

T3 - Magnetic Luminex Kit

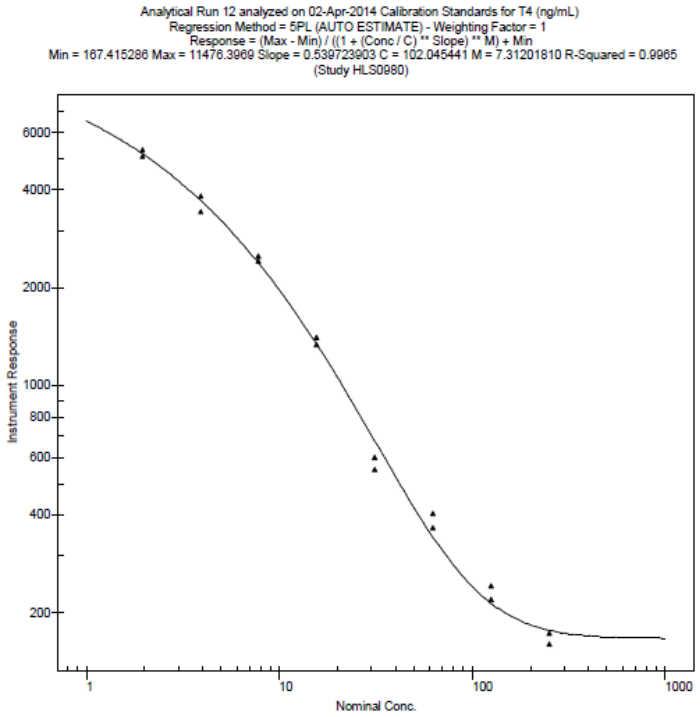


Run number	Concentration (ng/mL) 0.391	0.781	1.56	3.13	6.25	12.5	25.0	50.0
12	0.372	0.826	1.49	3.24	6.63	11.4	25.2	57.3
14	0.356	0.772	1.61	2.95	6.36	13.3	26.7	44.3
15	0.389	0.785	1.61	3.00	6.43	12.7	24.3	50.7
16	0.406	0.741	1.65	3.09	6.10	13.1	25.5	46.7
17	0.404	0.735	1.62	3.10	5.96	13.5	27.6	42.6
20	0.360	0.802	1.54	3.16	6.45	11.5	27.3	50.6
21	0.396	0.769	1.58	3.10	6.15	13.0	25.8	46.3
22	**0.516**	0.802	1.52	2.88	6.91	13.9	22.3	*28.1
23	**0.640**	0.703	1.62	3.08	6.04	13.6	26.7	43.5
24	0.397	0.763	1.60	3.20	5.90	12.8	27.1	48.7
25	0.383	0.800	1.52	3.23	6.14	12.3	26.7	48.5
26	0.397	0.777	1.57	3.15	6.21	12.6	24.8	50.6
29	0.376	0.789	1.65	2.98	6.19	13.3	25.8	46.4
Mean	0.415	0.774	1.58	3.09	6.27	12.8	25.8	48.0
SD	0.0785	0.0330	0.0519	0.111	0.284	0.768	1.47	4.02
CV (%)	18.9	4.3	3.3	3.6	4.5	6.0	5.7	8.4
RE (%)	6.0	-0.9	1.3	-1.4	0.3	2.5	3.2	-4.0
N	13	13	13	13	13	13	13	12

Bold Result from one replicate only
*technical error during analysis

Sample identification	Observed concentration (ng/mL)
C14/00620	2.46
C14/00621	1.93
C14/00622	1.39
C14/00623	1.78
C14/00624	5.33
C14/00625	2.43
C14/00626	1.69
C14/00627	1.73
C14/00628	1.33
C14/00629	2.58

T4 – Magnetic Kit



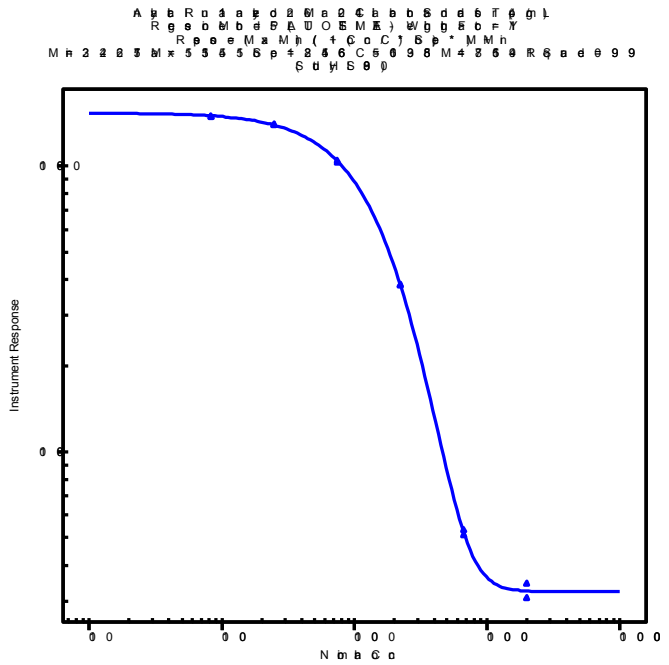
Run number	Concentration (ng/mL) 1.95	3.91	7.81	15.6	31.3	62.5	125	250
12	1.93	4.09	7.50	15.3	36.6	55.1	109	**313**
14	2.97	3.91	8.02	14.2	32.7	76.2	130	193
15	1.98	3.90	8.02	14.8	32.3	65.1	133	213
16	1.95	3.89	7.92	15.5	30.7	67.2	135	212
17	3.05	3.90	7.95	15.0	31.2	70.4	135	203
20	1.90	4.03	7.63	15.8	33.2	53.0	144	380
23	1.94	4.01	7.51	16.5	30.1	61.8	131	250
24	**2.54**	3.93	7.80	15.1	30.6	75.3	131	198
29	1.91	4.29	7.47	15.1	33.9	63.1	120	236
Mean	2.24	3.99	7.76	15.2	32.3	65.2	129	244
SD	0.481	0.132	0.232	0.645	2.04	8.07	10.1	63.0
CV (%)	21.5	3.3	3.0	4.2	6.3	12.4	7.8	25.8
RE (%)	14.8	2.1	-0.7	-2.3	3.3	4.4	3.5	-2.4
N	9	9	9	9	9	9	9	9

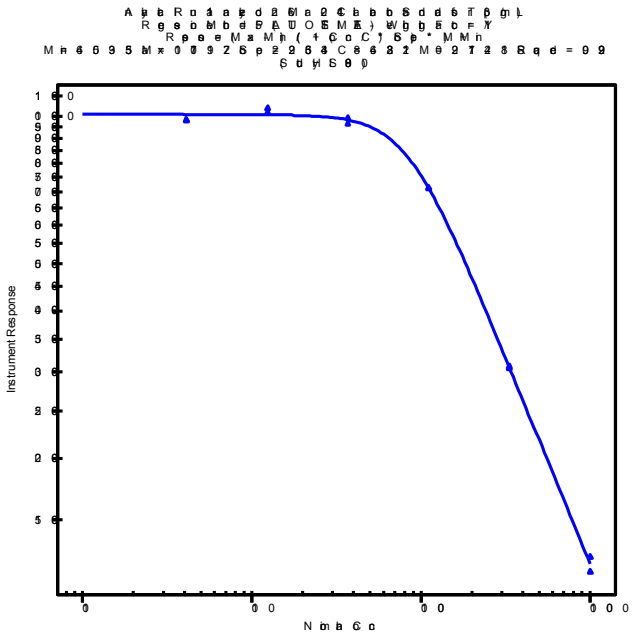
Bold Result from one replicate only

Sample identification	Observed concentration (ng/mL)
C14/00620	11.1
C14/00621	19.6
C14/00622	15.9
C14/00623	12.8
C14/00624	30.5
C14/00625	23.2
C14/00626	15.6
C14/00627	14.5
C14/00628	23.9
C14/00629	18.6

T3 and T4 Development Continued

- Flat curves seen and not resolved
- Matrix effects still an issue even with 1:50 dilution
- Luminex unable to read some wells giving no results for some and good results for others.





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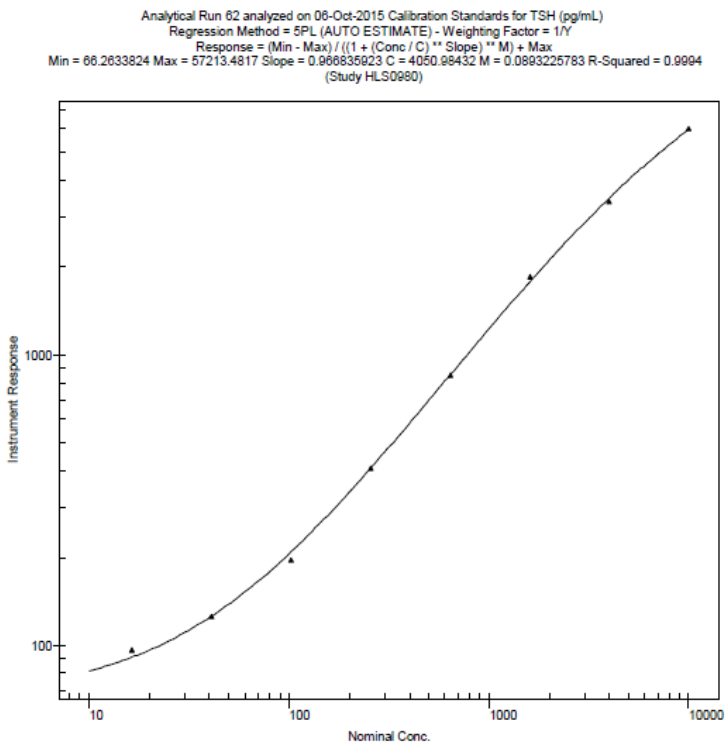
Pushing for Sensitivity

- Truncated the lower end of the curve but unable to get below 41 pg/ml



Run number	Concentration (pg/mL) 4.57	13.79	41.2	123	370	1110	3330	10000
40	4.77	13.5	41.1	122	378	1080	3380	9960
41	2.83	15.3	42.1	125	365	CV	3330	10000
42	NP	15.9	39.5	125	409	1040	3390	9960
43	4.13	6.95	43.7	137	373	1080	3370	9980
46	6.11	14.2	40.0	115	381	1120	3300	10000
47	6.34	14.7	39.3	114	387	1100	3330	10000
48	NP	13.3	41.0	143	351	1110	3340	9970
51	8.57	10.1	39.0	126	370	CV	3330	10000
52	3.74	11.6	40.4	134	375	1080	3370	9980
53	NP	12.6	43.5	137	380	1060	3400	9920
54	5.74	13.4	41.5	117	383	1100	3340	10000
55	3.57	13.3	38.4	138	358	1110	3340	9990
57	NP	10.3	53.1	143	343	1130	3330	9100
58	NP	11.1	47.9	125	381	1080	3390	9750
59	5.05	11.2	39.9	134	362	1110	3340	9990
60	NP	12.9	53.4	125	355	1120	3330	10000
61	3.00	16.1	44.4	114	373	1120	3300	10000
Mean	4.90	12.7	42.8	128	372	1100	3350	9970
SD	1.71	2.35	4.58	9.82	15.6	25.3	30.3	60.9
%CV	35.0	18.5	10.7	7.7	4.2	2.3	0.9	0.6
%RE	7.1	-7.1	4.0	4.0	0.5	-1.3	0.5	-0.3
N	11	17	17	17	17	15	17	17

Run number	Concentration (pg/mL) 16.4	41.0	102	256	640	1600	4000	10000
62	20.0	40.8	92.5	255	639	1680	3840	10100
63	14.0	43.7	103	259	626	1620	3980	10000
66	13.5	39.0	112	258	616	1640	3970	10000
67	9.53	43.4	112	265	591	1690	3910	10100
68	13.2	42.4	108	249	648	1590	4020	9980
71	16.0	39.1	108	258	621	1630	3980	10000
Mean	14.4	41.4	106	257	624	1640	3950	10000
SD	3.47	2.08	7.36	5.24	19.8	37.6	64.5	54.8
%CV	24.1	5.0	7.0	2.0	3.2	2.3	1.6	0.5
%RE	-12.4	1.0	3.8	0.5	-2.6	2.6	-1.3	0.3
N	6	6	6	6	6	6	6	6

Sample Handling

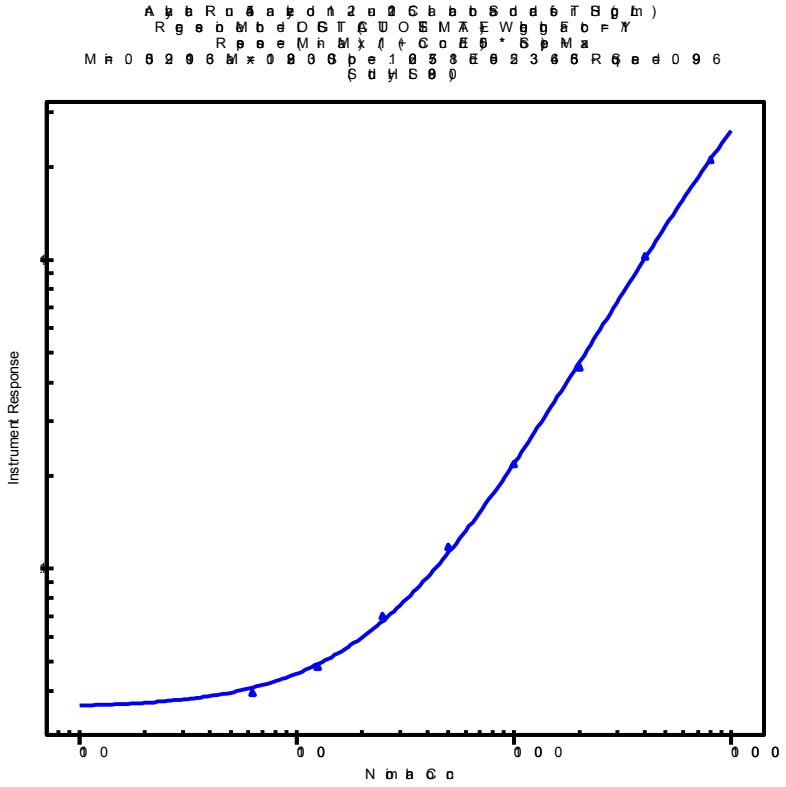
- Processing of the samples from blood acquisition to sampling was shown to be critical
- Variation in observed concentrations without centrifugation of plasma prior to analysis

	Result (pg/mL)				
	Run 40	Run 41 (selectivity)	Run 42 (selectivity)	HLS	Millipore rep
Sample	14-Apr	16-Apr	27-Apr	06-May	06-May
C14/02898	679	1410	1346	1136	1119
C14/02899	1230	473	622	605	576
C14/02900	1032	962	1353	1174	1158
C14/02901	1040	1189	1121	1216	1218
C14/02902	1421	1421	1232	1547	1233
C14/02903	1680	844	824	1102	929
C14/02904	621	555	636	667	663
C14/02905	502	666	405	530	463
C14/02906	873	663	755	599	549
C14/02907	625	749	730	721	749
C14/02119 (Pool)	Not tested	1445	1007	938	840
C14/02120 (Pool)	Not tested	1635	849	809	891

Further Advancement in Kits

- Magnetic pituitary kit (currently in use)
  - TSH worked in this kit
  - Endogenous levels in the middle of the curve
- New 3 plex kit developed
  - Considered use of this kit as limited on sample volume.
  - Completed a ‘look see’ experiment
  - TSH not suitable in this kit either so no real advancement
- Also had a brief look at Alpco ELISA but assay range was poor and sensitivity of detection in samples was low.

Alpco Kit Performance Data



Sample Identification	Observed Concentration (pg/mL)
C14/00620	<std
C14/00621	<std
C14/00622	2700
C14/00623	<std
C14/00624	<std
C14/00625	<std
C14/00626	<std
C14/00627	4750
C14/00628	<std
C14/00629	4490

<std = Result below the bottom standard point 2500 pg/mL

Other Thoughts

- Did not know levels we were going to assess
  - variable levels reported in the literature
- Maturity of animals
- T3 / T4 bound to proteins and masking epitopes
- Service from provider regards background knowledge
- Rat Spiking material for TSH coming from single world source.
- Route of taking samples is essential as clean samples are required
- Serum vs. Plasma; levels do differ

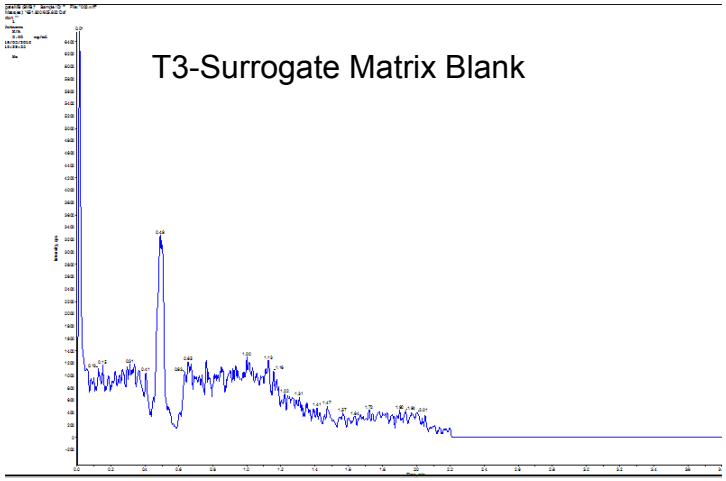
T3 and T4 in rat serum by LC-MS/MS

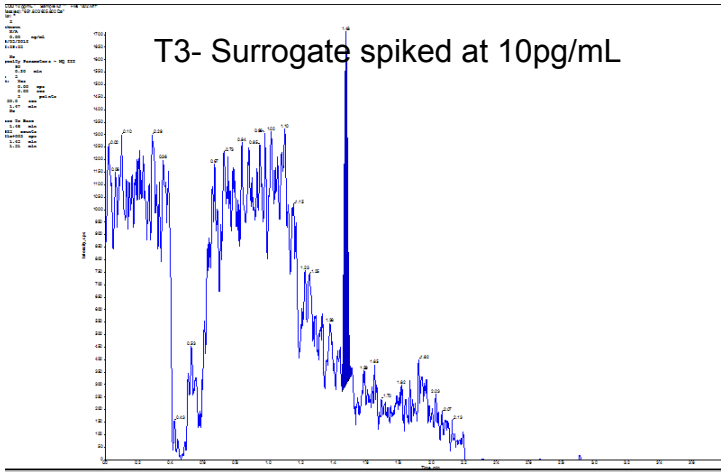
- Feasibility test
  - triple quad MS (Acquity-Sciex 5500)
  - calibration standards in surrogate matrix (BSA/PBS solution)
  - extraction was performed using 50 µL sample volume
  - gradient elution

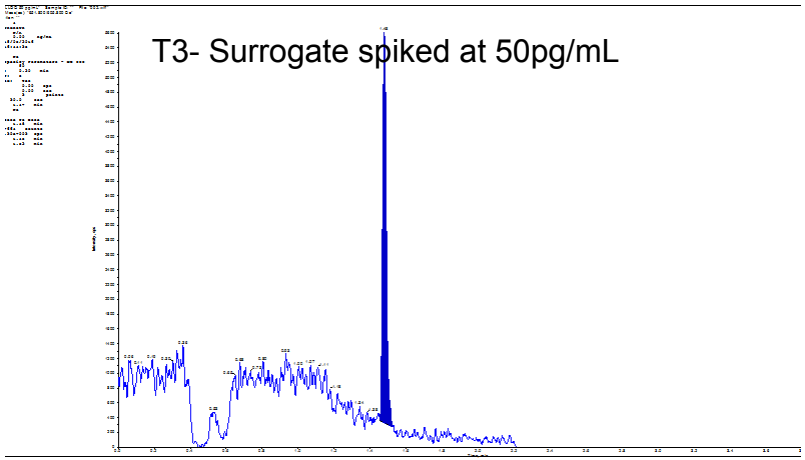
- Scientific validation
  - 25 pg/mL established, lower can be achieved from further method development
  - suitable concentration range selected = 25 to 25000 pg/mL
  - labelled internal standards included
  - LLOQ and LoQC prepared in surrogate matrix
  - *MidQC prepared by blank rat serum diluted with surrogate matrix with ISTD
  - *HiQC prepared by spiking blank rat serum

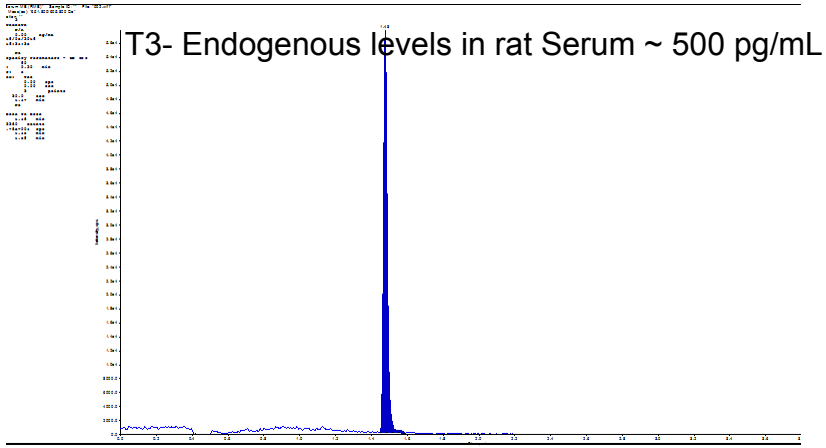
* at least 6 replicates of blank rat serum analysed to determine the endogenous levels to set spiking concentrations

T3 – LC-MS/MS

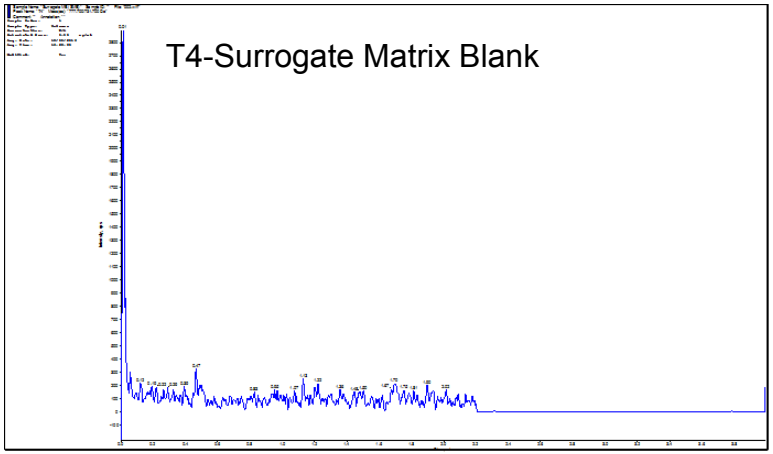

T3-Surrogate Matrix Blank


T3- Surrogate spiked at 10pg/mL

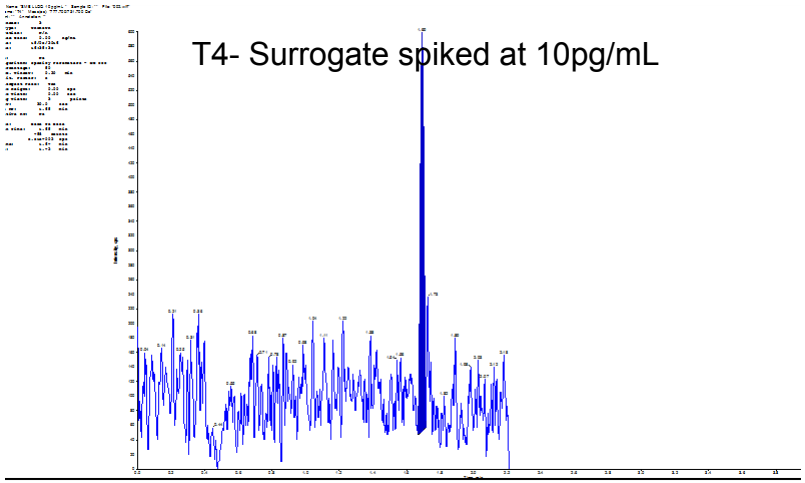

T3- Surrogate spiked at 50pg/mL


T3- Endogenous levels in rat Serum ~ 500 pg/mL

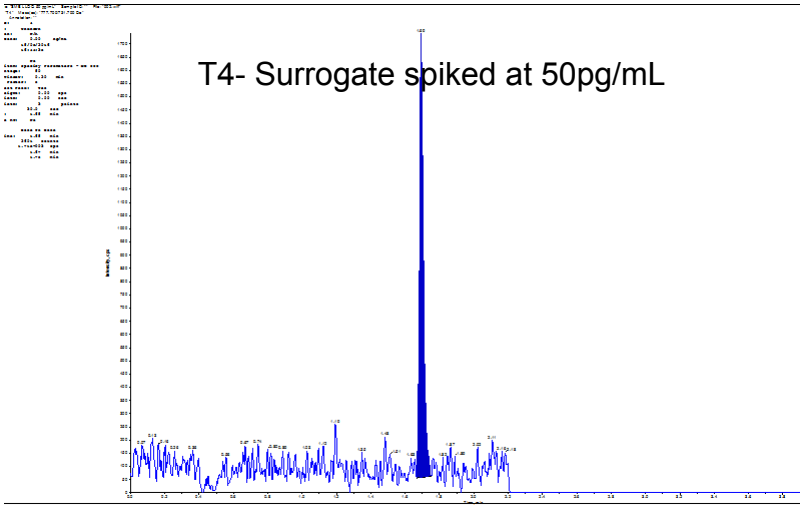
T4 – LC-MS/MS



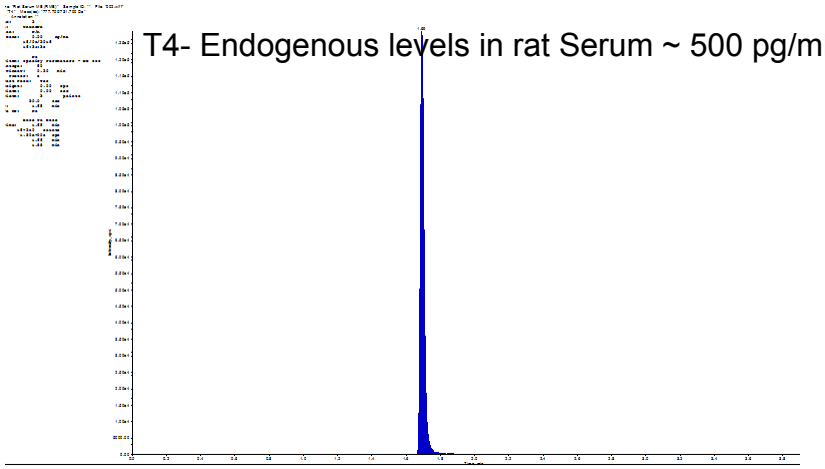
T4-Surrogate Matrix Blank



T4- Surrogate spiked at 10pg/mL



T4- Surrogate spiked at 50pg/mL



T4- Endogenous levels in rat Serum ~ 500 pg/mL

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Consideration on the Use of the Data

Biomarker Strategy

- How much do you complete, how far do you take the development of the assay in the Discovery phase.
- Many teams want to back compare data and be consistent with an assay through a products life cycle.
- Needs in Discovery are different to later stage development
- Consider cost
- Consider Technology

BMV Biomarker Strategy Considerations

- Majority of Biomarker validations are ‘Fit for Purpose’ covering:
  - Selectivity
  - Inter and Intra accuracy and precision
  - Linearity
  - Stability
  - Parallelism
  - Robustness
  - Endogenous assessment
- Lee et al paper and many other papers considering the ‘Fit for Purpose’ strategy

Discovery Technologies & Translation to later stages

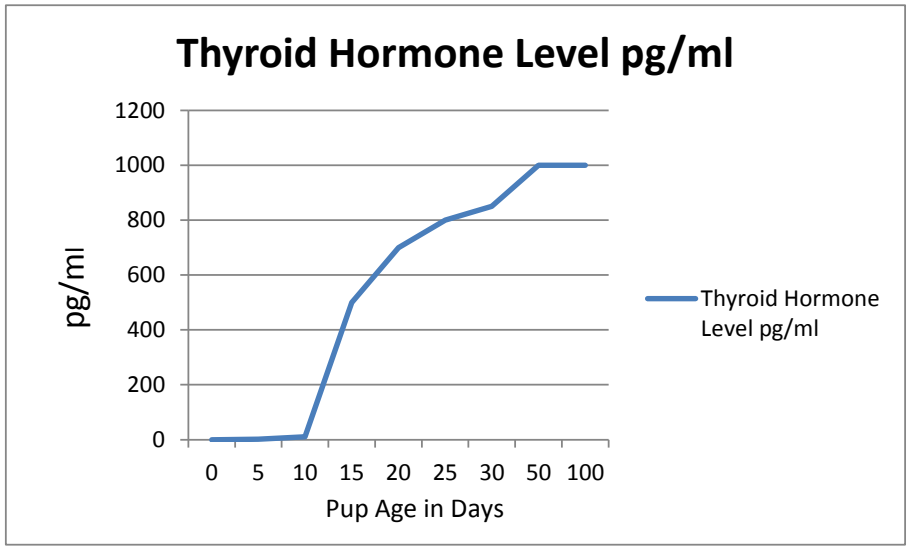
- Cost of analysis a consideration due to some discovery platforms being expensive per test.
- Assay QC's used less routinely in addition to less instrument QC.
- Less standardisation of the operating procedures and less detailed documentation.
- Assay procedure can sometimes be more complex
- Robustness of the technology
  - wider platform base with less regulated control

Disease state and Assay dynamic range

- Assay range should be linked to the Biological range.
- This could differ within Discovery based assays vs. later stage regulated studies.
- Biomarker could be used for mechanistic purposes in one phase and a safety marker in another stage.
  - Increase or decrease in levels to be measured?
- More awareness of the models will develop as the project develops and this may mean that any LLOQ and ULOQ set may change.
- Ranges may change depending on the exact disease state e.g. Asthma vs. COPD.

Understanding of Thyroid Hormone Development and Projects Data Usage

- As the projects progressed there was a realisation for data generated in the in-vivo discovery models that:
  - Thyroid hormones in the Rat develop during the course of growth.
  - PD projects could not potentially utilise the same assay as any future Toxicological studies due to requirement for a different dynamic range.



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Conclusions

- How far do we consider projects when developing assays with Discovery?
  - Which Platform
  - Data Usage
  - Sample Handling
  - Primary aim for the assay
  - Status of the level of the validation applied
  - Switching assays and the comparison of data
  - Disease state and concentration levels at the stages required

Thank you

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