Determination of Ustekinumab in Human Serum Using High Sensitivity PK ELISA.

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Introduction

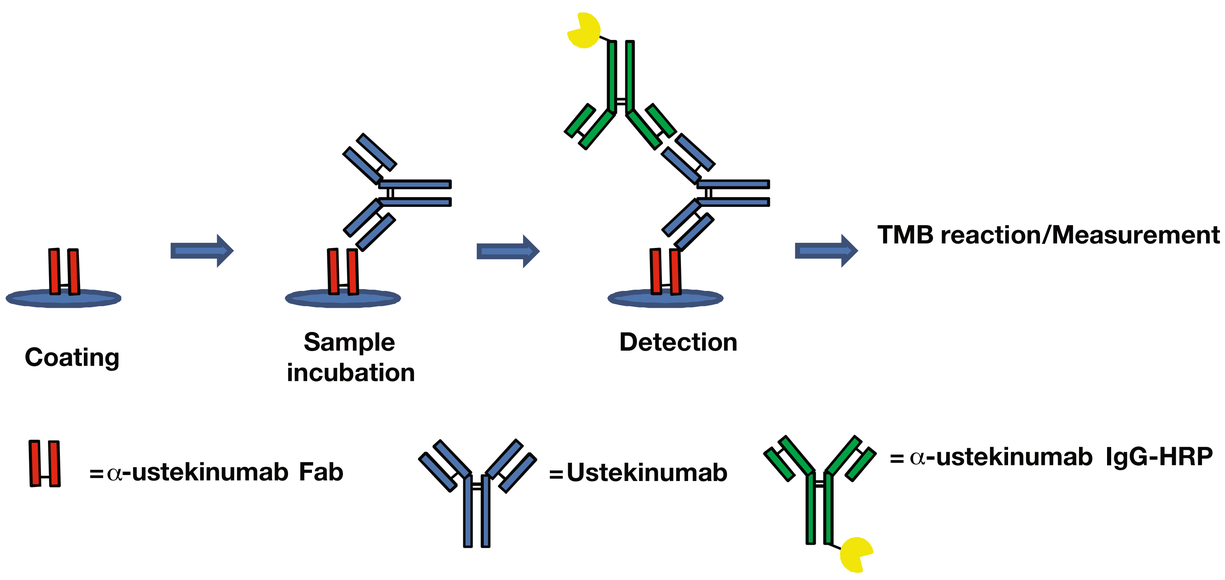
Psoriasis is a chronic inflammatory condition affecting 2-4% of human population. Scientific evidence suggests that it is an autoimmune disease caused by a combination of genetic and environmental factors. Psoriasis manifests itself as skin inflammation and scaling however it may also lead to arthritis and death. Though incurable, psoriasis can be effectively treated in order to reduce its symptoms. Therapeutic approaches include topical agents (e.g. steroids), phototherapy and systemic drugs (e.g. ustekinumab).

The goal of this study was to set up a robust and highly sensitive protocol for the detection of ustekinumab - a human monoclonal antibody used for the treatment of psoriasis. The major challenge was to design the assay in a way to use low sample volumes, as other species, including small animals, were planned for testing. The target sensitivity was supposed to be ≤ 100 ng/mL.

Assay Development

The total ustekinumab concentration in human serum was measured using quantitative sandwich ELISA (Figure 1). The assay utilizes two anti-ustekinumab antibodies recognizing distinct epitopes on the analyte. One of these antibodies (Fab fragment) is used to capture ustekinumab on the ELISA plate whereas the second one, a HRP-conjugate, serves as detection reagent. The assay is developed using TMB and the resulting colorimetric reaction is measured with spectrophotometer.

Figure 1: Principle of the assay used to measure ustekinumab concentration



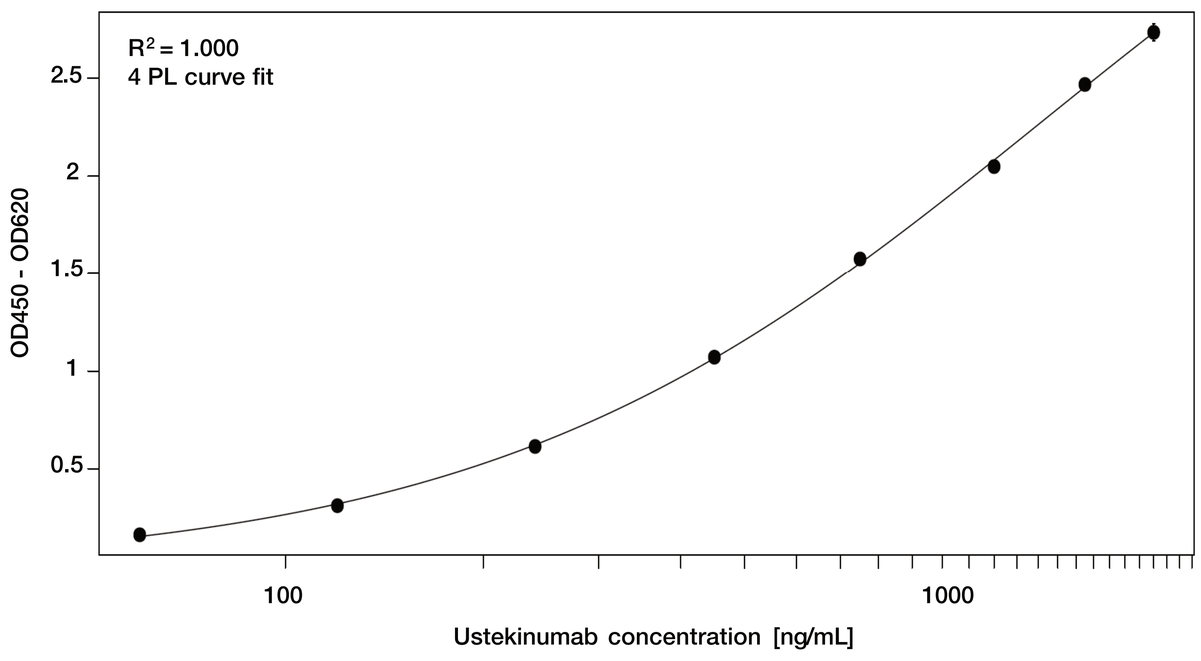
Results

Assay development involved testing of the optimal concentration of coating (0.5 vs 1 µg/mL) and detection (0.05 vs 0.1 vs 0.15 vs 0.2 µg/mL) reagents as well as evaluation of the minimal required dilution (MRD 10 vs 20 vs 100). Coating at 1 µg/mL and detection at 0.05 µg/mL enabled to reach high signal to background ratio as well as favorable parameters of calibration curve. MRD 30 was chosen as a good compromise between assay sensitivity and the volume of sample to be utilized.

Using the above-mentioned conditions the analytical range was established between 60 and 2100 ng/mL. (Figure 2).

Figure 2: Analytical range of the assay (A- example of 1 curve) and standard curve parameters (B). 3 independent runs were performed. Reported is the average of a duplicate measurement.

A



B

Run/ Nominal [ng/mL]	STD01 60	Nom %	STD02 120	Nom %	STD03 240	Nom %	STD04 450	Nom %	STD05 750	Nom %	STD06 1200	Nom %	STD07 1650	Nom %	STD08 2100	Nom %
R13	63.05	105.08	116.58	97.15	236.54	98.56	452.64	100.59	766.11	102.15	1166.77	97.23	1668.41	101.12	2102.98	100.14
R14	59.49	99.15	119.65	99.71	240.74	100.31	454.76	101.06	739.17	98.56	1202.61	100.22	1668.60	101.13	2084.14	99.24
R15	55.95	93.25	122.29	101.91	243.45	101.44	456.49	101.44	724.07	96.54	1224.91	102.08	1663.02	100.79	2079.38	99.02

Selectivity assessment showed no interference related to matrix content (Figure 3). The precision and accuracy were proved at 5 different QC levels in 3 independent runs (Figure 4). Finally, room temperature and freeze and thaw stability was also demonstrated (Figure 5).

Figure 3: Selectivity of the assay. Selectivity was tested using 10 individual human plasma samples (5 females, 5 males) and mixed gender human plasma pool as control. Each sample was tested unspiked, spiked with 60 ng/mL (Low Spike) or with 1500 ng/mL (High Spike) of ustekinumab. Maximal allowed %Bias is indicated. Reported is the average of a duplicate measurement.

R13 Sample	Blank	Sample	Low Spike [ustekinumab] Nominal [ng/mL] 60.000	Nom %	High Spike [ustekinumab] Nominal [ng/mL] 1500.000	Nom %
CM/17-1559	BLQ	CM/17-1559	62.72	104.53	1479.80	98.65
CM/17-1561	BLQ	CM/17-1561	64.92	108.20	1528.51	101.90
CM/17-1562	BLQ	CM/17-1562	64.31	107.18	1500.44	100.03
CM/17-1563	BLQ	CM/17-1563	63.84	106.39	1514.34	100.96
CM/17-1564	BLQ	CM/17-1564	62.37	103.95	1486.31	99.09
CM/17-1584	BLQ	CM/17-1584	64.41	107.35	1491.68	99.45
CM/17-1585	BLQ	CM/17-1585	60.71	101.19	1438.43	95.90
CM/17-1586	BLQ	CM/17-1586	61.30	102.17	1474.50	98.30
CM/17-1587	BLQ	CM/17-1587	63.03	105.05	1520.44	101.36
CM/17-1588	BLQ	CM/17-1588	64.16	106.93	1496.17	99.74
pool: CMP/1600429	BLQ	pool: CMP/1600429	64.01	106.68	1612.73	107.52
		mean [ng/mL]	63.18		1493.06	
		SD	1.40		25.98	
		CV [%]	2.22		1.74	
Total BLQ	10	Nominal [%]	105.29		99.54	
n	10	n	10		10	
BLQ: Below Limit of Quantitation						
		± maximal %Bias	25		20	

	Blank		Low Spike [ustekinumab]	High Spike [ustekinumab]
individuals	10 / 10 BLQ	individuals	10 / 10 acceptable	10 / 10 acceptable
pool	BLQ	pool	acceptable	acceptable

Figure 4: Precision and accuracy of QC samples. 3 independent runs, each containing 2 QC sets were performed. Reported is the average of a duplicate measurement.

Nominal [ng/mL]	QC LLOQ 60	Nom %	QC Low 180	Nom %	QC Med 840	Nom %	QC High 1500	Nom %	QC ULOQ 2100	Nom %
R13	61.36	102.26	175.03	97.24	821.44	97.79	1502.52	100.17	1931.22	91.96
	60.87	101.45	171.51	95.28	791.24	94.20	1419.95	94.66	1993.14	94.91
mean [ng/mL]	61.11		173.27		806.34		1461.23		1962.18	
SD	0.35		2.49		21.35		58.39		43.78	
CV [%]	0.57		1.44		2.65		4.00		2.23	
Nominal [%]	101.85		96.26		95.99		97.42		93.44	
n	2		2		2		2		2	
R14	62.05	103.42	184.78	102.65	894.82	106.53	1646.31	109.75	2254.64	107.36
	59.62	99.36	179.96	99.98	839.32	99.92	1567.20	104.48	2097.27	99.87
mean [ng/mL]	60.83		182.37		867.07		1606.75		2175.95	
SD	1.72		3.40		39.24		55.94		111.28	
CV [%]	2.83		1.87		4.53		3.48		5.11	
Nominal [%]	101.39		101.32		103.22		107.12		103.62	
n	2		2		2		2		2	
R15	57.93	96.55	179.54	99.74	865.85	103.08	1482.23	98.82	1964.42	93.54
	58.44	97.39	174.75	97.08	785.42	93.50	1387.36	92.49	1992.19	94.87
mean [ng/mL]	58.18		177.14		825.63		1434.80		1978.31	
SD	0.36		3.38		56.88		67.09		19.64	
CV [%]	0.62		1.91		6.89		4.68		0.99	
Nominal [%]	96.97		98.41		98.29		95.65		94.21	
n	2		2		2		2		2	
ANOVA evaluation										
Mean Observed Conc. [ng/mL]	60.04		177.59		833.02		1500.93		2038.81	
Nominal [%]	100.07		98.66		99.17		100.06		97.09	
Number of Runs	3		3		3		3		3	
Number of replicates	6		6		6		6		6	
Between Run Precision (%CV)	2.40		2.25		1.15		5.47		5.31	
Within Run Precision (%CV)	1.72		1.76		5.01		4.04		3.43	
Total Variation (%CV)	2.96		2.86		5.14		6.80		6.32	

Figure 5: Stability evaluation of ustekinumab in human serum. Ustekinumab was spiked into mixed gender human serum pool at QC Low and QC high levels (2 aliquots for each QC level and test condition). Next, each test sample was submitted to 3 different conditions: BenchTop ON incubation, 3 cycles of freeze/thaw at -20°C or -80°C. Reported is the average of a duplicate measurement.

Conditions	BenchTop, ON				Freeze / Thaw 3 cycles, -20°C				Freeze / Thaw 3 cycles, -80°C			
Nominal [ng/mL]	QC Low 180	Nom %	QC High 1500	Nom %	QC Low 180	Nom %	QC High 1500	Nom %	QC Low 180	Nom %	QC High 1500	Nom %
R14	188.13	104.51	1482.33	98.82	179.33	99.63	1456.95	97.13	189.91	105.51	1587.30	105.82
	161.60	89.78	1422.61	94.84	169.87	94.37	1435.15	95.68	168.71	93.73	1420.56	94.70
mean [ng/mL]	174.87		1452.47		174.60		1446.05		179.31		1503.93	
SD	18.75		42.22		6.69		15.41		14.99		117.90	
CV [%]	10.72		2.91		3.83		1.07		8.36		7.84	
Nominal [%]	97.15		96.83		97.00		96.40		99.62		100.26	
n	2	2	2	2	2	2	2	2	2	2	2	2

Discussion

In our protocol we utilized two BIO-RAD antibodies (two distinct clones) in a PK ELISA assay. The first one, a Fab fragment, was used to capture ustekinumab on plate. The second one, a HRP-conjugate, was chosen as detection reagent. In a stepwise fashion we have optimized the concentration of coating (1 µg/mL) and detection (0.05 µg/mL) antibodies. Further, by applying minimal required dilution of 1:30 we managed to generate a protocol that utilizes as little as ~7 µL of sample per duplicate measurement. Using the above-mentioned setup the analytical range was between 60 and 2100 ng/mL thus fulfilling the initial criteria.

Conclusion

The PK assays described here showed high sensitivity, reliability and reproducibility. Such assays are routinely developed at Celerion Switzerland AG. They offer important tools for early clinical development of novel antibody therapeutics as well as biosimilars.

Poster presentation at EBF 2017