

## SUPPLEMENTAL DATA

Steroid	Transition	Retention time (min)	Fragmentor Voltage (eV)	Collision Energy (eV)
Cortisone	361.3>163.3	4.65	125	25
Cortisone-d6	369.3>168.1		150	25
Cortisol	363.2>121.1	5.5	125	24
Cortisol-d4	367.1>121.1		120	24
21OH	347.2>311.2	7.2	145	12
21OH-d8	355.1>319.1		155	12
Cortico	347.3>121	8	125	22
Cortico-d8	355.1>125.1		135	20
11OH	347.2>109	8.3	140	24
11OH-d5	352.2>113		135	26
D4	287.2>109.1	10.2	125	25
D4-d7	294.1>100		155	22
DOC	331.2>108.9	10.9	125	20
DOC-d8	339.2>100		150	28
Testo	289.2>109	11.3	135	24
Testo-d4	293.2>97.9		135	26
17OHP	331.2>109.1	12.1	120	22
17OHP-d8	339.4>113.1		160	30
DHEA	304.2>253.1	3.6	98	14
DHEA-d6	310.2>259.2		148	16
Pregnenolone	332.2>86	4.95	125	30
Pregnenolone-d4	335.2>89		129	28
17Pregn	348.2>330.2	3.25	75	8
17Pregn-d3	351.2>33.2		95	8

**Supplemental data 1.** Characteristics of the steroids and internal standards used

	17Pregn	Pregnenolone	17OHP	DHEA	Cortisol	21OH	Corticosterone	11OH	D4	DOC	Testosterone	Cortisone
<b>Intra-Assay Precision (CV%)</b>												
QC 1	7.2	3.8	5.3	2.3	1.4	1.6	3.8	6.2	4.0	5.1	2.9	1.3
QC 2	3.4	3.0	2.8	3.0	1.6	3.1	1.6	2.6	2.1	1.5	1.7	1.8
<b>Inter-Assay Precision (CV%)</b>												
QC 1	11.57	11.84	8.79	14.85	8.12	8.40	4.31	5.78	5.71	8.44	5.97	4.07
QC 2	1.79	6.47	9.03	4.46	6.83	7.80	5.28	4.34	6.78	5.57	4.50	6.18
<b>LOD (nmol/L)</b>	0.03	0.25	0.063	0.063	0.13	0.063	0.063	0.063	0.063	0.063	0.063	0.13
<b>LOQ (nmol/L)</b>	0.13	0.50	0.063	0.13	0.13	0.063	0.063	0.063	0.063	0.063	0.063	0.13
<b>LOQ (CV%)</b>	5.2	4.6	9.43	7.1	6.66	12.47	7.44	5.49	8.87	6.45	10.54	3.29
<b>Linearity (nmol/L)</b>	0.13-10	0.50-10	0.063-50	0.13-10	0.13-1000	0.063-50	0.13-50	0.063-10	0.063-50	0.063-50	0.063-50	0.13-1000

**Supplemental data 2.** Method validation criteria

<b>Ratio of Steroid hormones (nmol/L)</b>							
<b>All controls</b>	<b>N</b>	<b>Mean</b>	<b>SD</b>	<b>Min</b>	<b>Max</b>	<b>5th</b>	<b>95th</b>
17OHP/17Pregn	128	1.7	0.095	0.25	8.609	0.651	3.785
D4/DHEA	128	1.703	0.112	0.239	11.44	0.467	3.438
11OH/17OHP	145	0.152	0.022	0.05	3.143	0.077	0.187
Cortisol/11OH	145	57.991	3.917	5.625	393	17.847	130.911
Cortisol/Cortisone	145	1.334	0.071	0.343	6.157	0.416	2.587
Testosterone/D4	145	0.316	0.023	0.038	1.496	0.062	0.816
<b>Foetus 46,XY</b>	<b>N</b>	<b>Mean</b>	<b>SD</b>	<b>Min</b>	<b>Max</b>	<b>5th</b>	<b>95th</b>
17OHP/17Pregn	67	1.805	0.112	0.636	4.582	0.773	3.789
D4/DHEA	67	2.077	0.18	0.707	11.44	0.797	4.392
11OH/17OHP	76	0.175	0.042	0.05	3.143	0.076	0.181
Cortisol/11OH	76	55.545	4.317	5.625	230.45	18.024	122.226
Cortisol/Cortisone	76	1.298	0.075	0.343	3.375	0.463	2.513
Testosterone/D4	76	0.497	0.032	0.117	1.496	0.18	1.182
<b>Foetus 46,XX</b>	<b>N</b>	<b>Mean</b>	<b>SD</b>	<b>Min</b>	<b>Max</b>	<b>5th</b>	<b>95th</b>
17OHP/17Pregn	61	1.585	0.157	0.25	8.609	0.502	2.742
D4/DHEA	61	1.293	0.106	0.239	4.536	0.421	2.901
11OH/17OHP	69	0.127	0.004	0.076	0.227	0.084	0.187
Cortisol/11OH	69	60.686	6.74	12.643	393	18.158	143.672
Cortisol/Cortisone	69	1.373	0.126	0.343	6.157	0.382	2.771
Testosterone/D4	69	0.116	0.007	0.038	0.364	0.055	0.233

**Supplemental data 3.** Distribution of ratios of hormone values, all and according to foetal karyotype

<b>Steroid hormone (nmol/L)</b>									
<b>All 21OHD</b>	<b>N</b>	<b>Mean</b>	<b>SD</b>	<b>Min</b>	<b>Max</b>	<b>5th</b>	<b>95th</b>	<b>Trend</b>	<b>p-value</b>
Cortisone	16	21.281	1.793	10.7	38.2	12.35	33.4	+	0.0464
Cortisol	21	27.897	3.769	5.6	86.1	7.8	44.7		0.249
Corticosterone	23	1.052	0.193	0.2	4.4	0.204	2.455	-	0.0003
11-desoxycortisol	23	0.649	0.11	0.1	2.5	0.207	1.588	+	0.0001
Delta4-Androstenedione	23	12.833	2.268	0.914	43.9	1.61	27.21	+	<0.0001
Deoxycorticosterone	23	0.578	0.091	0	2.2	0.208	0.993		0.9445
Testosterone	23	0.964	0.219	0.1	4.6	0.111	2.456	+	<0.0001
17-hydroxyprogesterone	23	23.345	4.36	1.6	78.3	2.13	53.18	+	<0.0001
17-hydroxypregnenolone	14	3.595	0.757	0.2	10.5	0.395	8.55	+	0.0409
Dehydroepiandrosterone	18	1.272	0.213	0.2	3.4	0.37	2.822		0.4525
Pregnenolone	14	6.601	0.995	1.8	16	1.865	12.145	+	0.0083
<b>Foetus 46,XY</b>	<b>N</b>	<b>Mean</b>	<b>SD</b>	<b>Min</b>	<b>Max</b>	<b>5th</b>	<b>95th</b>	<b>Trend</b>	<b>p-value</b>
Cortisone	2	20.25	5.65	14.6	25.9	15.165	25.335	+	0.6169
Cortisol	3	20.173	6.268	7.8	28.1	9.482	27.752	-	0.8805
Corticosterone	4	0.493	0.158	0.2	0.8	0.206	0.79	-	0.0074
11-desoxycortisol	4	0.622	0.187	0.319	1.167	0.346	1.067	+	0.1145
Delta4-Androstenedione	4	16.922	3.496	9.539	26.4	10.456	24.855	+	<0.0001
Deoxycorticosterone	4	0.46	0.055	0.298	0.544	0.328	0.537	-	0.0802
Testosterone	4	0.996	0.165	0.612	1.4	0.655	1.351	+	0.5549
17-hydroxyprogesterone	4	27.065	4.493	16.41	37.9	17.638	36.617	+	<0.0001
17-hydroxypregnenolone	2	3.6	0.2	3.4	3.8	3.42	3.78	+	0.7556
Dehydroepiandrosterone	3	0.937	0.342	0.5	1.61	0.52	1.519	-	0.6435
Pregnenolone	2	5.7	0.2	5.5	5.9	5.52	5.88	-	0.7749
<b>Foetus 46,XX</b>	<b>N</b>	<b>Mean</b>	<b>SD</b>	<b>Min</b>	<b>Max</b>	<b>5th</b>	<b>95th</b>	<b>Trend</b>	<b>p-value</b>
Cortisone	14	21.429	1.969	10.7	38.2	12.13	34.04	+	0.0542
Cortisol	18	29.184	4.248	5.6	86.1	9.595	50.91	+	0.3638
Corticosterone	19	1.17	0.223	0.2	4.4	0.29	2.699	-	0.0086
11-desoxycortisol	19	0.655	0.129	0.1	2.5	0.19	1.721	+	0.0007
Delta4-Androstenedione	19	11.972	2.635	0.914	43.9	1.531	28.96	+	<0.0001
Deoxycorticosterone	19	0.602	0.109	0	2.2	0.18	1.12	+	0.533
Testosterone	19	0.957	0.265	0.1	4.6	0.103	2.696	+	<0.0001
17-hydroxyprogesterone	19	22.562	5.217	1.6	78.3	1.965	55.71	+	<0.0001
17-hydroxypregnenolone	12	3.594	0.889	0.2	10.5	0.365	8.85	+	0.0455
Dehydroepiandrosterone	15	1.339	0.246	0.2	3.4	0.34	2.924	+	0.2952
Pregnenolone	12	6.752	1.162	1.8	16	1.855	12.738	+	0.0106

**Supplemental Data 4.** Results of hormone measurements for the samples obtained in case of 21-hydroxylase deficiency and comparison to the reference sample using a polynomial linear regression to adjust for gestational age, maternal age and foetal sex. Trend is + if 21-hydroxylase deficiency levels are significantly higher than reference values, and – if 21-hydroxylase deficiency levels are significantly lower than reference values.

# Steroid hormone (nmol/L)

Total cohort	Direction	AUC	AUC 95.CI	Cut-off value		
				Cost.50	Cost.10	Cost.1
Cortisone	controls < cases	0.642	[ 0.507 ; 0.777 ]	10.55	38.03	Inf
Cortisol	controls < cases	0.609	[ 0.493 ; 0.726 ]	16.43	81.25	Inf
Corticosterone	controls > cases	0.783	[ 0.676 ; 0.89 ]	1.31	0.76	Inf
11-desoxycortisol	controls < cases	0.641	[ 0.513 ; 0.768 ]	0.46	0.98	2.21
Delta4-Androstenedione	controls < cases	0.908	[ 0.83 ; 0.986 ]	3.15	5.28	12.04
Deoxycorticosterone	controls > cases	0.554	[ 0.409 ; 0.699 ]	3.16	Inf	Inf
Testosterone	controls < cases	0.664	[ 0.551 ; 0.778 ]	0.17	1.89	4.17
17-hydroxyprogesterone	controls < cases	0.836	[ 0.722 ; 0.949 ]	8.01	9.69	23.37
17-hydroxypregnenolone	controls < cases	0.61	[ 0.427 ; 0.793 ]	2.46	7.2	Inf
Dehydroepiandrosterone	controls < cases	0.523	[ 0.356 ; 0.691 ]	0.4	2.48	Inf
Pregnenolone	controls < cases	0.631	[ 0.465 ; 0.798 ]	5.46	15.29	Inf
Foetus 46,XY	Direction	AUC	AUC 95.CI	Cost.50	Cost.10	Cost.1
Cortisone	controls < cases	0.595	[ 0.093 ; 1 ]	14.32	Inf	Inf
Cortisol	controls < cases	0.465	[ 0.112 ; 0.817 ]	7.56	Inf	Inf
Corticosterone	controls > cases	0.967	[ 0.92 ; 1 ]	0.82	0.82	0.3
11-desoxycortisol	controls < cases	0.645	[ 0.37 ; 0.919 ]	0.31	1.06	1.06
Delta4-Androstenedione	controls < cases	1	[ 1 ; 1 ]	7.15	7.15	7.15
Deoxycorticosterone	controls > cases	0.734	[ 0.562 ; 0.905 ]	0.55	0.3	Inf
Testosterone	controls < cases	0.648	[ 0.411 ; 0.885 ]	0.61	Inf	Inf
17-hydroxyprogesterone	controls < cases	1	[ 1 ; 1 ]	12.99	12.99	12.99
17-hydroxypregnenolone	controls < cases	0.821	[ 0.715 ; 0.926 ]	3.37	3.37	Inf
Dehydroepiandrosterone	controls > cases	0.627	[ 0.206 ; 1 ]	0.72	Inf	Inf
Pregnenolone	controls < cases	0.649	[ 0.517 ; 0.782 ]	5.39	Inf	Inf
Foetus 46,XX	Direction	AUC	AUC 95.CI	Cost.50	Cost.10	Cost.1
Cortisone	controls < cases	0.67	[ 0.521 ; 0.819 ]	10.55	35.95	Inf
Cortisol	controls < cases	0.66	[ 0.529 ; 0.792 ]	16.26	81.25	Inf
Corticosterone	controls > cases	0.716	[ 0.584 ; 0.849 ]	1.31	0.75	Inf
11-desoxycortisol	controls < cases	0.665	[ 0.515 ; 0.815 ]	0.46	0.8	2.21
Delta4-Androstenedione	controls < cases	0.925	[ 0.858 ; 0.992 ]	1.59	3.31	12.04
Deoxycorticosterone	controls > cases	0.481	[ 0.315 ; 0.648 ]	3.16	Inf	Inf
Testosterone	controls < cases	0.903	[ 0.831 ; 0.974 ]	0.16	0.45	0.7
17-hydroxyprogesterone	controls < cases	0.815	[ 0.688 ; 0.942 ]	7.06	7.06	24.16
17-hydroxypregnenolone	controls < cases	0.566	[ 0.36 ; 0.772 ]	2.45	6.96	Inf
Dehydroepiandrosterone	controls < cases	0.568	[ 0.378 ; 0.757 ]	0.4	2.48	Inf
Pregnenolone	controls < cases	0.616	[ 0.427 ; 0.806 ]	1.78	15.29	Inf

**Supplemental Data 5.** Areas under the curve (AUC) and their confidence interval obtained using hormones to diagnose 21OH deficiency. And optimal cut-off values for three different relative costs of a false negative and a false positive: 1; 10 and 50.