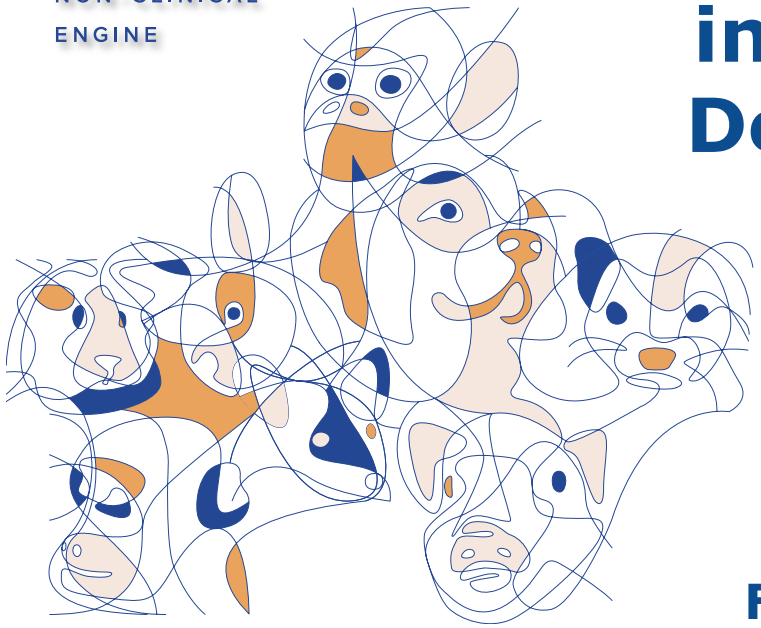
THE NON-CLINICAL ENGINE

Hormone Monitoring in Preclinical Development



Federico Pastori

(LabWare on behalf of ERBC)

EBF – November 2023

LABWARE® Results Count

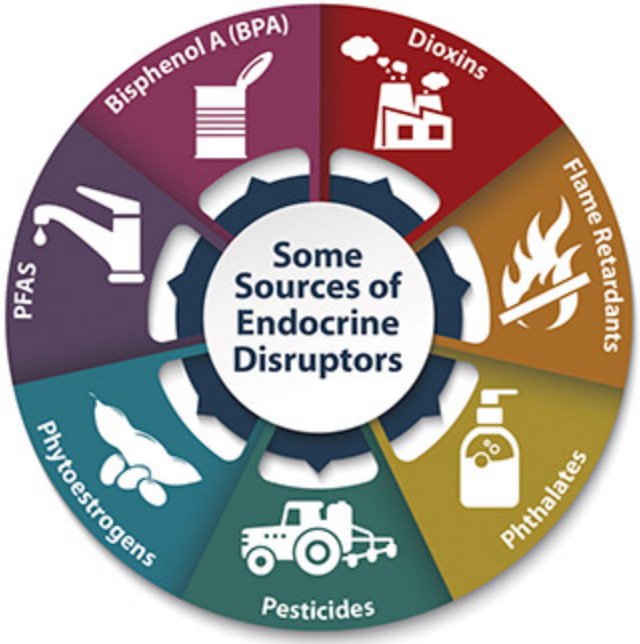
Hormone Monitoring in Preclinical Development

Main objective of the Preclinicaldevelopment is to:

Determine and address the primary toxicity endpoints and all effects correlated with the New Chemical Entities (NCE) administration.

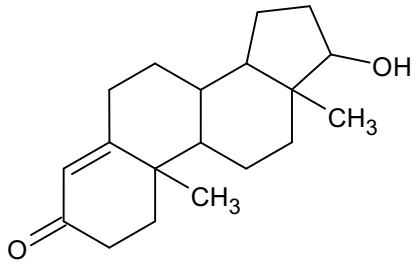
For chemical products, parallel evaluation of the **secondary toxicity effects on the endocrine system** is equally important.

The **panel** of the non-clinical studies to be designed **could be strongly affected** in the case in which a relevant secondary toxic effects occur.



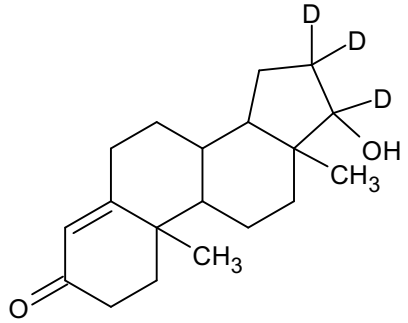
52 weeks Chronic Toxicity Study – Hormone Panel

TESTOSTERONE (TTE)



Molecular Formula: $C_{19}H_{28}O_2$
Formula Weight: 288.431
RDBE: 6
Monoisotopic Mass: 288.20893 Da
Nominal Mass: 288 Da
[M+H]+: 289.216207 Da

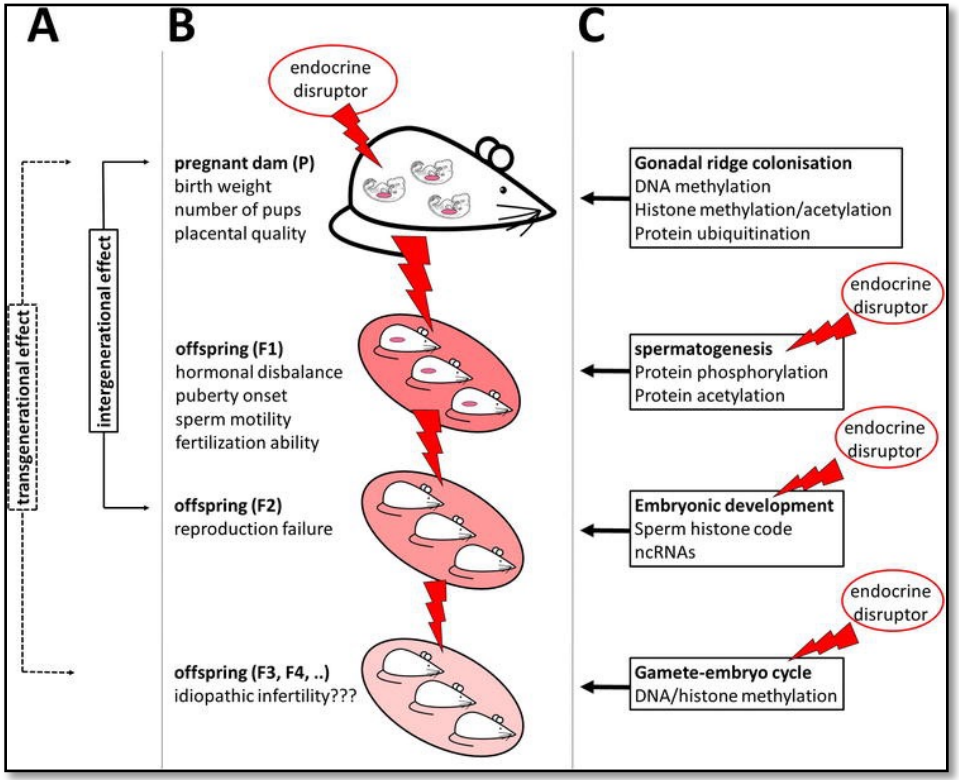
TESTOSTERONE-D3 (TTE-D3,IS)



Molecular Formula: $C_{19}H_{25}D_3O_2$
Formula Weight: 291.449306
RDBE: 6
Monoisotopic Mass: 291.22776 Da
Nominal Mass: 291 Da
[M+H]+: 292.235037 Da

In this case
"**Endocrine disrupting chemicals** (EDCs) can interfere with normal hormonal balance and may exert adverse consequences on humans. **The male reproductive system may be susceptible** to the effects of such environmental toxicants."

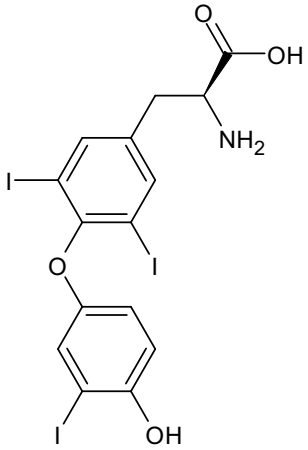
In this case to properly assess the risk for human could be necessary to complete the **Chronic Toxicity** evaluation with a **Reproduction Toxicity Study** (OECD 421 or 423) to evaluate the impact of the disruptor on the reproductive system and the eventual teratogenic effects.



Exposure to endocrine disrupting chemicals and male reproductive health https://www.frontiersin.org/articles/10.3389/fpubh.2014.00055/full

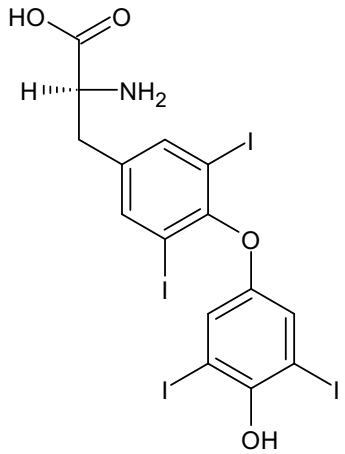
52 weeks Chronic Toxicity Study – Hormone Panel

3,3',5-Triiodo-L-thyronine (T3)



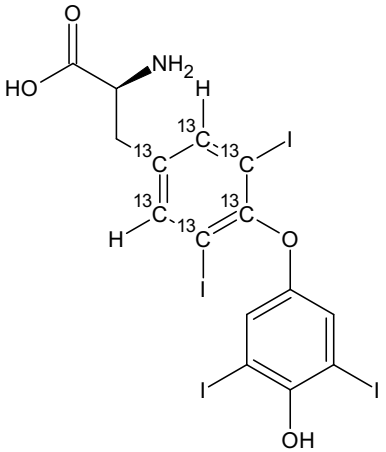
Molecular Formula: $C_{15}H_{12}I_3NO_4$
Formula Weight: 650.97741
RDBE: 9
Monoisotopic Mass: 650.790013 Da
Nominal Mass: 651 Da
[M+H]+: 651.797289 Da

L-Thyroxine (T4)



Molecular Formula: $C_{15}H_{11}I_4NO_4$
Formula Weight: 776.87388
RDBE: 9
Monoisotopic Mass: 776.686648 Da
Nominal Mass: 777 Da
[M+H]+: 777.693924 Da

L-Thyroxine-13C6 (T3-13C6, IS)



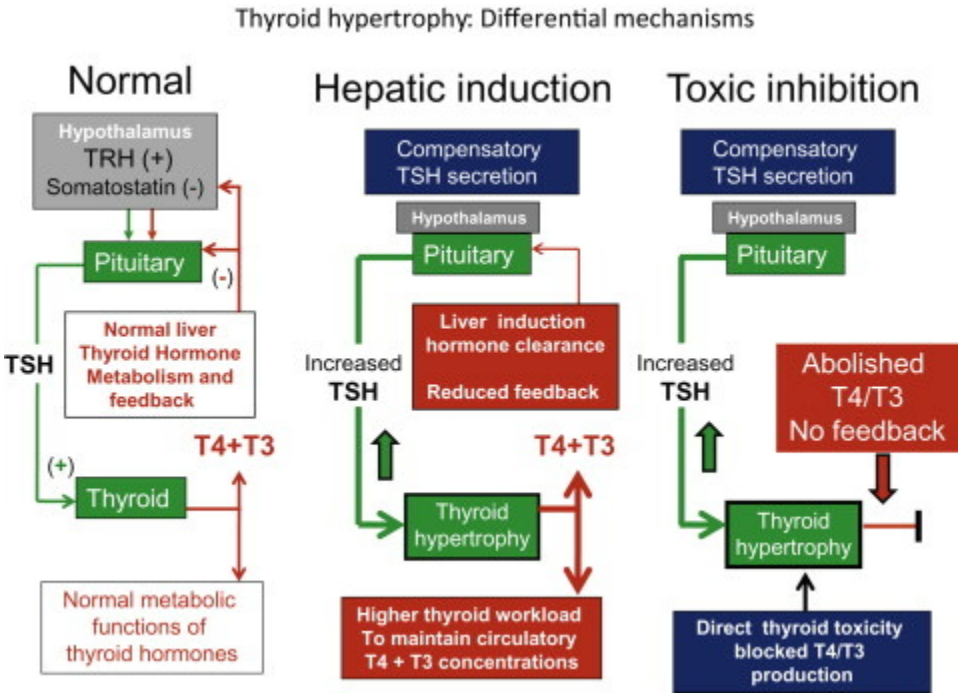
Molecular Formula: $C_{15}H_{11}I_4NO_4$
Formula Weight: 782.828009
RDBE: 9
Monoisotopic Mass: 782.706777 Da
Nominal Mass: 783 Da
[M+H]+: 783.714053 Da

The **profile of TSH, T4, and T3** varies with the mechanism of toxicity affecting the thyroid.

Functional levels of **T3** and **T4** correctly **regulates** the concentration of **TSH**

Depletion of T3 and T4 lead to elevation of TSH could lead to **cancerogenic effect** on the thyroid.

T3 and T4 monitoring helps to properly assess the risk for human.
Chronic Toxicity studies could be completed with **Toxicity Study to evaluate carcinogenic effects**.



Endocrine Disruption of Thyroid Function: Chemicals, Mechanisms, and Toxicopathology
Catherine Sutcliffe and Philip W. Harvey http://dx.doi.org/10.1016/B978-0-12-801139-3.00011-9

Hormone Monitoring in Preclinical Development

Bioanalytical Method was set to determine the unconjugated compounds in serum samples:

Application Ranges:

TTE: 0.1 – 25 ng/mL, ISTD: TTE-D3

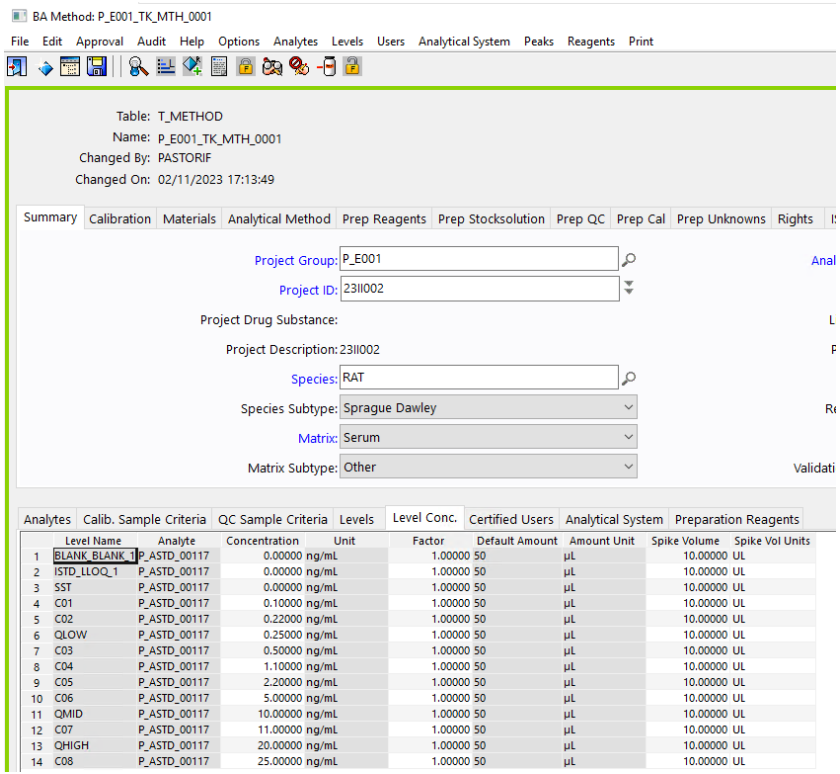
T3: 0.15 – 15 nmol/L, ISTD: T3-13C6

T4: 5 – 125 nmol/L, ISTD: T3-13C6

Matrix: SERUM **Sample Volume:** 50uL

Sample Preparation:

- 50ul Serum sample
- 100ul of Acetonitrile
- Vortex
- Centrifugation 13000 rpm
- Injection



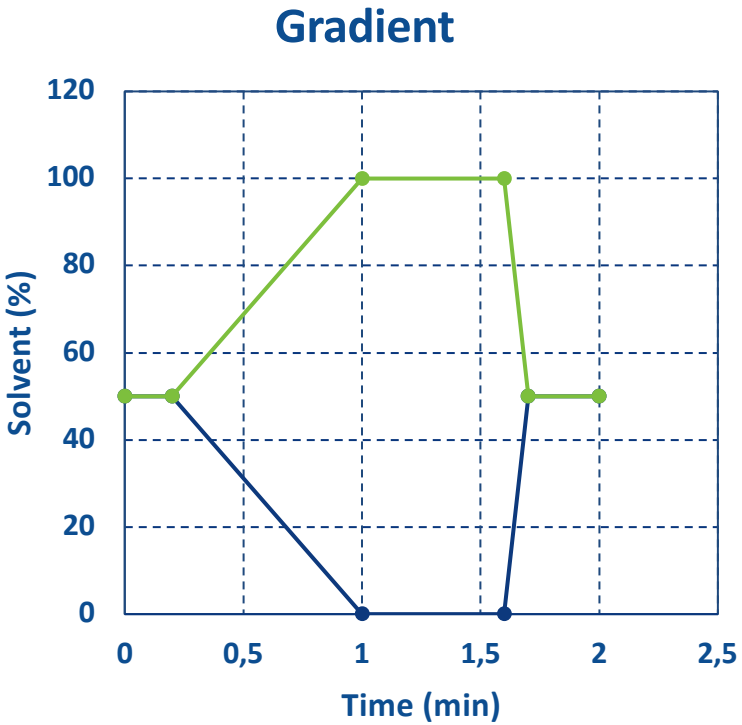
	Level Name	Analyte	Concentration	Unit	Factor	Default Amount	Amount Unit	Spike Volume	Spike Vol Units
1	BLANK_BLANK_1	P_ASTD_00117	0.00000	ng/mL	1.00000	50	µL	10.00000	UL
2	ISTD_LLOQ_1	P_ASTD_00117	0.00000	ng/mL	1.00000	50	µL	10.00000	UL
3	SST	P_ASTD_00117	0.00000	ng/mL	1.00000	50	µL	10.00000	UL
4	C01	P_ASTD_00117	0.10000	ng/mL	1.00000	50	µL	10.00000	UL
5	C02	P_ASTD_00117	0.22000	ng/mL	1.00000	50	µL	10.00000	UL
6	QLOW	P_ASTD_00117	0.25000	ng/mL	1.00000	50	µL	10.00000	UL
7	C03	P_ASTD_00117	0.50000	ng/mL	1.00000	50	µL	10.00000	UL
8	C04	P_ASTD_00117	1.10000	ng/mL	1.00000	50	µL	10.00000	UL
9	C05	P_ASTD_00117	2.20000	ng/mL	1.00000	50	µL	10.00000	UL
10	C06	P_ASTD_00117	5.00000	ng/mL	1.00000	50	µL	10.00000	UL
11	QMID	P_ASTD_00117	10.00000	ng/mL	1.00000	50	µL	10.00000	UL
12	C07	P_ASTD_00117	11.00000	ng/mL	1.00000	50	µL	10.00000	UL
13	QHIGH	P_ASTD_00117	20.00000	ng/mL	1.00000	50	µL	10.00000	UL
14	C08	P_ASTD_00117	25.00000	ng/mL	1.00000	50	µL	10.00000	UL

Chromatography Settings:
ACQUITY UPLC BEH C18 1.7 µm 1x50mm (Waters)

Eluent A: 0.1% Formic acid in water
Eluent B: 0.1% Formic acid in acetonitrile
Injection Volume: 1.5uL
Gradient for analysis of TTE and TTE D3 in rat serum

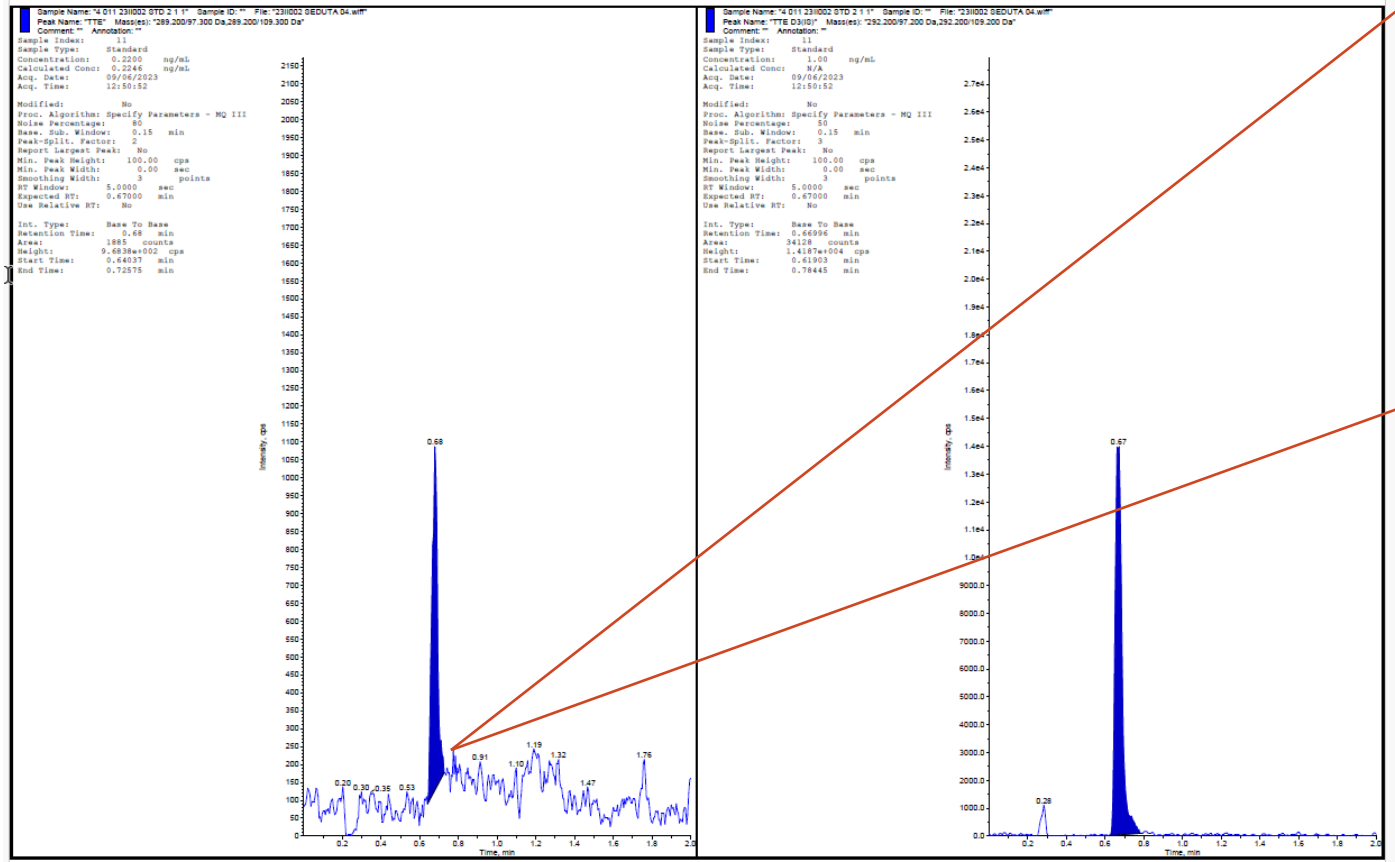
Time (min)	Flow (µL/min)	A (%)	B (%)
0	80	50	50
0.2		50	50
1.0		0	100
1.6		0	100
1.7		50	50
2		50	50

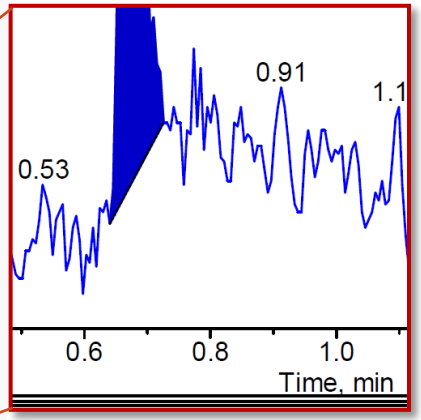
P = 62.5



Great **gain in sensitivity**, due to:
Micro LC M5 (SCIEX), I.D. and **Particle Size** of the column.

Hormone Monitoring in Preclinical Development





Peak Time

t_1-t_0 = 0.74 - 0.63 min = 0.11 min = 6.6 sec

To grant 10 points for a good peak shape acquisition instrument duty cycle time should be less than 660 msec

Hormone Monitoring in Preclinical Development

Low-Resolution MS System:

- No. 2: M3 - API 4000
- No. 1: M5 - API 5500
- No. 2: M5 - API 7500

A comparable sensitivity with RIA obtained with LC-MS will be then used to scale-up the method more sensible MS System, scaling down the Sample Volume

High-Resolution MS Systems:

- No. 1: M5 - API 5600
- No. 2: M5 - API 6600+

The Pilot GLP Study for hormone monitoring was performed on the entry level SCIEX API 4000.

Full Validation: Sample and Data Processing

Bioanalytical process Flow

Validation Protocol Definition

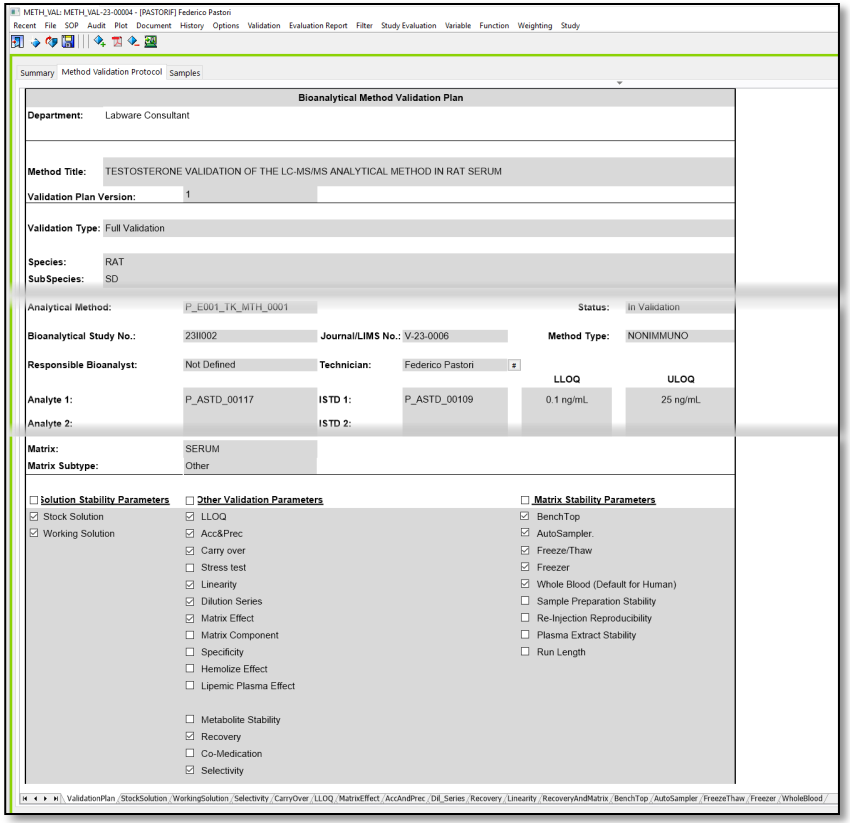
Sample Planning and Login

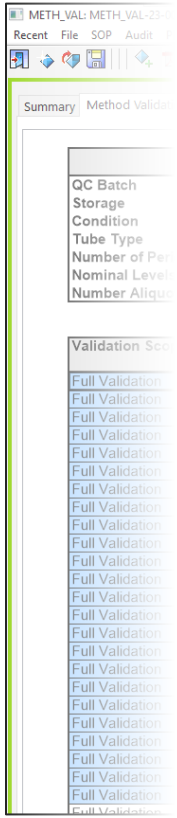
Worklist Preparation

Bi-directional interfacing between LabWare and SCIEX Analyst

Regression in LabWare

Reporting





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Reporting

METH_VAL: METH_VAL-23-00004 - [PASTORIF] Federico Pastori

Recent File SOP Audit Plot Document History Options Validation Evaluation Report Filter Study Evaluation Variable Function Weighting Study

Summary | Method Validation Protocol | Samples

Accuracy and Precision		
QC Batch	Set Batch	QC_2023-FPA-00048
Storage	Set Storage	RT
Condition	Set Condition	NORMAL_LIGHT
Tube Type	Set Tube Type	EP
Number of Periods/Days	Set Number of Periods/Days	3
Nominal Levels	Set Nominal Levels	QLLOQ, QHIGH, QLOW, QMID
Number Aliquots/Replicates	Set Aliquots	6

Validation Scope	Period/Day Number	QC Batch	Nominal Level	Aliquot	Sample Name
Full Validation	1	QC_2023-FPA-00048	QLLOQ	1	V_AP1_QC_2023-FPA-00048_QLLOQ_1
Full Validation	1	QC_2023-FPA-00048	QLLOQ	2	V_AP1_QC_2023-FPA-00048_QLLOQ_2
Full Validation	1	QC_2023-FPA-00048	QLLOQ	3	V_AP1_QC_2023-FPA-00048_QLLOQ_3
Full Validation	1	QC_2023-FPA-00048	QLLOQ	4	V_AP1_QC_2023-FPA-00048_QLLOQ_4
Full Validation	1	QC_2023-FPA-00048	QLLOQ	5	V_AP1_QC_2023-FPA-00048_QLLOQ_5
Full Validation	1	QC_2023-FPA-00048	QLLOQ	6	V_AP1_QC_2023-FPA-00048_QLLOQ_6
Full Validation	1	QC_2023-FPA-00048	QHIGH	1	V_AP1_QC_2023-FPA-00048_QHIGH_1
Full Validation	1	QC_2023-FPA-00048	QHIGH	2	V_AP1_QC_2023-FPA-00048_QHIGH_2
Full Validation	1	QC_2023-FPA-00048	QHIGH	3	V_AP1_QC_2023-FPA-00048_QHIGH_3
Full Validation	1	QC_2023-FPA-00048	QHIGH	4	V_AP1_QC_2023-FPA-00048_QHIGH_4
Full Validation	1	QC_2023-FPA-00048	QHIGH	5	V_AP1_QC_2023-FPA-00048_QHIGH_5
Full Validation	1	QC_2023-FPA-00048	QHIGH	6	V_AP1_QC_2023-FPA-00048_QHIGH_6
Full Validation	1	QC_2023-FPA-00048	QLOW	1	V_AP1_QC_2023-FPA-00048_QLOW_1
Full Validation	1	QC_2023-FPA-00048	QLOW	2	V_AP1_QC_2023-FPA-00048_QLOW_2
Full Validation	1	QC_2023-FPA-00048	QLOW	3	V_AP1_QC_2023-FPA-00048_QLOW_3
Full Validation	1	QC_2023-FPA-00048	QLOW	4	V_AP1_QC_2023-FPA-00048_QLOW_4
Full Validation	1	QC_2023-FPA-00048	QLOW	5	V_AP1_QC_2023-FPA-00048_QLOW_5
Full Validation	1	QC_2023-FPA-00048	QLOW	6	V_AP1_QC_2023-FPA-00048_QLOW_6
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Full Validation	1	QC_2023-FPA-00048	QMID	2	V_AP1_QC_2023-FPA-00048_QMID_2
Full Validation	1	QC_2023-FPA-00048	QMID	3	V_AP1_QC_2023-FPA-00048_QMID_3
Full Validation	1	QC_2023-FPA-00048	QMID	4	V_AP1_QC_2023-FPA-00048_QMID_4
Full Validation	1	QC_2023-FPA-00048	QMID	5	V_AP1_QC_2023-FPA-00048_QMID_5
Full Validation	1	QC_2023-FPA-00048	QMID	6	V_AP1_QC_2023-FPA-00048_QMID_6

Hormone Monitoring in Preclinical Development

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Bioanalytical process Flow

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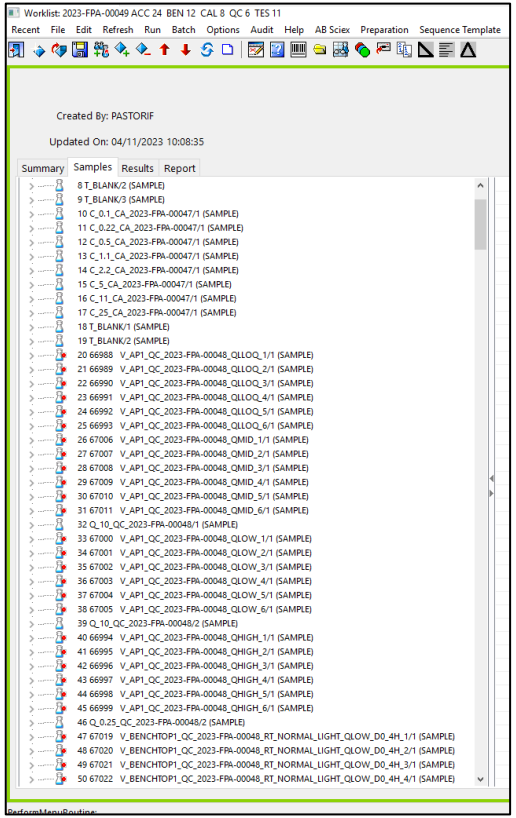

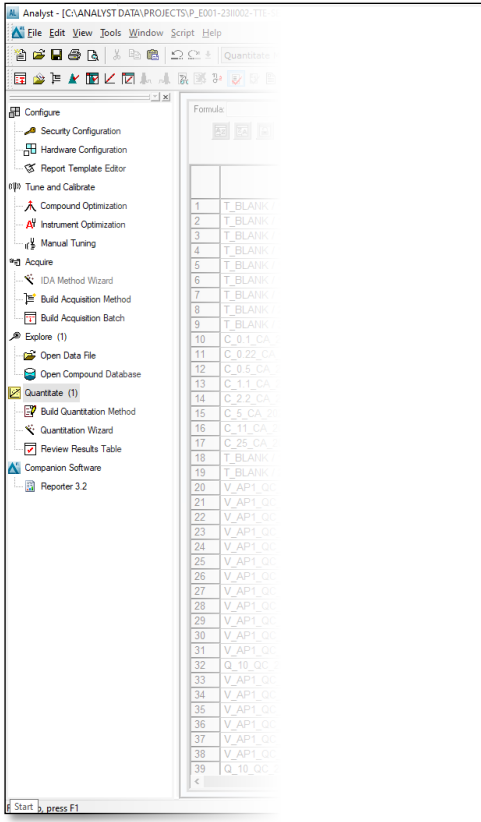

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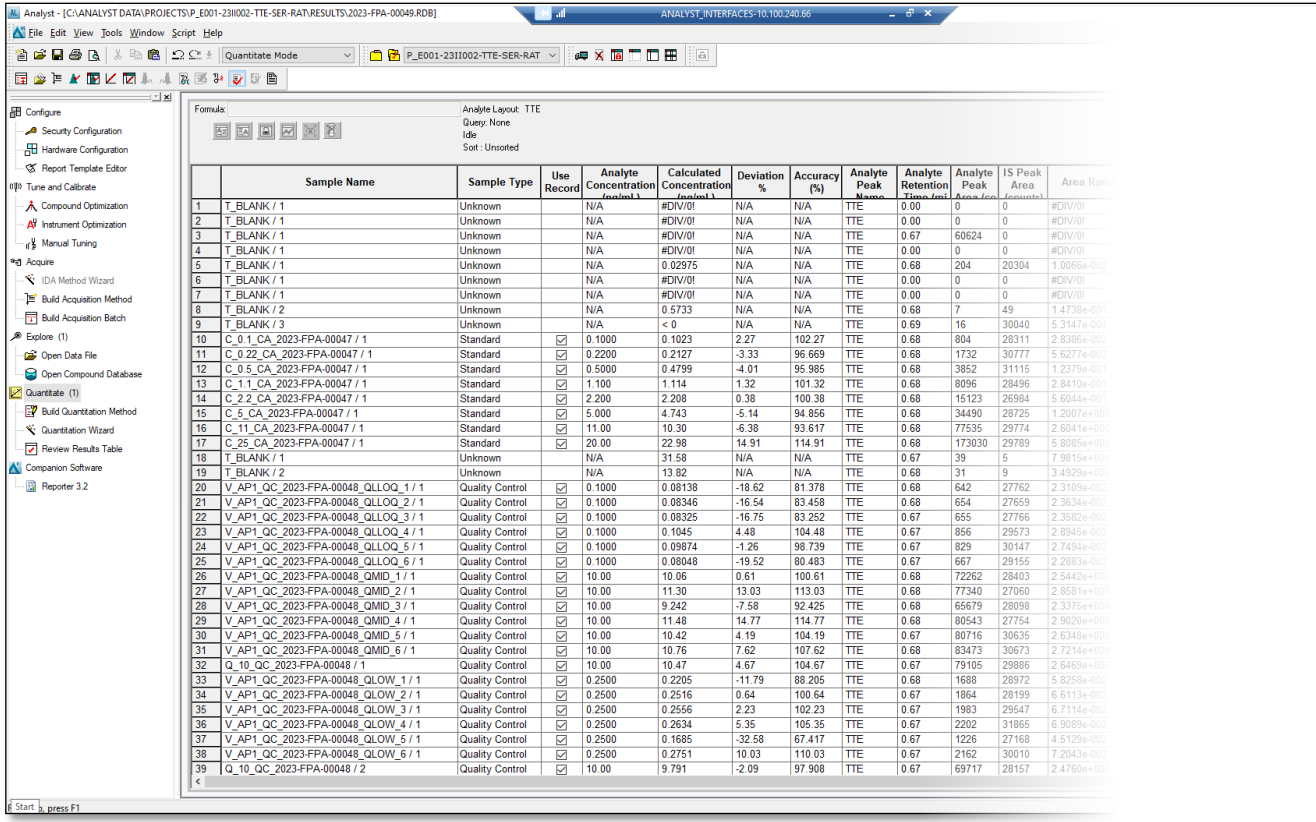


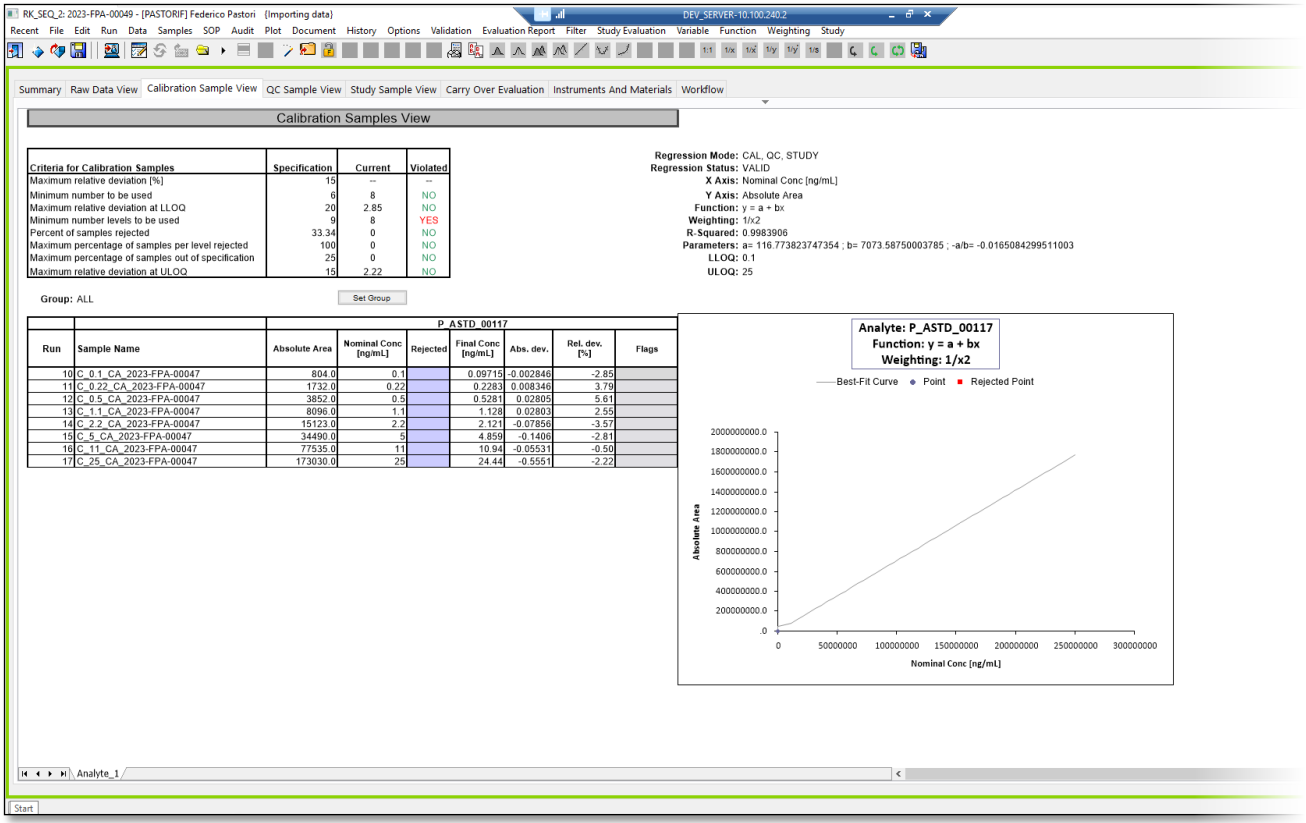
	Sample Name	Sample Type	Use Record	Analyte Concentration (ng/mL)	Calculated Concentration (ng/mL)	Deviation %	Accuracy (%)	Analyte Peak Name	Analyte Retention Time (min)	Analyte Peak Area (counts)	IS Peak Area (counts)	Area Ratio
1	T_BLANK / 1	Unknown		N/A	#DIV/0!	N/A	N/A	TTE	0.00	0	0	#DIV/0!
2	T_BLANK / 1	Unknown		N/A	#DIV/0!	N/A	N/A	TTE	0.00	0	0	#DIV/0!
3	T_BLANK / 1	Unknown		N/A	#DIV/0!	N/A	N/A	TTE	0.67	60624	0	#DIV/0!
4	T_BLANK / 1	Unknown		N/A	#DIV/0!	N/A	N/A	TTE	0.00	0	0	#DIV/0!
5	T_BLANK / 1	Unknown		N/A	0.02975	N/A	N/A	TTE	0.68	204	20304	1.0066e-002
6	T_BLANK / 1	Unknown		N/A	#DIV/0!	N/A	N/A	TTE	0.00	0	0	#DIV/0!
7	T_BLANK / 1	Unknown		N/A	#DIV/0!	N/A	N/A	TTE	0.00	0	0	#DIV/0!
8	T_BLANK / 2	Unknown		N/A	0.5733	N/A	N/A	TTE	0.68	7	49	1.4738e-001
9	T_BLANK / 3	Unknown		N/A	< 0	N/A	N/A	TTE	0.69	16	30040	5.3147e-004
10	C_0.1_CA_2023-FPA-00047 / 1	Standard	☑	0.1000	0.1023	2.27	102.27	TTE	0.68	804	28311	2.8386e-002
11	C_0.22_CA_2023-FPA-00047 / 1	Standard	☑	0.2200	0.2127	-3.33	96.669	TTE	0.68	1732	30777	5.6277e-002
12	C_0.5_CA_2023-FPA-00047 / 1	Standard	☑	0.5000	0.4799	-4.01	95.985	TTE	0.68	3852	31115	1.2379e-001
13	C_1.1_CA_2023-FPA-00047 / 1	Standard	☑	1.100	1.114	1.32	101.32	TTE	0.68	8096	28496	2.8410e-001
14	C_2.2_CA_2023-FPA-00047 / 1	Standard	☑	2.200	2.208	0.38	100.38	TTE	0.68	15123	26984	5.6044e-001
15	C_5_CA_2023-FPA-00047 / 1	Standard	☑	5.000	4.743	-5.14	94.856	TTE	0.68	34490	28725	1.2007e+000
16	C_11_CA_2023-FPA-00047 / 1	Standard	☑	11.00	10.30	-6.38	93.617	TTE	0.68	77535	29774	2.6041e+000
17	C_25_CA_2023-FPA-00047 / 1	Standard	☑	20.00	22.98	14.91	114.91	TTE	0.68	173030	29789	5.8085e+000
18	T_BLANK / 1	Unknown		N/A	31.58	N/A	N/A	TTE	0.67	39	5	7.9815e+000
19	T_BLANK / 2	Unknown		N/A	13.82	N/A	N/A	TTE	0.68	31	9	3.4929e+000
20	V_AP1_QC_2023-FPA-00048_QLLOQ_1 / 1	Quality Control	☑	0.1000	0.08138	-18.62	81.378	TTE	0.68	642	27762	2.3109e-002
21	V_AP1_QC_2023-FPA-00048_QLLOQ_2 / 1	Quality Control	☑	0.1000	0.08346	-16.54	83.458	TTE	0.68	654	27659	2.3634e-002
22	V_AP1_QC_2023-FPA-00048_QLLOQ_3 / 1	Quality Control	☑	0.1000	0.08325	-16.75	83.252	TTE	0.67	655	27766	2.3582e-002
23	V_AP1_QC_2023-FPA-00048_QLLOQ_4 / 1	Quality Control	☑	0.1000	0.1045	4.48	104.48	TTE	0.67	856	29573	2.8945e-002
24	V_AP1_QC_2023-FPA-00048_QLLOQ_5 / 1	Quality Control	☑	0.1000	0.09874	-1.26	98.739	TTE	0.67	829	30147	2.7494e-002
25	V_AP1_QC_2023-FPA-00048_QLLOQ_6 / 1	Quality Control	☑	0.1000	0.08048	-19.52	80.483	TTE	0.67	667	29155	2.2883e-002
26	V_AP1_QC_2023-FPA-00048_QMID_1 / 1	Quality Control	☑	10.00	10.06	0.61	100.61	TTE	0.68	72262	28403	2.5442e+000
27	V_AP1_QC_2023-FPA-00048_QMID_2 / 1	Quality Control	☑	10.00	11.30	13.03	113.03	TTE	0.68	77340	27060	2.8581e+000
28	V_AP1_QC_2023-FPA-00048_QMID_3 / 1	Quality Control	☑	10.00	9.242	-7.58	92.425	TTE	0.68	65679	28098	2.3375e+000
29	V_AP1_QC_2023-FPA-00048_QMID_4 / 1	Quality Control	☑	10.00	11.48	14.77	114.77	TTE	0.68	80543	27754	2.9020e+000
30	V_AP1_QC_2023-FPA-00048_QMID_5 / 1	Quality Control	☑	10.00	10.42	4.19	104.19	TTE	0.67	80716	30635	2.6348e+000
31	V_AP1_QC_2023-FPA-00048_QMID_6 / 1	Quality Control	☑	10.00	10.76	7.62	107.62	TTE	0.68	83473	30673	2.7214e+000
32	Q_10_QC_2023-FPA-00048 / 1	Quality Control	☑	10.00	10.47	4.67	104.67	TTE	0.67	79105	29886	2.6469e+000
33	V_AP1_QC_2023-FPA-00048_QLOW_1 / 1	Quality Control	☑	0.2500	0.2205	-11.79	88.205	TTE	0.68	1688	28972	5.8258e-002
34	V_AP1_QC_2023-FPA-00048_QLOW_2 / 1	Quality Control	☑	0.2500	0.2516	0.64	100.64	TTE	0.67	1864	28199	6.6113e-002
35	V_AP1_QC_2023-FPA-00048_QLOW_3 / 1	Quality Control	☑	0.2500	0.2556	2.23	102.23	TTE	0.67	1983	29547	6.7114e-002
36	V_AP1_QC_2023-FPA-00048_QLOW_4 / 1	Quality Control	☑	0.2500	0.2634	5.35	105.35	TTE	0.67	2202	31865	6.9089e-002
37	V_AP1_QC_2023-FPA-00048_QLOW_5 / 1	Quality Control	☑	0.2500	0.1685	-32.58	67.417	TTE	0.67	1226	27168	4.5129e-002
38	V_AP1_QC_2023-FPA-00048_QLOW_6 / 1	Quality Control	☑	0.2500	0.2751	10.03	110.03	TTE	0.67	2162	30010	7.2043e-002
39	Q_10_QC_2023-FPA-00048 / 2	Quality Control	☑	10.00	9.791	-2.09	97.908	TTE	0.67	69717	28157	2.4760e+000

Hormone Monitoring in Preclinical Development

Full Validation: Sample and Data Processing

Bioanalytical process Flow

- Validation Protocol Definition
- Sample Planning and Login
- Worklist Preparation
- Bi-directional interfacing between LabWare and SCIEX Analyst
- Regression in LabWare
- Reporting



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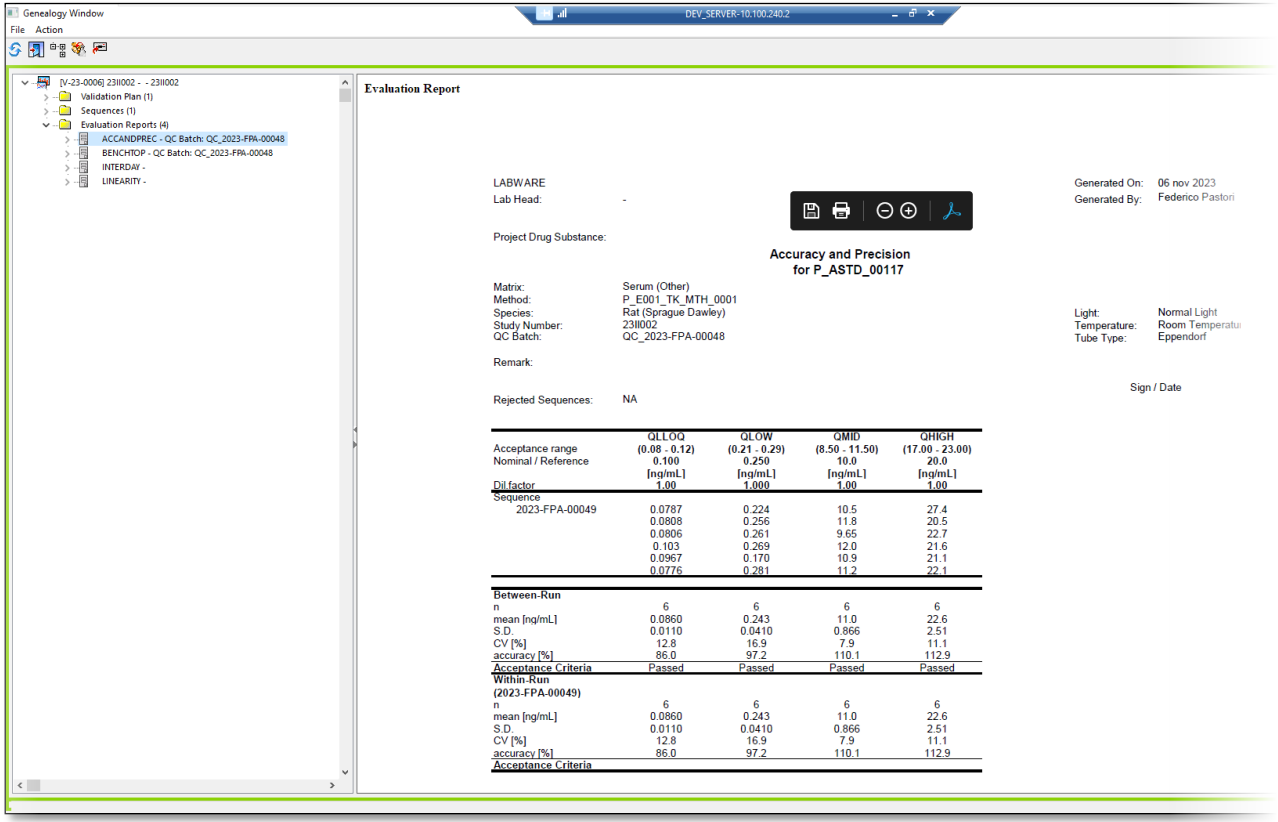
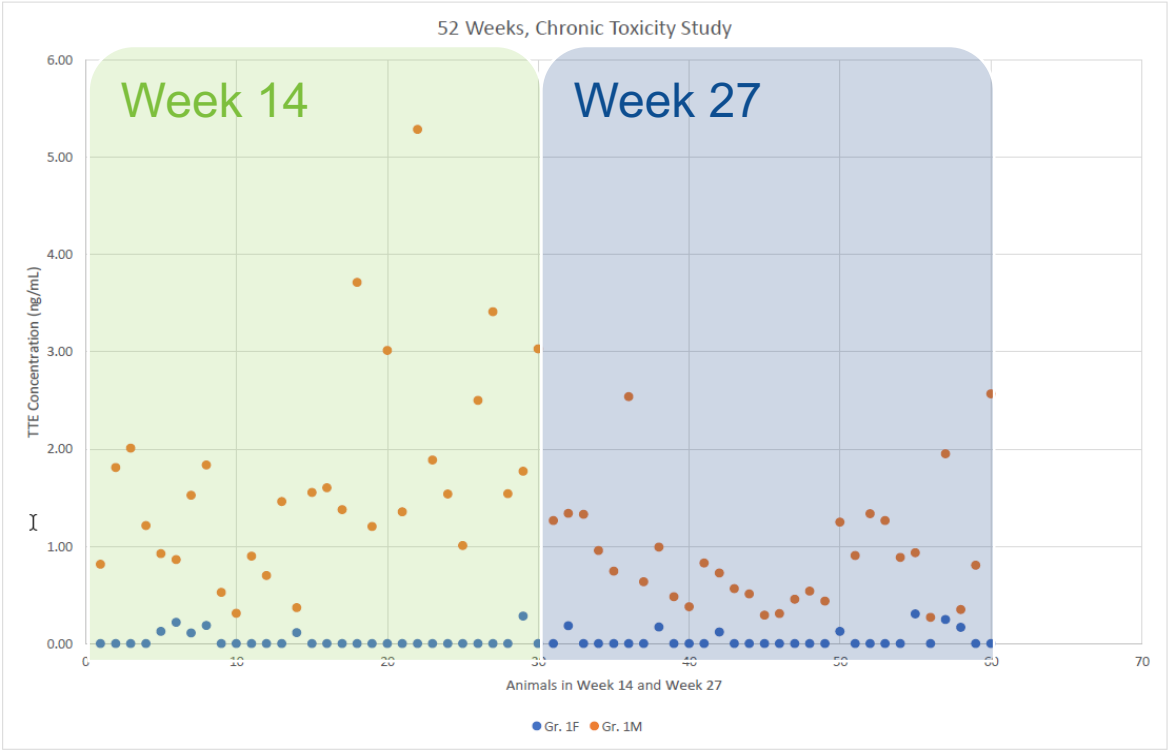


Test Item - Treatment Schema:

Gr. No.	Dose Level (mg/kg/day)	Level	No. of Animals
1	0	Control	30
2	1000	Low	20
3	2000	Medium	20
4	4000	High	30

Results of the Control Group are used as baseline to detect eventual increase of the TTE.

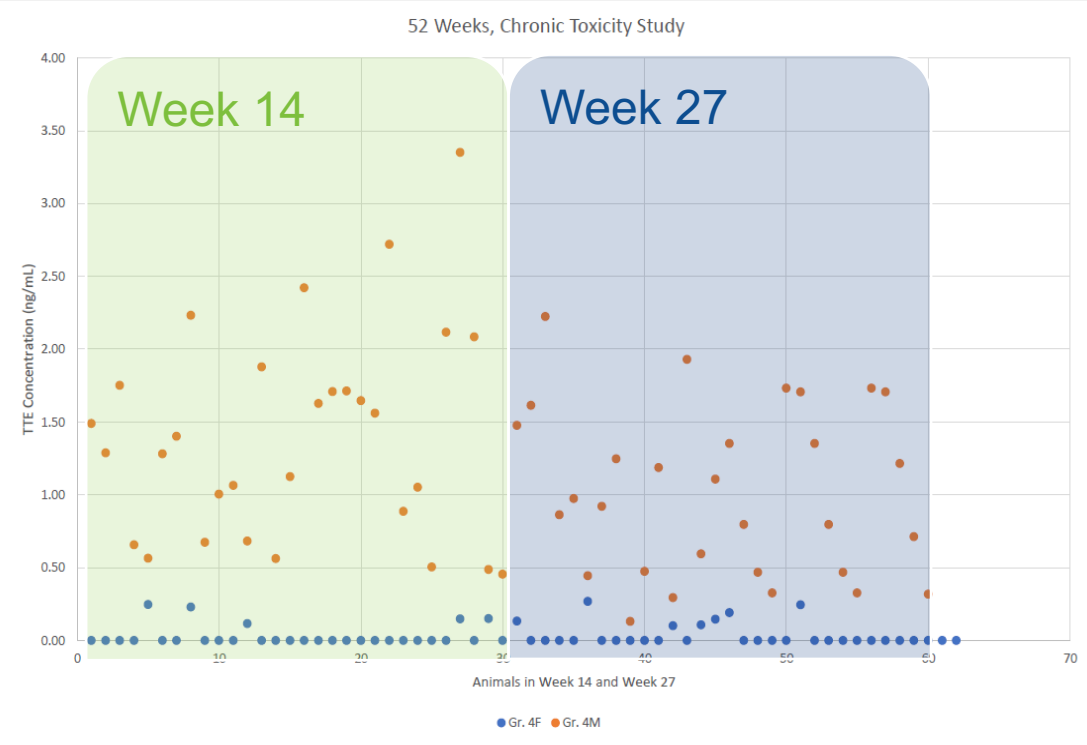
50ul of Serum were sampled in the **Main Groups** and not from a satellite ones.



Results of the Control Group Does not show any alteration of the physiological level of TTE.

The study is on-going and samples of 52 Weeks are not still sampled.

Group 4 Samples

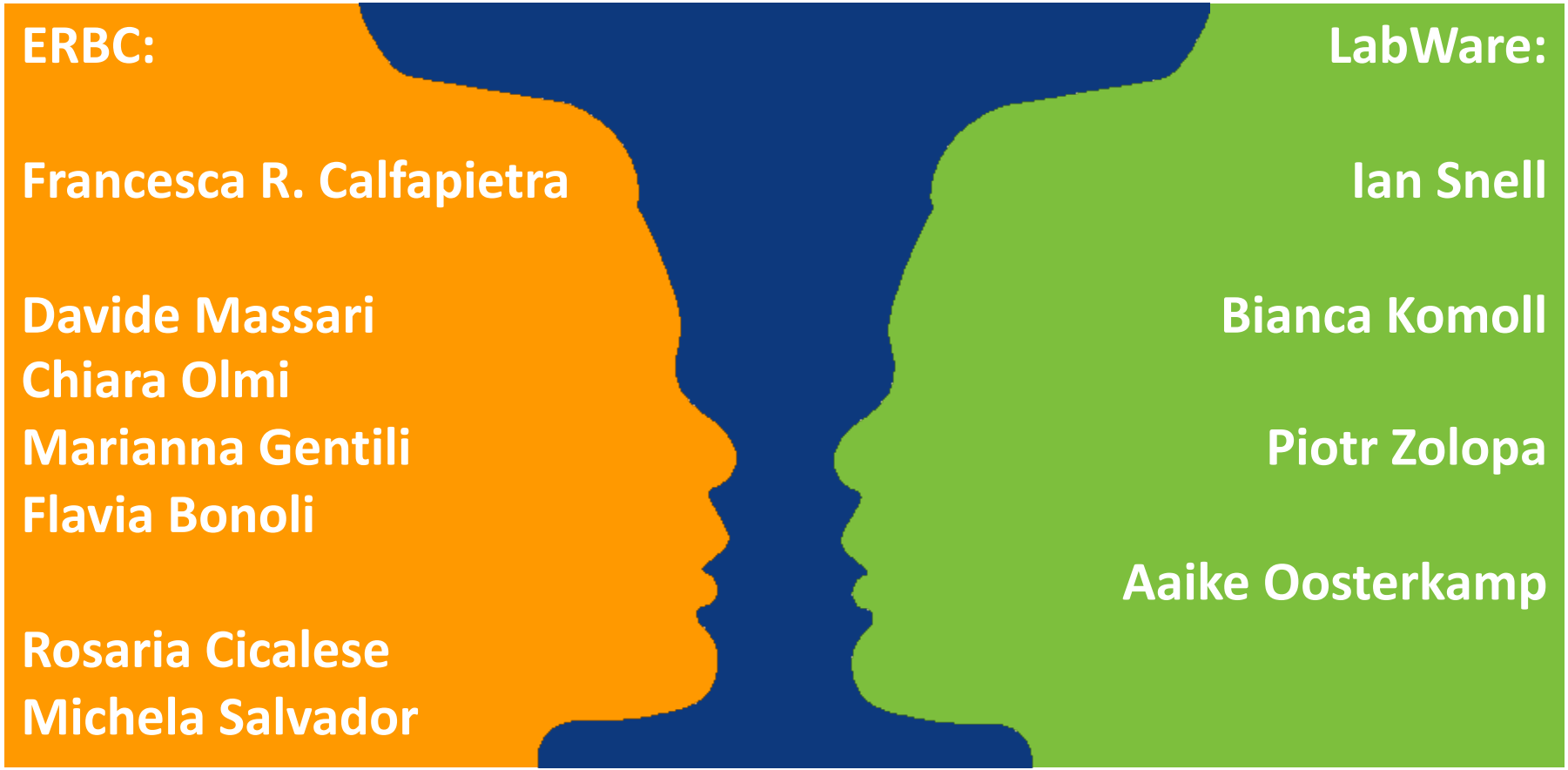


Conclusion

- Pilot Validation Study shown very good achievements in sensitivity using: Micro HPLC and M5 – ACQUITY BEH 1.7um 1x50mm – API4000.
- **Physiological Levels of TTE in Serum** have been properly described.
- **2 min chromatographic run** is a key to **reduce the analysis time impact** on the lab workload.
- Data management under **GxP compliance** from the Validation to the Sample analysis was realized with **LabWare LIMS**.

- TTE, T3 and T4 monitoring with LC-MS will be used in **all the Chronic Toxicity Studies in ERBC** substituting RIA Analytical Method.
- **Hormone panel will be extended** with potential ones that could be target of Test Item acting as endocrine disruptor.
- **Sensitivity will be increased** using more sensible **machine therefore the Volume of the Serum could be decreased**.
- Volume decrease is fundamental to reduce the animal stress. (3R-Reduction)
- **Volume Reduction** will be fundamental to sample blood from the litter in the reproductive Studies using **capillary sampling**.
- **LabWare LIMS** will always be there to help this process.

Special Thanks



“Differences should be much more appreciated and could only help us in doing Better”