

# The development of robust cortisol assays for sports-based applications



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# Project aims



- Address the measurement issues associated with monitoring the effectiveness of an athlete's training regime “trackside”.
- Project consortium has included UK Sport/ English Institute of Sport, BOA and RFU.
- Devise novel immunoassays for the detection of stress biomarkers: cytokine & steroidal hormones, particularly cortisol.
- Overcome problems with existing assays for both total & free cortisol in serum assays.
- For meaningful changes in the physiological state for these analytes, a CV of  $\leq 20\%$  and preferably  $<10\%$ , is required for the assays.

# Cortisol assay construction using open platform technologies

- Robust immunoassays were constructed for the detection of total cortisol within serum using the MSD, and subsequently with the Aushon platform.



**Meso Scale SECTOR Imager 6000**

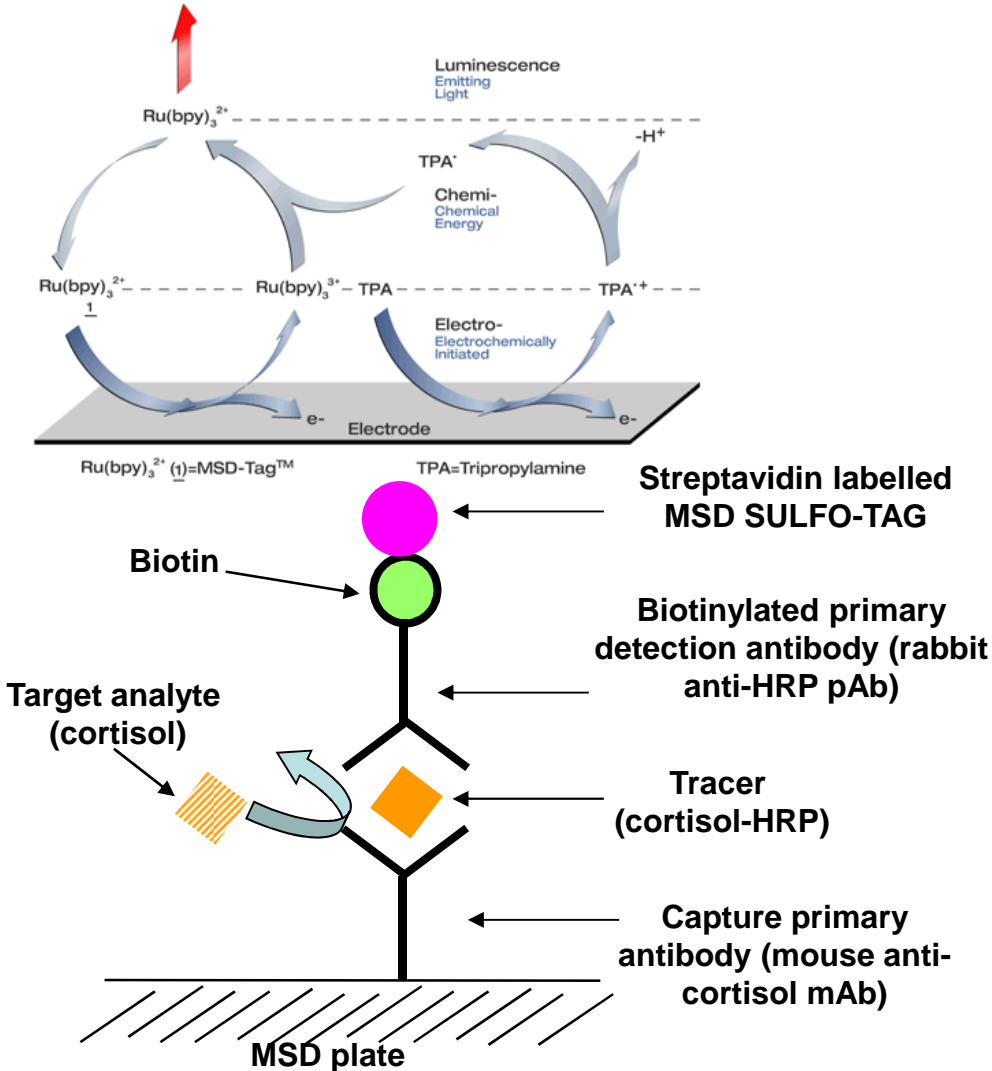


**Aushon Cirascan**

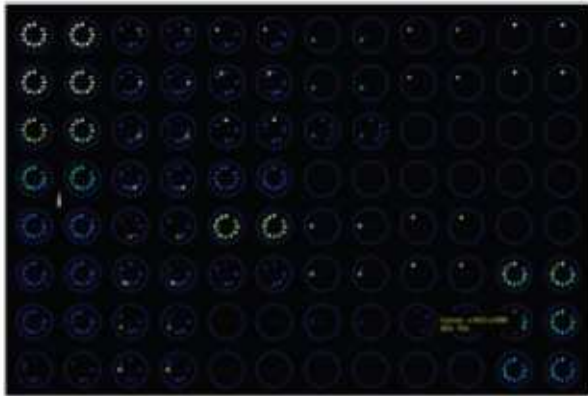
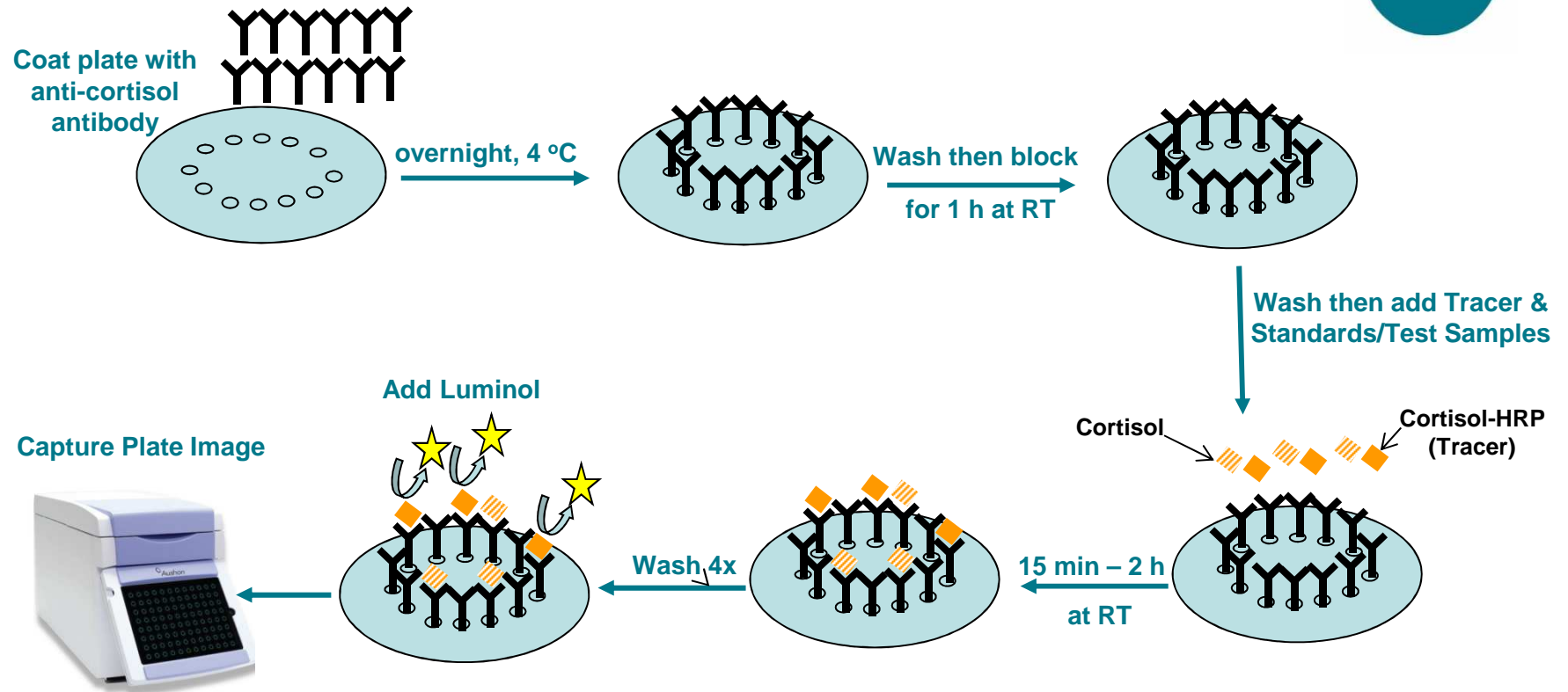
# Cortisol assay using the MSD platform



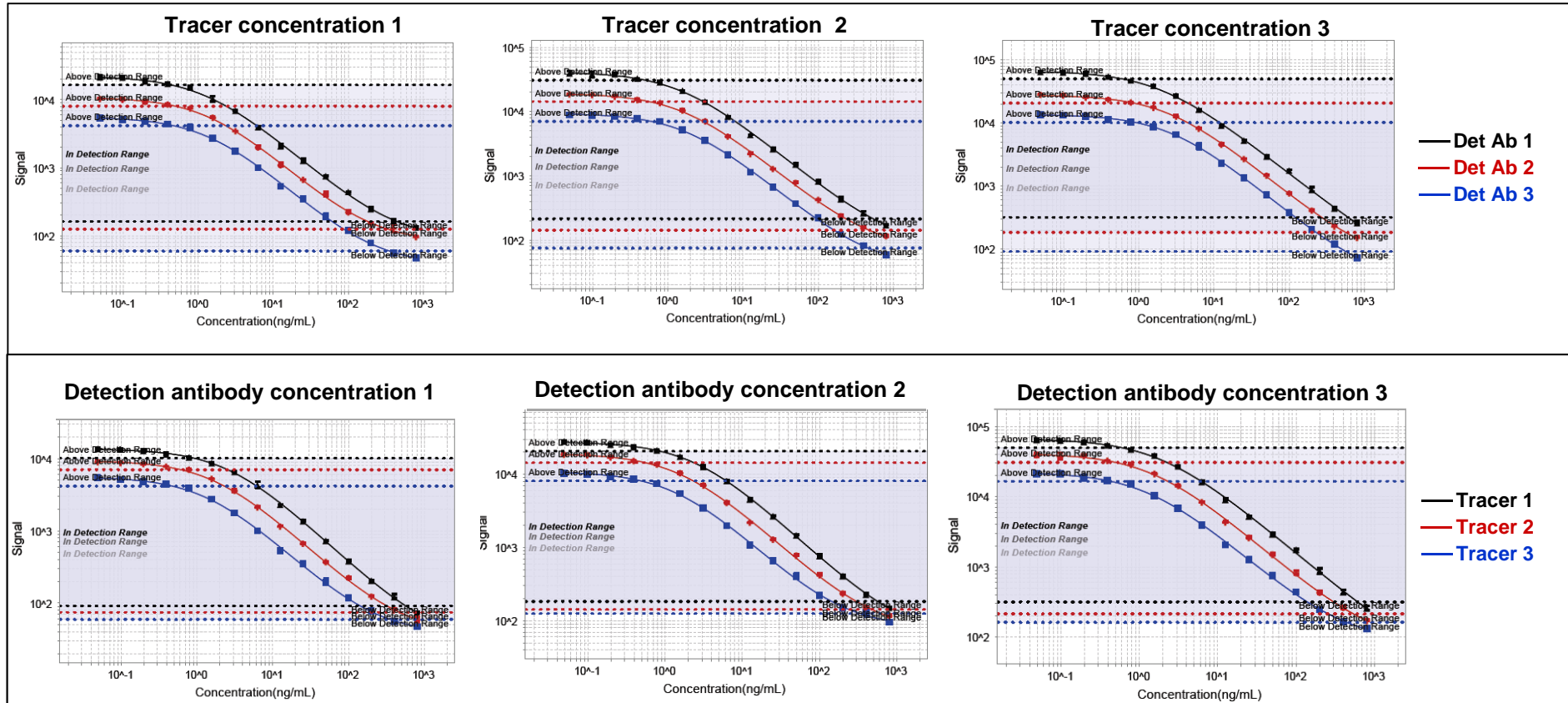
- Capture antibody  
- Overnight at 4 °C
- Blocking reagent  
- Incubate for 1 h & wash
- Tracer with Standards & Samples  
- Incubate for 1 h & wash
- Biotinylated 1° detection antibody  
- Incubate for 1 h & wash
- Streptavidin MSD SULFO-TAG  
- Incubate for 1 h & wash
- Capture the plate image



# Schematic for Aushon assays



# Titration of reagents for the construction of the MSD cortisol assay



- The capture antibody, tracer and detection antibody were all titrated to ascertain the optimum working conditions.

# Initial attempt to detect cortisol in serum



Test Serum	Dilution Factor	% Recovery of Cortisol
Serum 1	2	32.54
Serum 2	2	11.31
Serum 3	2	9.62
Serum 1	4	75.52
Serum 2	4	29.25
Serum 3	4	29.07
Serum 1	8	135.92
Serum 2	8	100.06
Serum 3	8	120.61

- Dilution of the serum evidently increased the recovery of cortisol.
- However, even with an 8-fold dilution, the variability in the range of recovery was not acceptable.



## Methods cited for the recovery of cortisol from serum

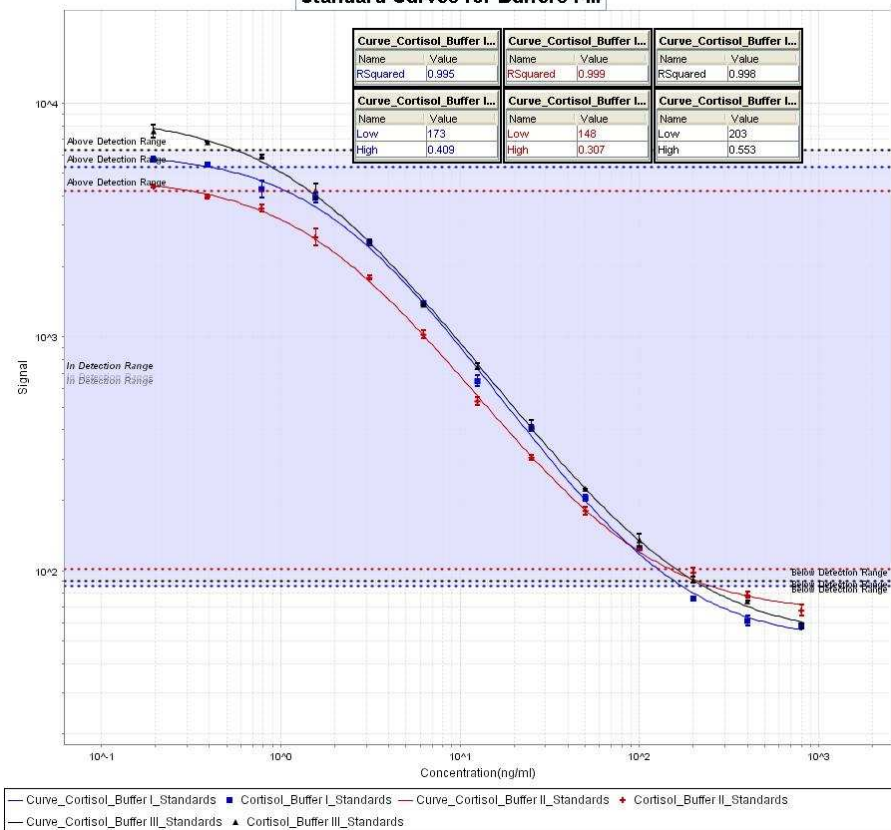
- Dilution of serum
- Heating the sample in an alkaline buffer
- Adjusting the pH of the sample
- Use of chemical reagents





# Calculation of the % cortisol recovered using 3 distinct assay buffers

Standard Curves for Buffers I-III



Test Serum	Dilution Factor	% Recovery of Cortisol		
		Buffer I	Buffer II	Buffer III
Serum 1	X	15.01	39.76	38.96
Serum 2	X	14.90	45.38	42.61
Serum 3	X	19.06	51.65	47.63
Serum 1	Y	20.95	61.78	49.52
Serum 2	Y	20.32	59.19	52.76
Serum 3	Y	22.12	63.14	58.50
Serum 1	Z	24.03	62.31	42.77
Serum 2	Z	22.62	68.19	48.08
Serum 3	Z	24.91	68.77	61.06

- This preliminary study indicated that Buffer II gives rise to better cortisol recovery from serum than the other two buffers evaluated.

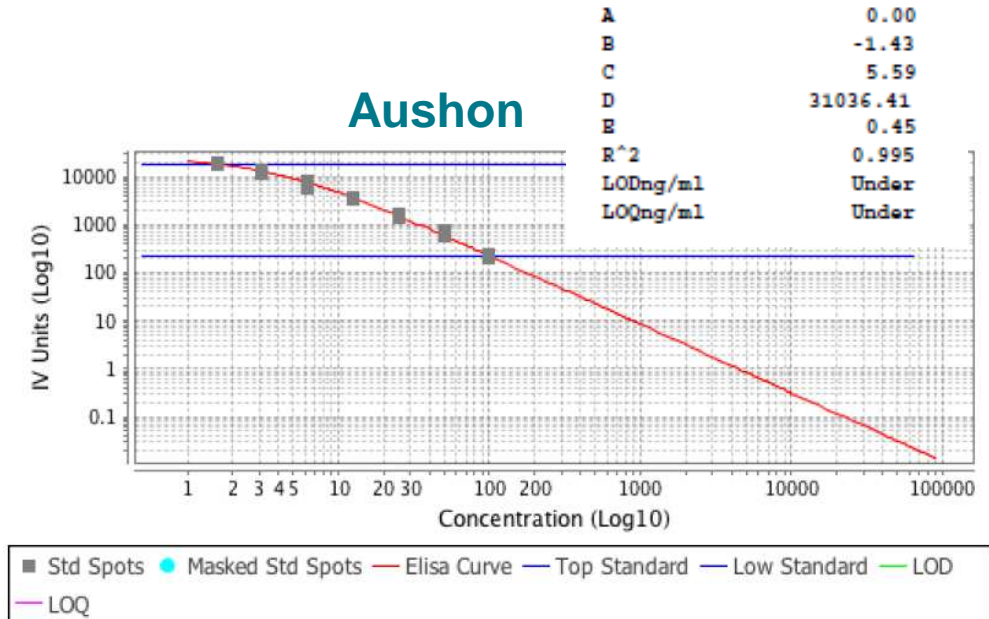
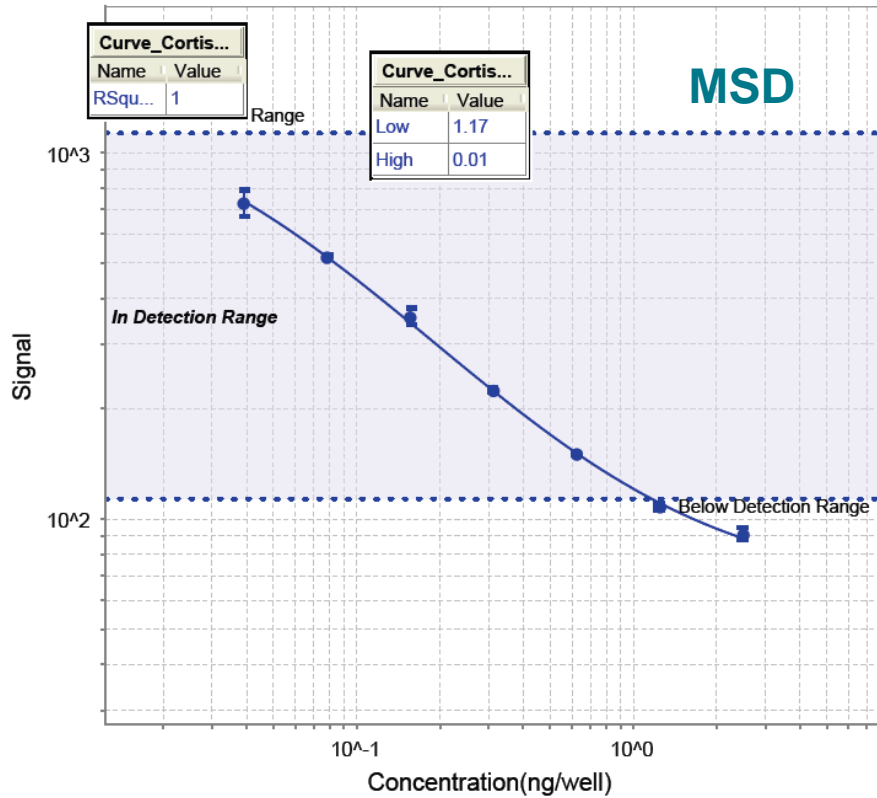


# Optimisation of cortisol assay buffer II conditions

- Further dilution factors of the serum were evaluated.
- The concentration of the reagents within Buffer II were also refined.

Dilution of Serum	% Recovery of Cortisol	
	Buffer II Concentration 1	Buffer II Concentration 2
A	96.6 - 104.1 %	95.6 - 106.7 %
B	95.1 - 103.2 %	88.4 - 111.7 %
C	92.8 - 111.4 %	90.9 - 108.8 %

# Total cortisol assays for serum using the MSD & Aushon platforms



● Cortisol\_Standard — Curve\_Cortisol\_Standard

Platform for the total serum cortisol assay	Mean % Recovery of total cortisol in male NIST sera (CRM)	Intra-assay CV	Inter-assay CV
Meso Scale Discovery SECTOR Imager 6000	113.24	≤ 6.7 % (n=3)	8.6 % (n=5)
Aushon cirascan imager	109.71	≤ 10.2 % (n=3)	5.0 % (n=4)

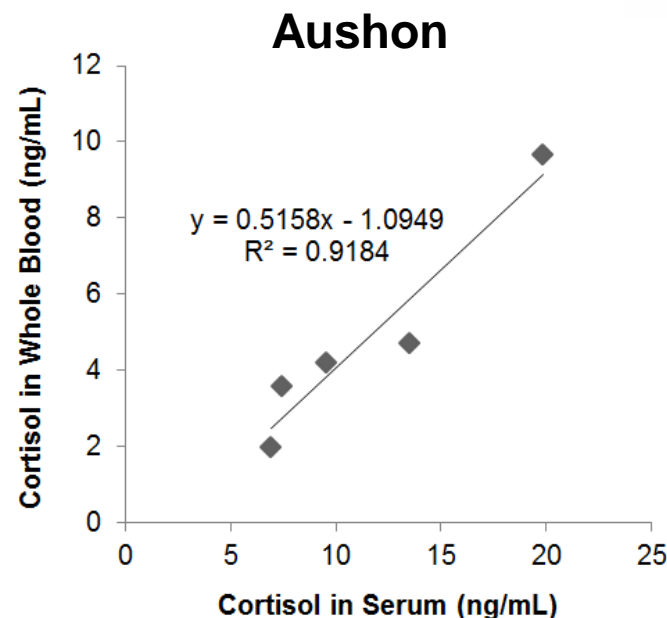
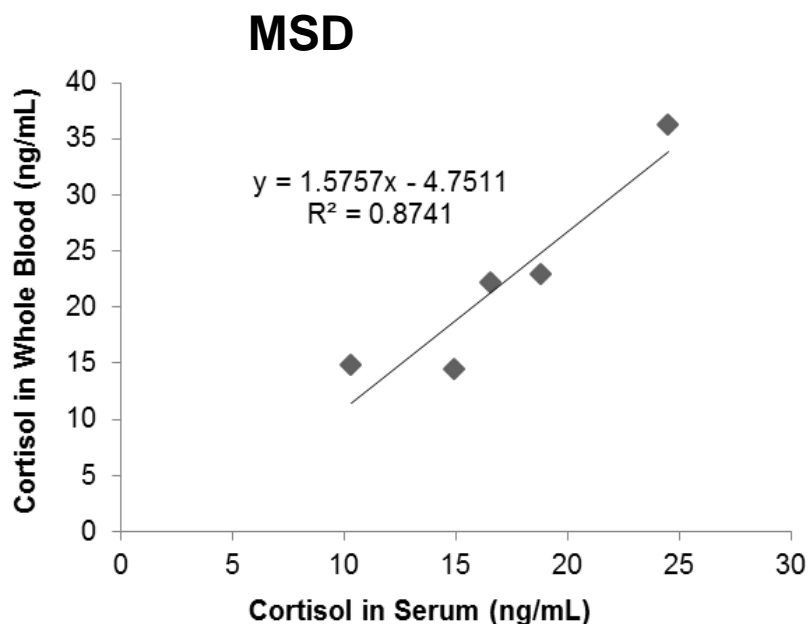
# Total cortisol assay data



Sample	Predicted Cortisol (ng/mL)	MSD			Aushon		
		Observed Cortisol (ng/mL)	Intra-assay CV (%)	% Recovery	Observed Cortisol (ng/mL)	Intra-assay CV (%)	% Recovery
Serum 1	702.469	826.664	3.46	117.68	726.72	1.10	103.45
Serum 2	402.469	482.315	6.96	119.84	435.84	1.00	108.29
Serum 3	252.469	287.307	2.20	113.80	283.92	2.80	112.46
Serum 4	177.469	205.149	3.82	115.60	203.04	2.40	114.41
Serum 5	139.969	157.131	2.87	112.26	157.92	2.60	112.83
Serum 6	121.219	135.365	2.23	111.67	140.16	3.80	115.63
NIST male serum	102.469	113.234	6.71	110.51	105.36	0.70	102.82

- Acceptable recovery range of analytes for ELISAs: 80-120 %.
- The total cortisol assays on both platforms encompass the full physiological concentration range within male and female test sera (20-600 ng/mL).

# Total cortisol assays: serum vs whole blood



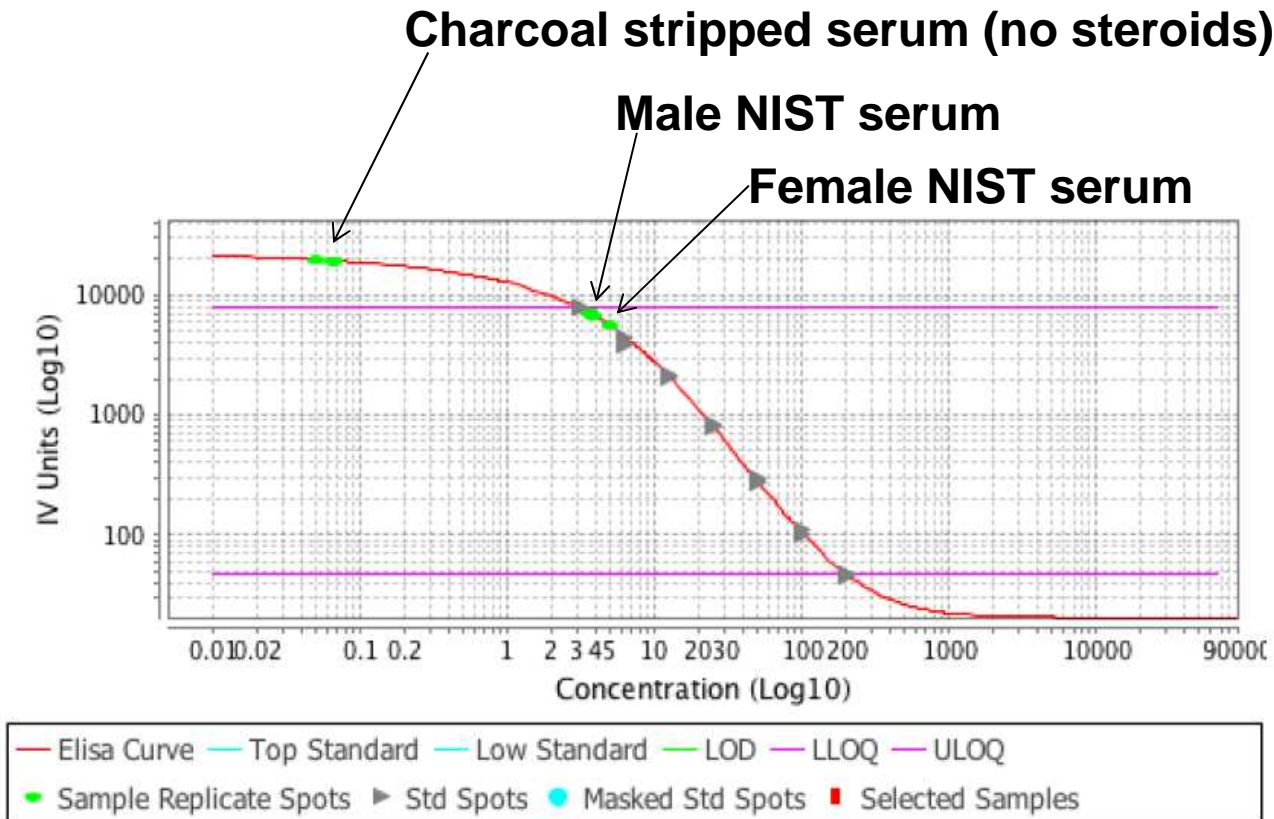
- There appears to be a rough correlation between the total cortisol in the serum and whole blood.
- Preliminary work indicates it may be worthwhile exploring the use of capillary blood for the total cortisol assays.

# Cortisol: free and protein-bound hormone



- Our collaborators requested the development of an assay for free cortisol as it is the biologically active form of the hormone.
- Most of the serum cortisol is bound to carrier proteins, primarily cortisol binding globulin.
- In normal human serum, < 5 % cortisol in serum is free.
- Existing free cortisol assays for serum are laborious, and to date, there is no direct free cortisol immunoassay that is suitable for serum.

# Direct free cortisol Aushon assay for serum





# Key performance indicators of the free cortisol assays

Platform for the free cortisol assay	Mean % Free Cortisol in male NIST sera	Anticipated % free cortisol in male NIST sera	Intra-assay CV	Inter-assay CV
Meso Scale Discovery SECTOR Imager 6000	4.11	< 5 % for normal sera	≤ 3.4 % (n=3)	9.8 % (n=3)
Aushon cirascan imager	3.63	< 5 % for normal sera	≤ 3.7 % (n=3)	4.3 % (n=2)

- The NIST male serum (NIST 921) comprised of pooled normal male serum.
- For normal subjects, the % free cortisol is expected to be < 5 %.
- Serum samples taken from athletes pre- and post-training will be required to validate the free cortisol assay.

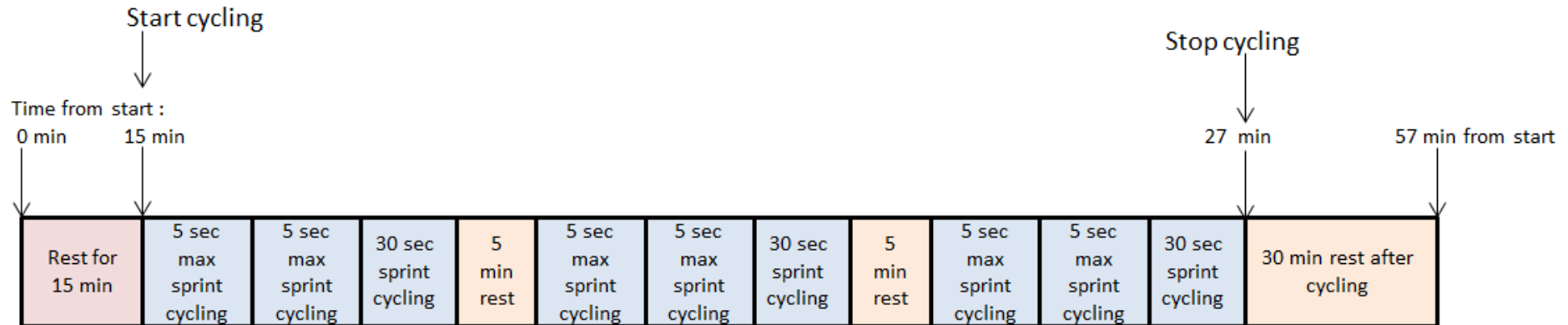


# Final optimisation of the assay prior to the field-based experiment



- The analyte/tracer incubation step for the Aushon assay was originally 2 h.
- Attempts to reduce this incubation step to 1 h, 30 min and 15 min were successful.
- Two-factor ANOVAs showed that there was no statistical difference in the back fitted results between the different assay conditions; the p-value for interaction term was  $> 0.05$ .

# Exercise regime for field-based experiment



Time point 0: Immediately before exercise after 15 min at rest

Collect saliva (s), venous blood (v) and capillary blood (c)

Time point 1: Immediately after 3x sprint cycling, & 27 min from start time

Collect saliva (s), venous blood (v) and capillary blood (c)

Time point 2: 30 min after cycling, & 57 min from start time

Collect saliva (s), venous blood (v) and capillary blood (c)

## Key

Athlete ID: **A, B, C, D, E, F**

Sampling time point: **0** = pre-training time point 0; **1** = 0 min post training time point 1; **2** = 30 min post-training time point 2

Sample type: **v** = venous blood, **c** = capillary blood, **s** = saliva

e.g. Athlete C's 1<sup>st</sup> post-training capillary blood sample is C1c.

# Conclusions



- We have successfully devised robust total and free cortisol assays that encompass the full physiological range of cortisol in human sera.
- The analyte incubation step could be reduced from 2 h to 15 minutes without affecting the recovery or reproducibility of either the free or total cortisol assays.
- The assays were initially constructed using the lab-based MSD platform but have been transferred to the Aushon platform which has been used for a field-based study.
- We have eliminated the sample processing step for the detection of free cortisol in serum.

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