Real Time and Accelerated Stability Studies of Testosterone Calibrators

Linda Nagore, Ravi Orugunty,
Isil Dilek, and Uma Sreenivasan
Cerilliant Corporation

Introduction

Development of accuracy-based calibrators in biological matrices for clinical diagnostic applications requires reference measurement calibrators and materials with high accuracy and sensitivity. Testosterone presents a unique challenge with the wide range of endogenous levels across female, male and age-based patient populations. A method for quantitation of Testosterone across the entire therapeutic range from 20 pg/mL to 20,000 pg/mL in serum by Liquid Chromatography Mass Spectrometry (LC-MS/MS) was developed and validated. Given the 1000-fold range of the calibrators the analysis is divided into three groups. Data presented will provide evidence on the quality of the product and show that the Testosterone calibrators will remain stable under normal use and recommended storage conditions.

T-107 Calibrator Kit					
Low pg/mL		Medium pg/mL		High pg/mL	
T-096	20	T-100	350	T-103	5000
T-097	40	T-101	525	T-104	7500
T-098	90	T-102	1500	T-105	20000
T-099	175				

Study Design

The method consists of Value Assignment Calibrator Curves that are used to quantitate testosterone in serum calibrators with the addition of a known amount of internal standard (Testosterone-$^{13}C_3$). Based on a NIST method, a multi-step liquid-liquid extraction of testosterone from the serum calibrators followed by analyzation by LC-MS/MS was performed. Data was analyzed with Agilent MassHunter Workstation Quantitation Software. NIST SRM-971 was used as quality control samples during all stages of testing.

LC-MS Parameters	
HPLC System	Agilent 1290
MS/MS System	Agilent 6490
Mobile Phase A	0.1% Formic Acid in Water
Mobile Phase B	0.1% Formic Acid in Acetonitrile
Column	Thermo Hypersil Gold C-18, 3µ, 3 x 50 mm
Gas Flow Temp (˚C)	290
Gas Flow	14
Nebulizer Pressure	45
Sheath Gas Temp (˚C)	400
Sheath Gas Flow	12
Capillary Voltage	3000
Vcharging	1500
Positive High Pressure RF	100
Positive Low Pressure RF	120

Testosterone LC Gradient (Flow Rate 0.700 mL/min)					
Time	0.0	0.5	16.0	16.1	19.1
% A	90	90	5	90	90
% B	10	10	95	10	10

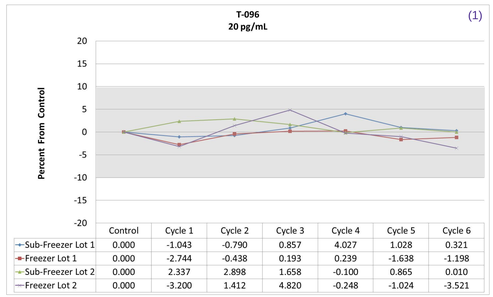
Native and Labeled Testosterone MRM Transitions			
Compound Name	Precursor Ion	Product Ion	CE (volts)
Testosterone	289.5	97	20
Testosterone	289.5	109	28
Testosterone-$^{13}C_3$	292.5	100	20
Testosterone-$^{13}C_3$	292.5	112	28

Freeze-Thaw

- The effect of repeated freezing (at -20° C and -80° C) and thawing (at room temperature) on two lots Testosterone calibrators was studied.
- Fourteen T-107 Calibrator kits were placed at sub-freezer and freezer for each lot.
- T-107 calibrator kits were removed from their storage condition at each time interval, thawed, uncapped for approximately two minutes then closed again and placed back into the original storage condition.
- No significant changes were found over six cycles of freeze-thaw in at any level of calibrator.
- Figures 1, 2, and 3 are representative of analysis at low, medium, and high concentrations during freeze/thaw experiments.

Stress Conditions	Control	24 Hrs	48 Hrs	72 Hrs	96 Hrs	120 Hrs	144 Hrs
Sub-Freezer	14	12	10	8	6	4	2
Freezer	14	12	10	8	6	4	2

(1)



	Control	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6
Sub-Freezer Lot 1	0.000	-1.043	-0.790	0.857	4.027	1.028	0.321
Freezer Lot 1	0.000	-2.744	-0.438	0.193	0.239	-1.638	-1.198
Sub-Freezer Lot 2	0.000	2.337	2.898	1.658	-0.100	0.865	0.010
Freezer Lot 2	0.000	-3.200	1.412	4.820	-0.248	-1.024	-3.521

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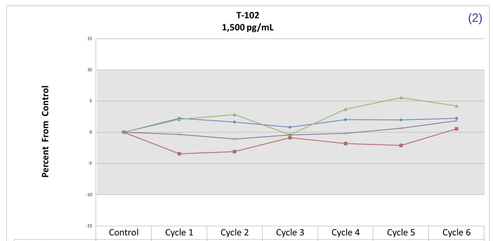

	Control	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6
Sub-Freezer Lot 1	0.000	2.235	1.639	0.801	2.038	1.977	2.259
Freezer Lot 1	0.000	-3.438	-3.088	-0.878	-1.803	-2.089	0.559
Sub-Freezer Lot 2	0.000	2.077	2.851	-0.389	3.710	5.554	4.213
Freezer Lot 2	0.000	-0.322	-1.070	-0.427	-0.157	0.633	1.852

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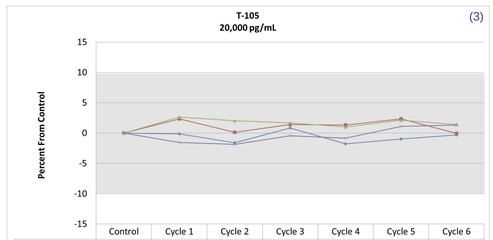

	Control	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6
Sub-Freezer Lot 1	0.000	-0.133	-1.610	0.861	-1.769	-0.992	-0.304
Freezer Lot 1	0.000	2.347	0.152	1.431	1.306	2.351	-0.065
Sub-Freezer Lot 2	0.000	2.655	2.017	1.668	1.023	2.110	1.407
Freezer Lot 2	0.000	-1.538	-1.836	-0.445	-0.841	1.099	1.312

Temperature Stress

- Ten T-107 kits were placed in each of the stress conditions and four T-107 kits were placed in the freezer as a control.
- At each time interval two kits were removed from each of the storage conditions along with two controls.
- Refrigerate and 20º C conditions concentrations remained within 10% of the original value through 8 weeks and were consistent with control samples.
- Significant changes were observed at 30° C over an eight week period.
- Figures 4, 5, and 6 are representative of analysis at low, medium, and high concentrations during temperature stress experiments.

Stress Conditions	3 Days	1 Week	2 Weeks	4 Weeks	8 Weeks
Freezer (Control)	N/A	2	2	2	2
Refrigerate (2°C-8°C)	2	2	2	2	2
20 °C	2	2	2	2	2
30°C	2	2	2	2	2

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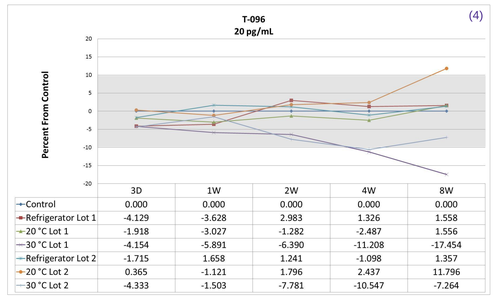

	3D	1W	2W	4W	8W
Control	0.000	0.000	0.000	0.000	0.000
Refrigerator Lot 1	-4.129	-3.628	2.983	1.326	1.558
20 °C Lot 1	-1.918	-3.027	-1.282	-2.487	1.556
30 °C Lot 1	-4.154	-5.891	-6.390	-11.208	-17.454
Refrigerator Lot 2	-1.715	1.658	1.241	-1.098	1.357
20 °C Lot 2	0.365	-1.121	1.796	2.437	11.796
30 °C Lot 2	-4.333	-1.503	-7.781	-10.547	-7.264

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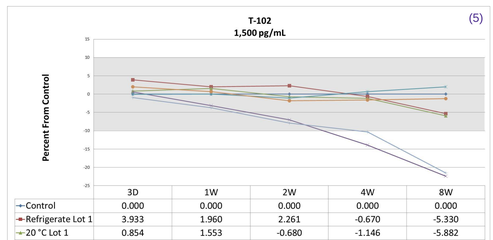

	3D	1W	2W	4W	8W
Control	0.000	0.000	0.000	0.000	0.000
Refrigerate Lot 1	3.933	1.960	2.261	-0.670	-5.330
20 °C Lot 1	0.854	1.553	-0.680	-1.146	-5.882
30 °C Lot 1	0.568	-3.152	-7.003	-13.923	-22.389
Refrigerate Lot 2	-0.089	-0.018	-1.108	0.701	2.005
20 °C Lot 2	1.965	0.675	-1.784	-1.606	-1.244
30 °C Lot 2	-0.908	-3.707	-7.924	-10.303	-21.500

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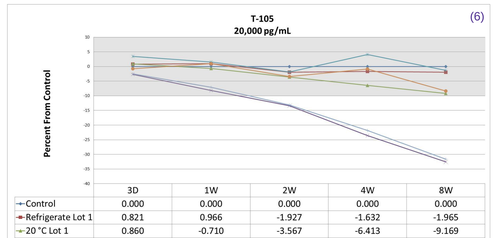
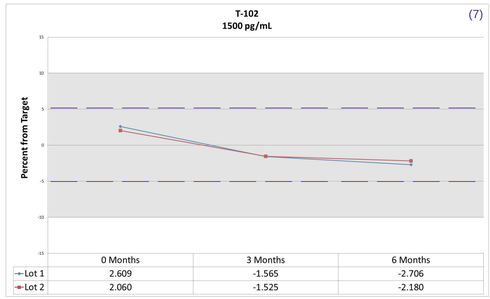


	3D	1W	2W	4W	8W
Control	0.000	0.000	0.000	0.000	0.000
Refrigerate Lot 1	0.821	0.966	-1.927	-1.632	-1.965
20 °C Lot 1	0.860	-0.710	-3.567	-6.413	-9.169
30 °C Lot 1	-2.652	-8.174	-13.441	-23.601	-32.513
Refrigerate Lot 2	3.492	1.491	-1.885	4.094	-1.266
20 °C Lot 2	-0.734	0.964	-3.418	-0.811	-8.337
30 °C Lot 2	-2.532	-7.057	-13.087	-21.789	-31.658

Real Time Stability

- Nine T-107 kits from each lot were placed at -20º C. Three replicates at each time intervals were analyzed.
- Long-term stability testing was found to be consistent with accelerated stability findings.
- Results are within the expanded uncertainties of certification at time 0 and stability testing.
- Expanded uncertainties for the products are approximately 4.2% and included preparation, certification and homogeneity. (95% Confidence Interval)
- Expanded uncertainty of LC-MS method is 4.1%.
- Representative example shown in Figure 7.

(7)



	0 Months	3 Months	6 Months
Lot 1	2.609	-1.565	-2.706
Lot 2	2.060	-1.525	-2.180

Chromatography

Chromatography throughout the analysis had an elution time of approximately 6.2. Separation and detection were reproducible and robust throughout the project.

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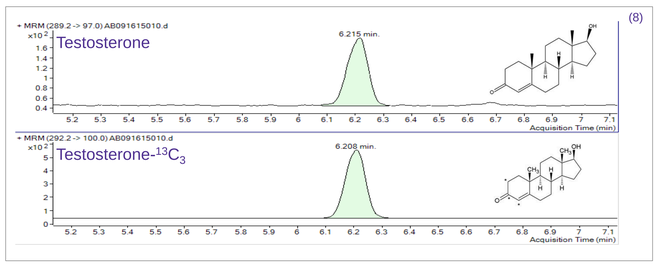

Workflow

Weigh out 1 mL of Serum → Spike with appropriate amount of IS → Add Ammonium Acetate → Incubate for 2 hours → Extract 2X with Ethyl Acetate/Hexane → Evaporate to Dryness → Reconstitute in Ammonium Carbonate → Extract 2X with Hexanes → Evaporate to Dryness → Reconstitute in 50% Acetonitrile in Water → Analyze by LC/MS Against Appropriate VAC → Data Analysis

Summary

- Accuracy-based calibrators for testosterone were manufactured in accordance with ISO 17025 and ISO Guide 34.
- Data obtained proved to be consistent with control samples with little to no degradation and fell well within specifications.
- Forced degradation studies provide ample information to conclude that Testosterone calibrators are stable under most conditions.
- Ongoing real time stability indicates no significant changes outside of analytical variability.

Corresponding Author: Linda.Nagore@Cerilliant.com
www.Cerilliant.com | www.SigmaAldrich.com

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