Qualification of Steroid Markers for Preclinical Studies

Steroid hormones mediate a wide variety of crucial physiological functions ranging from anti-inflammatory response to regulation of events during pregnancy. Steroids are mostly synthesized and secreted into the bloodstream by the adrenal cortex and the gonads. Imbalances in hormone levels may occur in genetic diseases or upon exposure to toxins.

This poster describes multiplex panels that detect structurally-similar steroids specifically and quantifiably, even at low levels. These panels were developed for preclinical studies: they have been qualified to facilitate testing in regulated laboratories. Our Steroid Hormone Panel 1 (human/mouse/rat) measures estradiol, testosterone, progesterone and DHEA simultaneously from a single sample. Each analyte is measured using a competitive immunoassay. Luteinizing Hormone (LH), a glycoprotein, plays an important role in reproduction. Levels of LH in blood and urine samples can be measured using our Singleplex assay.

These panels have advantages that are typical of assays from Meso Scale Discovery (MSD): greater sensitivity, reduced sample volume, a greater dynamic range (both endogenous and elevated levels can be measured at a single dilution factor) and improved throughput. The Human Luteinizing Hormone (LH) is now available for purchase from MSD as a qualified kit.

Description of Markers (For use in a wide range of species, unless otherwise noted)

The following markers are highly conserved across species.

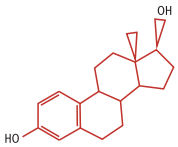
Testosterone is synthesized in the testes, adrenal cortex, and the ovary. Alterations lead to abnormal development & maintenance of male sex tissues as well as altered muscle mass and skeletal growth. In the female, elevations can lead to infertility and other side effects.

Estradiol is the most important form of estrogen. It is responsible for control of ovulatory cycle and female secondary sex characteristics. Estradiol is made and secreted from the ovaries, adrenal cortex, and placenta. Decreases can lead to menopausal symptoms and skeletal bone loss (osteoporosis, if severe). Increased incidences of certain cancers are linked to exposure to endocrine disruptor chemicals having estrogenic properties.

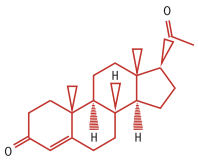
Dehydroepiandrosterone (DHEA) is synthesized in the adrenal gland and ovary. DHEA is suitable for evaluating abnormal adrenal androgen secretion.

Progesterone is produced in adrenal glands, the ovaries, and in the placenta. Progesterone is the hormone that prepares the uterus to implant and carry the embryo and fetus. Progesterone also influences water and sodium homeostasis. An altered hormonal milieu of progesterone and the estrogens may increase the risk of cardiovascular disease, cancer, bone resorption, and other ailments.

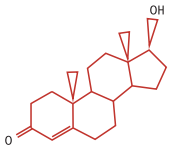
Luteinizing Hormone (LH) (Human) is produced in the pituitary and participates in regulation of testosterone and estrogen levels. Decreased LH can lead to both male and female infertility.



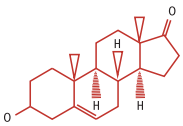
17-β-Estradiol



Progesterone



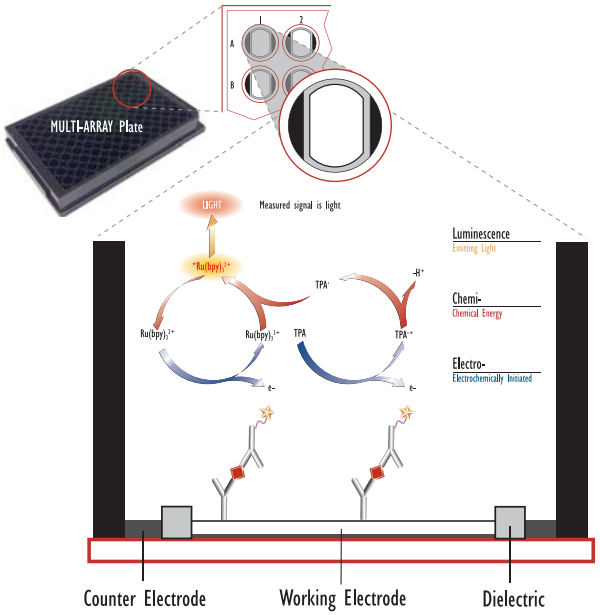
Testosterone



Dehydroepiandrosterone

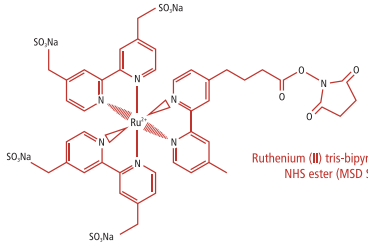
The MSD® Platform

MSD's electrochemiluminescence detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® and MULTI-SPOT® microplates.



Electrochemiluminescence Features:

- Minimal background signals and high signal to background ratios - the stimulation mechanism (electricity) is decoupled from the signal (light)
- Proximity - only labels bound near the electrode surface are detected, enabling non-washed assays
- Flexibility - labels are stable, non-radioactive, and are conveniently conjugated to biological molecules
- Emission at ~620 nm - eliminating problems with color quenching
- Signal amplification - multiple excitation cycles of each label enhance light levels and improve sensitivity



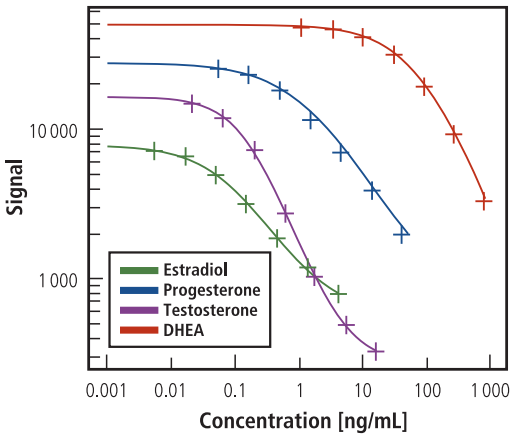
Ruthenium (II) tris-bipyridine-(4-methylsulfonate) NHS ester (MSD SULFO-TAG™ label)

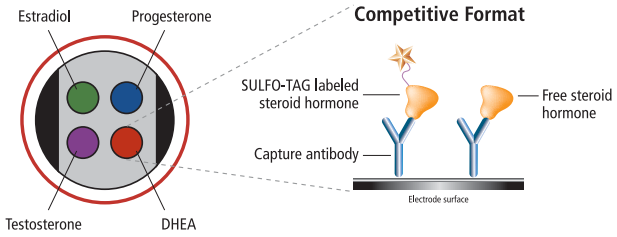
Steroid Hormone Panel 1 (human/mouse/rat): Estradiol, Progesterone, Testosterone, DHEA

MSD's Steroid Hormone Panel 1 (human/mouse/rat) measures estradiol, testosterone, progesterone and DHEA simultaneously from a single sample. We are in the process of qualification for this panel according to typical practices for pre-clinical biomarkers. The qualification procedure involved multi-day controls, establishment of limits of quantitation, spike recovery, dilutional linearity, and measurement of control and treated samples.

Standard Curve

Representative standard curves from a typical run are shown below. The lower limit of detection (LLOD) was determined as the concentration at 80% of the maximum signal for each hormone. The lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ) were assigned following a multi-day study. We assigned the LLOQ (or ULOQ) as the lowest (or highest) concentration where the %CV of the calculated concentration was less than 20% and the percent recovery of the concentration was between 80% and 120%.





Estradiol		
Concentration (ng/mL)	Mean	% CV
0	7538	6.5
0.005	7150	5.0
0.016	6577	2.9
0.05	5031	3.3
0.15	3160	3.6
0.44	1879	2.9
1.33	1185	2.8
4	795	4.4

Testosterone		
Concentration (ng/mL)	Mean	% CV
0	16532	7.8
0.02	14715	6.2
0.07	11807	6.5
0.20	7161	3.7
0.59	2749	7.5
1.78	1028	7.9
5.33	496	5.3
16	327	4.9

Progesterone		
Concentration (ng/mL)	Mean	% CV
0	26795	8.7
0.07	25318	4.6
0.21	23043	3.7
0.62	17816	4.4
1.85	11561	3.5
5.56	6943	2.3
16.7	3815	4.2
50	1979	4.8

DHEA		
Concentration (ng/mL)	Mean	% CV
0	49348	9.1
1.1	48724	2.8
3.3	47071	3.2
9.9	41481	2.3
30	32075	3.3
89	20229	4.1
267	9320	3.2
800	3431	6.6

	Estradiol (ng/mL)	Progesterone (ng/mL)	Testosterone (ng/mL)	DHEA (ng/mL)
LLOD	0.023	0.306	0.046	13.7
LLOQ	0.049	0.617	0.198	29.6
ULOQ	4	50	5.3	800

Standard Curve Data is from a representative run. Lower limit of detection (LLOD) was computed as 2.5 SD over the background signal.

Protocol:

1 Add 150 µL Blocker A solution, incubate 1 hour at RT.

2 Wash, add 50 µL of standard/sample, incubate 2 hours at RT.

3 Add 15 µL of tracer, incubate 30 min at RT.

4 Wash, add 150 µL of Read Buffer T, read.

Steroid Hormone Panel 1 (human/mouse/rat): Estradiol, Progesterone, Testosterone, DHEA

Precision: Multi-Day Study

A multi-day, multi-plate study over 5 plates was performed to show reproducibility. High, mid, and low controls were made by spiking assay calibrators into control rat plasma. The controls were tested on five plates run over 3 days. The average concentration of the controls and the interplate, interday CV of the concentration measurements are shown below. The high control for testosterone came in at a value greater than the assay ULOQ, and the low control for DHEA was less than the assay LLOQ (shown in gray). The other controls were recovered with acceptable reproducibility.

	Control	Plates	Concentration	Interday % CV (n=5 plates)
Estradiol (pg/mL)	High	5	1804	17.8
	Mid	5	570	13.8
	Low	5	41.0	12.6
Progesterone (ng/mL)	High	5	16.8	18.8
	Mid	5	1.05	17.1
	Low	5	0.58	14.4
Testosterone (ng/mL)	High	5	9.90	42.5
	Mid	5	1.48	12.3
	Low	5	0.17	8.1
DHEA (ng/mL)	High	5	485	6.1
	Mid	5	100	12.9
	Low	5	11.6	21.8

Dilutional Linearity

Rat lithium heparin plasma samples from male and female rats were pooled and tested at various dilutions on the Steroid Hormone Panel 1. Where the endogenous level was not sufficient to evaluate linearity of dilution, additional analyte was spiked into the plasma pools prior to dilution. Percent recovery of dilutional linearity is shown below. The concentrations shown below have been corrected for dilution. Percent recovery is calculated as the measured concentration divided by the concentration measured for the previous dilution (expected).

% Recovery = (measured * dilution factor) / expected * 100

	Estradiol			
Sample Group	Dilution	Conc. (pg/mL)	Calculated Conc. CV	% Recovery
Male (Spiked Sample)	1	1660	23.6	
	2	1834	23.1	111
	4	1436	13.6	78
	8	1376	3.3	96
Female (Spiked Sample)	1	1504	34.0	
	2	1402	15.6	93
	4	1604	27.0	114
	8	1216	5.6	76

	Testosterone			
Sample Group	Dilution	Conc. (ng/mL)	Calculated Conc. CV	% Recovery
Male (Spiked Sample)	1	3.42	20.2	
	2	2.80	5.6	82
	4	3.44	11.9	123
	8	4.00	1.1	117
Female (Spiked Sample)	1	3.52	22.8	
	2	2.50	13.7	71
	4	3.56	17.5	142
	8	4.00	5.5	113

	Progesterone			
Sample Group	Dilution	Conc. (ng/mL)	Calculated Conc. CV	% Recovery
Male	1	3.13	12.5	
	2	3.84	26.0	123
	4	4.24	14.7	110
	8	5.76	4.0	136
Female	1	26.9	21.5	
	2	22.0	22.1	82
	4	23.9	35.4	108
	8	28.2	9.2	118

	DHEA			
Sample Group	Dilution	Conc. (ng/mL)	Calculated Conc. CV	% Recovery
Male (Spiked Sample)	1	347	13.6	
	2	358	12.7	103
	4	401	16.0	113
	8	594	3.3	147
Female (Spiked Sample)	1	458	10.7	
	2	386	12.9	84
	4	436	16.4	112
	8	496	5.3	114

Steroid Hormone Panel 1 (human/mouse/rat): Estradiol, Progesterone, Testosterone, DHEA

Samples

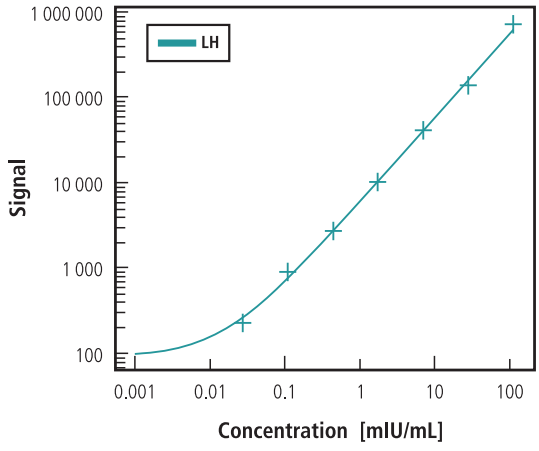
Lithium heparin rat plasma samples from normal Sprague-Dawley rats were tested on the Steroid Hormone Panel 1. Measurements that were below the LLOD are shown in green. For the other three analytes, all the measurements are in the quantitative range.

		Analyte Concentration			
Sex	Animal ID	Estradiol (pg/mL)	Progesterone (ng/mL)	Testosterone (ng/mL)	DHEA (ng/mL)
Male	1	13.3	1.3	0.41	36.6
	2	16.7	2.9	0.35	39.2
	3	11.4	4.9	0.31	38.6
	4	12.8	1.7	0.33	50.8
	5	6.7	4.0	0.42	43.1
Female	1	16.2	14.8	0.23	64.9
	2	32.6	17.6	0.25	87.3
	3	24.0	22.7	0.21	102.4
	4	18.6	21.2	0.22	117.7
	5	15.0	33.2	0.26	194.5
Male (n=5)	Median	12.8	2.9	0.35	39.2
	Range	6.7 - 16.7	1.3 - 4.9	0.3 - 0.4	36.6 - 50.8
Female (n=5)	Median	18.6	21.2	0.23	102.4
	Range	15.0 - 32.6	14.8 - 33.2	0.2 - 0.3	64.9 - 194.5

Highlighted measurements are < LLOD

Human Luteinizing Hormone (LH) Assay

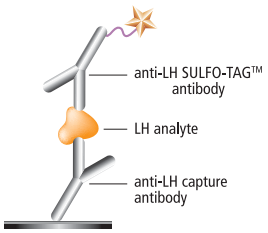
MSD offers a single-analyte assay for Human Luteinizing Hormone (LH). This assay is formatted as a sandwich immunoassay. A representative standard curve from a typical run is shown below. The lower limit of detection (LLOD) was determined by calculating 2.5 standard deviations above the average background (no analyte). The lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ) were assigned following a multi-day study. We assigned the LLOQ (or ULOQ) as the lowest (or highest) concentration where the %CV of the calculated concentration was less than 20% and the percent recovery of the concentration was between 80% and 120%.



LH		
Conc. (mIU/mL)	Mean	% CV
0	102	17
0.027	231	3.4
0.107	909	1.0
0.430	2749	1.5
1.719	10424	2.6
6.875	40938	1.4
27.50	137231	18
110	721718	6.4

	LH (mIU/mL)
LLOD	0.006
LLOQ	0.055
ULOQ	110

Sandwich Format



Protocol:

1 Add 25 µL of Diluent F.

2 Add 25 µL of standard/sample, incubate 2 hours at RT.

3 Add 25 µL of Detection Antibody, incubate 2 hours at RT.

4 Wash, add 150 µL of Read Buffer T, read.

Human Luteinizing Hormone (LH) Assay

Precision: Multi-Day Study

A multi-day, multi-plate study over 15 plates was performed to show reproducibility. In addition to the standard curves, control samples of a high, mid, and low levels of LH were measured on each plate. Each sample was run in triplicate. The average intra-plate %CV and inter-day %CV of the concentrations are shown below.

				Intra-plate	Inter-plate
	Control	Plates	Ave. Conc. (ng/mL)	Average % CV	% CV
LH	High	15	84.0	7.0	9.3
	Mid	15	31.2	7.4	9.8
	Low	15	6.24	5.8	10.7

Spike Recovery

Both male and female human serum samples were spiked with Human LH Calibrator at multiple levels throughout the range of the assay. All samples displayed good recovery and acceptable %CV for all spike levels. Results from 6 serum samples are shown in the table.

% Recovery = measured / expected * 100

	LH			
Human Serum Sample	Spike Level (mIU/mL)	Concentration (mIU/mL)	Conc. % CV	% Recovery
Male 1	0	3.9	6.7	
	15	17.5	5.8	92
	20	32.8	10.8	97
	60	62.1	9.6	97
Male 2	0	3.7	3.1	
	15	15.6	13.2	83
	20	29.6	11.3	88
	60	53.1	5.4	83
Male 3	0	5.3	3.9	
	15	19.1	8.1	94
	20	33.8	2.2	96
	60	60.8	6.2	93
Male 4	0	5.4	5.8	
	15	18.2	13.5	89
	20	34.7	10.8	98
	60	58.3	7.8	89
Male 5	0	4.8	8.6	
	15	18.8	7.9	95
	20	31.4	8.2	90
	60	65.0	4.4	100
Female 6	0	3.8	2.9	
	15	15.7	9.6	84
	20	28.2	11.3	83
	60	63.0	2.0	99

Dilutional Linearity

Multiple human serum samples were assayed at 2, 4, 8, and 16-fold dilutions to measure linearity. Samples with high, mid, and low level of LH were used to show linearity across the range of the assay. Linearity in five representative female serum samples is shown below. The concentrations shown below have been corrected for dilution. Percent recovery is calculated as the measured concentration divided by the concentration measured for the previous dilution (expected).

% Recovery = (measured * dilution factor) / expected * 100

	LH			
Human Serum Sample	Fold Dilution	Concentration (mIU/mL)	Conc. % CV	% Recovery
1	1	29.16	3.2	
	2	30.87	1.4	106
	4	30.25	15.2	98
	8	32.51	2.8	107
	16	33.36	7.3	103
2	1	53.87	10.7	
	2	54.84	8.2	102
	4	54.00	6.8	98
	8	48.31	2.2	89
	16	52.97	3.7	110
3	1	5.00	6.1	
	2	5.22	0.9	104
	4	5.62	0.6	108
	8	5.99	1.7	107
	16	5.82	4.2	97
4	1	3.17	1.8	
	2	3.16	7.8	100
	4	3.68	13.5	116
	8	3.45	1.8	94
	16	3.75	2.8	109
5	1	2.36	6.8	
	2	2.26	13.8	96
	4	2.20	15.8	97
	8	2.18	16.5	99
	16	2.14	6.6	98

Human Luteinizing Hormone (LH) Assay

Samples

Normal human serum samples from 10 females and 5 males were run neat on the Human LH assay. The median levels and ranges for concentration and %CV for each sex are presented in the table below.

		Endogenous Levels of Human Luteinizing Hormone			
	# Samples	Concentration (mIU/mL)		% CV	
		Median	Range	Median	Range
Female	10	5.2	2.2 - 55.2	11.4	3.4 - 19.1
Male	5	4.5	3.4 - 5.5	4.0	1.7 - 15.9

Conclusions

MSD has developed high performance, multiplex assays to measure steroid hormones. The combination of multiplexing, wide dynamic range, and increased throughput enables studies that measure many steroids from small pre-clinical samples. MSD has released certain panels as preconfigured, fully qualified kits. Other panels are available on a custom basis, albeit without full qualification.