



Pitfalls in the Establishment of Cut Points for ADA Assays Using the ECL Method

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Structure

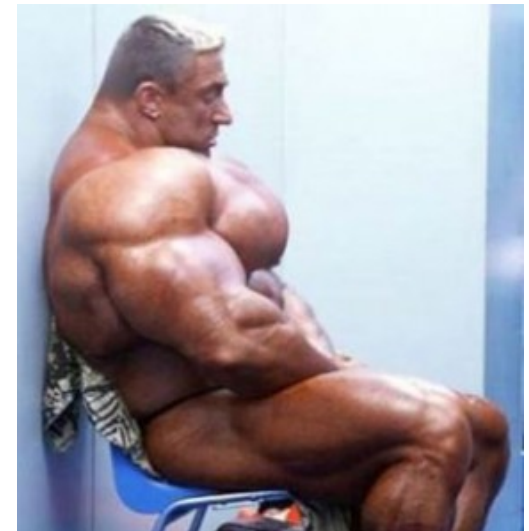
- ☞ Growth Hormone
- ☞ Study and Method Overview
- ☞ Validation Procedure
- ☞ Unexpected Trouble
- ☞ Troubleshooting

Human Growth Hormone (hGH)

- ☛ Peptide hormone (191 amino acids, 22 kDa)
- ☛ Secreted by pituitary gland
- ☛ Stimulates IGF-I secretion in the liver
- ☛ Involved in growth, glycogenolysis, lipolysis, as well as uptake and metabolization of amino acids
- ☛ Anabolic effects (Skeletal muscle, liver and bone)



<http://www.rcsb.org/pdb/explore/explore.do?structureId=1hgu>



Indications for hGH Treatment

Growth Hormone Deficiency (GHD)

☞ Frequency:

→ 1 : 5,000; m:f ca. 4:1

☞ Causes:

- Idiopathic
- Gene mutations (e.g. Typ1a)
- Prader-Willi syndrome, Turner syndrome
- Radiation therapy of the head in course of a tumor treatment

☞ Symptoms:

- Decreased growth velocity and height
- Decreased bone and skeletal muscle mass
- Cardiovascular Disease (in adults)

Different hGH Preparation and Immunogenicity

Preparation	Frequency of antibodies detected by Immunoassay	Reference
Genotropin	4/229 (1.7%), $C_{max} = 0.15$ mg/L 0/144	Lundin et al., Acta Paediatr Scand 372 (suppl), 1991
rhGH (Genentech) 1985-1994	2 (23000) <0.04%	Blethen et al., JCEM 1996 , 81:1704-1710
Valtropin Humatrope	3(98) 3.1% within 1 year 1(32) 2.0% within 1 year	Peterkova et al., Horm Res. 2007 ; 68:288-293
Omnitrope	3/44 (6.8%) transient over treatment period of 84 month	Romer et al., Horm Res 2009 , 72:359-369
China-made rhGH	17/45 (37.8%)	Jiang J et al. Chin Med Sci J. 2004 ; 19(3):225-9.
sr hGH (Genentech)	16(36) 44% bei 1.5 mg/kg 1x month 26(38) 68% bei 0.75 mg/kg 2x month	Reiter et al., JCEM 2000 , 86: 4700-061

Study and Method Description

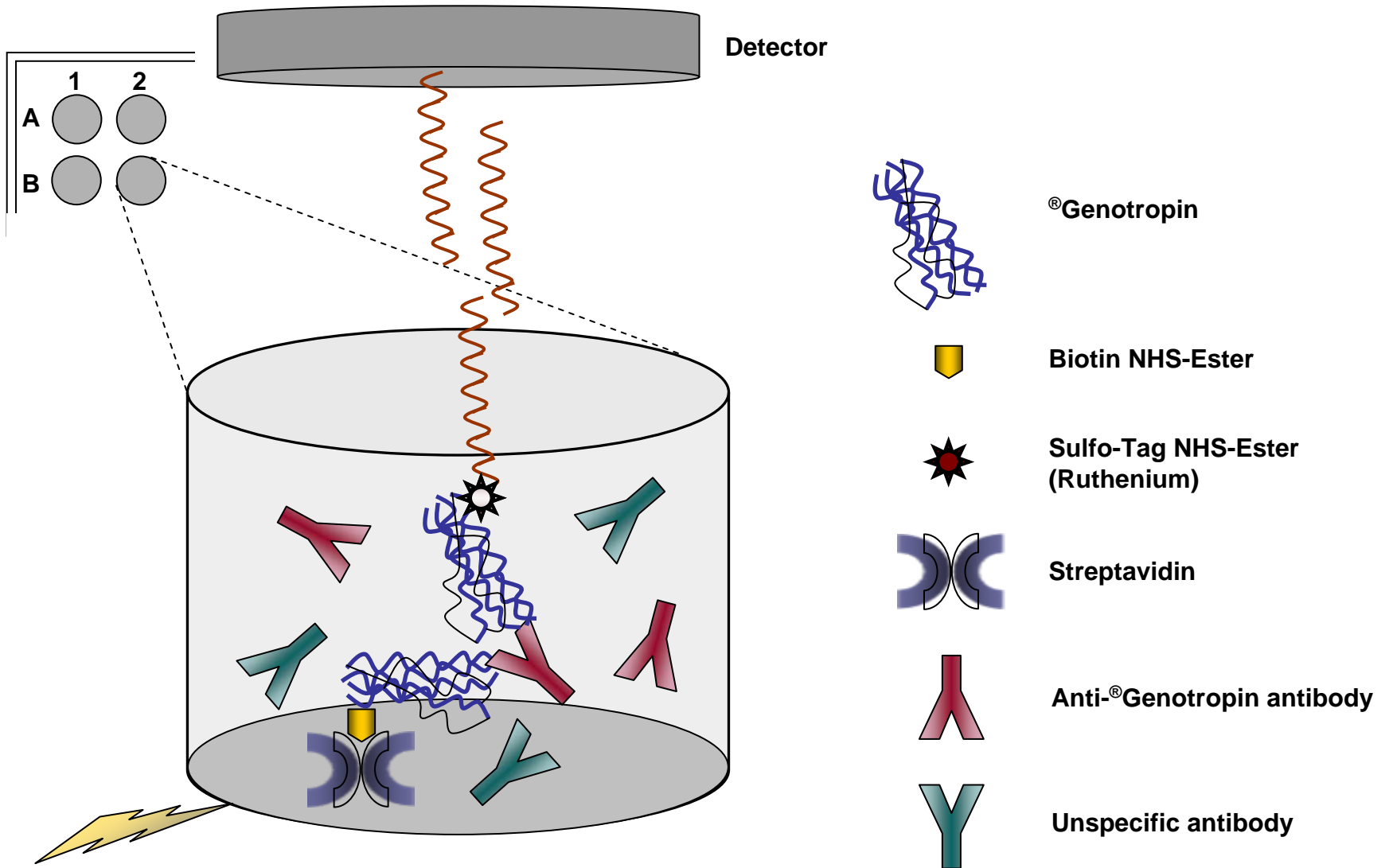
Study

- Phase 2 hGH treatment study
- GH deficient patients substituted with [®]Genotropin (control group)
- Validation of an anti-[®]Genotropin assay

ADA Assay Format

- Polyclonal anti-hGH antibody
- Sulfo-TAG and Biotin-labeled [®]Genotropin,
- Streptavidin Standard Plates (MSD)
- Sector Imager 2400 Reader
- Pool serum (commercially available) = Reference Control (**RQC**)
- Pool serum from healthy blood donors = Negative Quality Control (**NQC**)

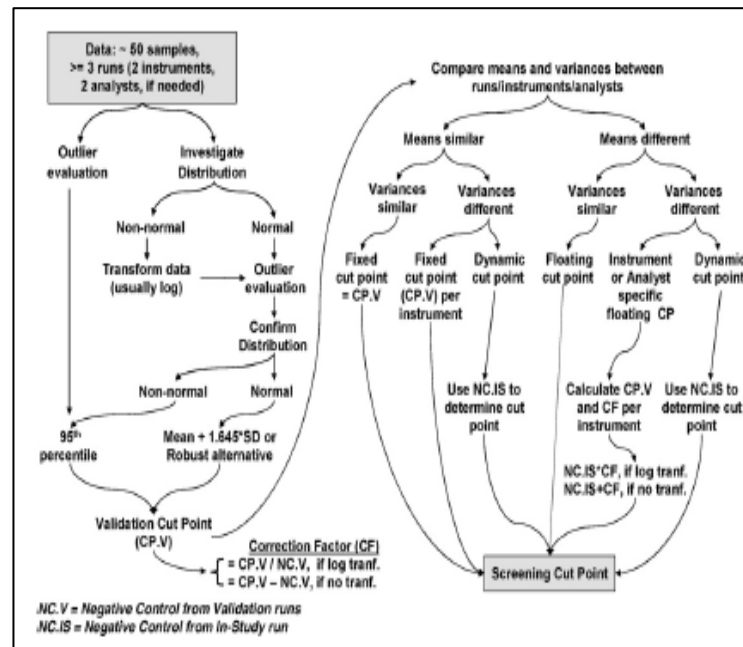
Anti-[®]Genotropin Assay



Cut Point Evaluation

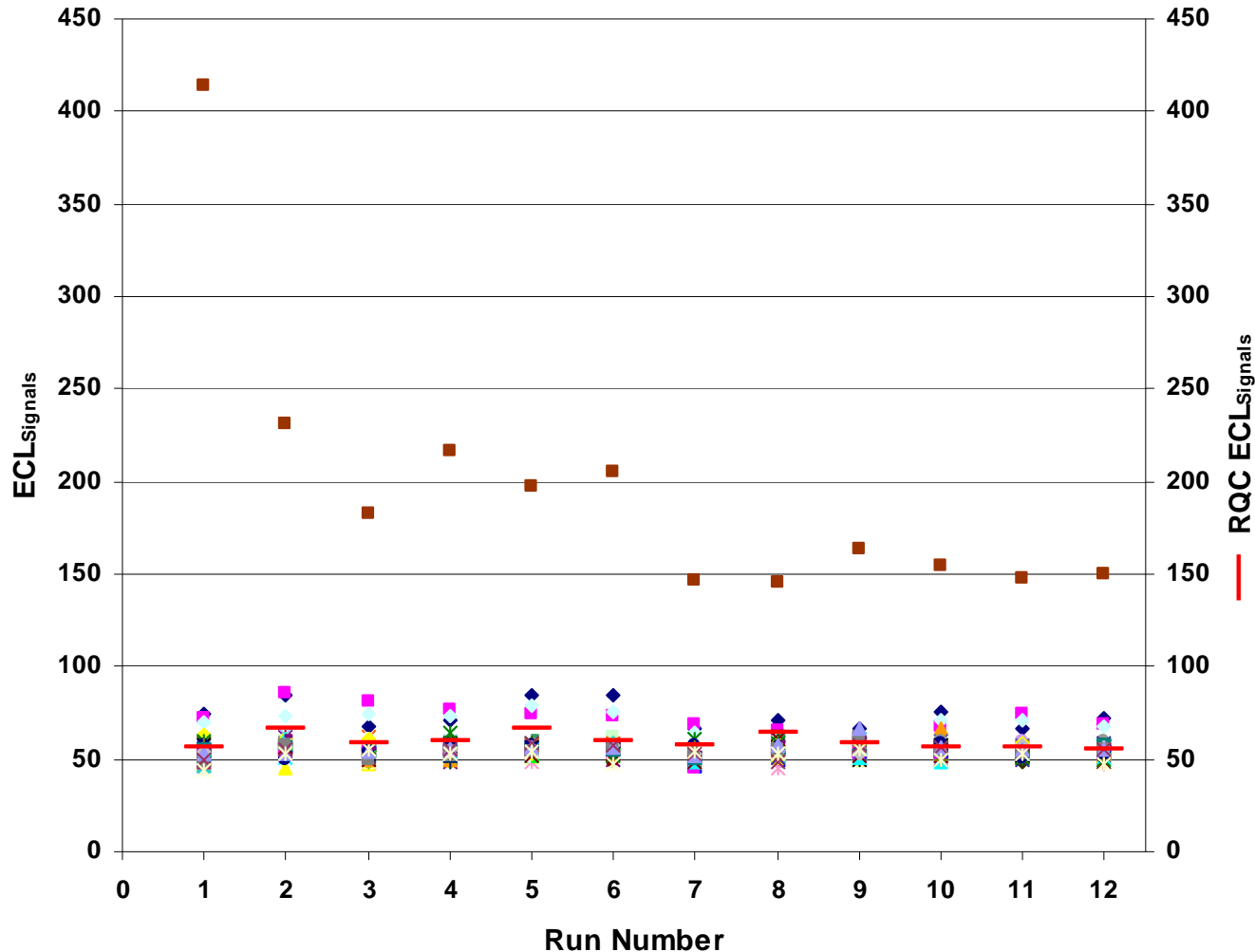
Screening Cut Point

- Established using sera of disease population (Adult GHD patients N=50, 25 female and 25 male patients)
- Calculation of screening cut point (Shankar et al. 2008)
- Objective and conservative approach, 5% false-positive rate



Shankar et al. J Pharm Biomed Anal. 2008 Dec 15;48(5):1267-81

Signal Distribution



Outlier evaluation was performed using box plot analysis.
Lower outlier: (25% quartile - 1.5 x IQR); Upper outlier: (75% quartile + 1.5 x IQR)

Outliers

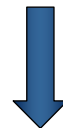
Female

Serum sample	Op1/Run 1	Op2/Run 1	Op1/Run 2	Op2/Run 2	Op1/Run 3	Op2/Run 3	Op1/Run 4	Op2/Run 4	Op1/Run 5	Op2/Run 5	Op1/Run 6	Op2/Run 6
1	75	85	68	72	85	85	67	71	66	76	67	72
2	72	86	81	77	74	73	69	65	61	68	75	69
3	55	45	48	50	59	58	54	54	59	59	52	60
4	51	51	56	50	55	55	51	56	61	58	57	57
5	53	62	49	48	57	50	49	52	59	53	55	52
6		58	55	55	57	48	49	52	57	57	54	50
7	52	57	56	53	56	60	49	53	56	58	53	58
8	57	55	56	53	55	59	43	47	53	56	54	54
9	52	58	56	51	59	57	51	49	61	54	57	50
10	70	73	74	74	79	76	65	63	62	71	71	68
11	53	60	52	50	55	62	54	55	59	55	55	54
12	50	59	48	55	51	57	48	51	50	52	53	56
13	57	63	56	60	59	58	53	53	54	51	53	56
14	49	59	52	58	49	57	51	46	55	53	50	53
15	58	57	53	53	55	56	48	51	58	53	60	57
16	54	57	58	56	56	57	48	57	54	55	56	56
17	49	55	54	48	59	60	50	52	50	56	57	58
18	43	60	52	52	49	58	51	48	54	50	53	58
19	55	61	49	48	54	59	51	56	56	56	56	52
20	51	57	55	54	52	56	51	50	54	52	54	49
21	49	56	50	50	59	54	50	56	57	66	52	55
22	51	58	62	56	57	59	55	58	56	58	57	56
23		59	53	52	54	56	49	57	54	60	58	54
24	51	55	60	51	54	58	53	59	53	60	56	60
25	54	61	53	49	58	59	53	54	53	51	50	54
26	56	56	57	61	63	59	58	62	51	54	54	55
27	51	57	55	61	56	57	53	64	59	58	52	60
28	63	58	55	53	56	53	53	60	62	57	49	51
29	414	231	183	216	198	205	147	146	164	154	148	150
30	48	55	51	56	59	57	53	57	55	50	56	54
31	50	56	52	57	57	56	48	51	54	56	50	59
32	57	54	54	56	57	53	51	56	50	58	52	55
33	52	58	61	54	51	57	48	56	56	52	56	55
34	52	55	53	61	53	54	47	53	58	49	52	48
35	54	55	55	56	49	56	48	56	52	52	54	53
36	54	53	55	56	56	56	49	54	51	51	52	54
37	63	61	61	57	53	57	53	59	56	53	59	56
38	53	53	55	54	55	54	45	56	53	53	52	52
39	55	61	55	59	57	59	49	57	51	49	54	51
40	51	57	50	57	52	54	49	61	52	52	51	49
41	60	53	56	64	59	52	61	59	58	57	51	50
42	58	50	54	58	57	56	58	57	53	59	51	51
43	57	58	51	53	56	53	57	52	58	50	54	50
44	56	62	56	56	57	58	53	59	61	56	55	56
45	58	59	51	56	56	52	48	53	54	53	51	60
46	54	58	52	55	55	57	53	55	54	54	58	57
47	49	57	50	55	54	55	56	56	63	55	53	55
48	53	53	55	55	56	56	52	57	67	56	55	56
49	50	56	55	56	59	58	54	48	58	57	53	55
50	44	53	56	53	54	47	54	53	55	50	54	48

Outliers (ECL Signals $>/< 1.5 \times \text{IQR}$)

Normality-Testing and Analysis of Variances

Screening Cut Point	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6	
	Operator 1	Operator 2	Operator 1	Operator 2	Operator 1	Operator 2	Operator 1	Operator 2	Operator 1	Operator 2	Operator 1	Operator 2
Shapiro-Wilk Normality Test for Data without Outliers												
N	44	45	46	45	46	44	46	48	49	45	46	46
W	0.976	0.987	0.971	0.969	0.951	0.977	0.974	0.988	0.958	0.961	0.985	0.967
P	0.477	0.901	0.303	0.270	0.053	0.521	0.399	0.897	0.080	0.138	0.800	0.217
Passed	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
One-Way Analysis of Variance												
P-Value	< 0.001											
N	12											
F	9.476											
Are means significant different?	Yes											
Levene Test of Equal Variance												
P-Value	<0.015											
N	12											
F	2.167											
Are the variances significant different?	Yes											

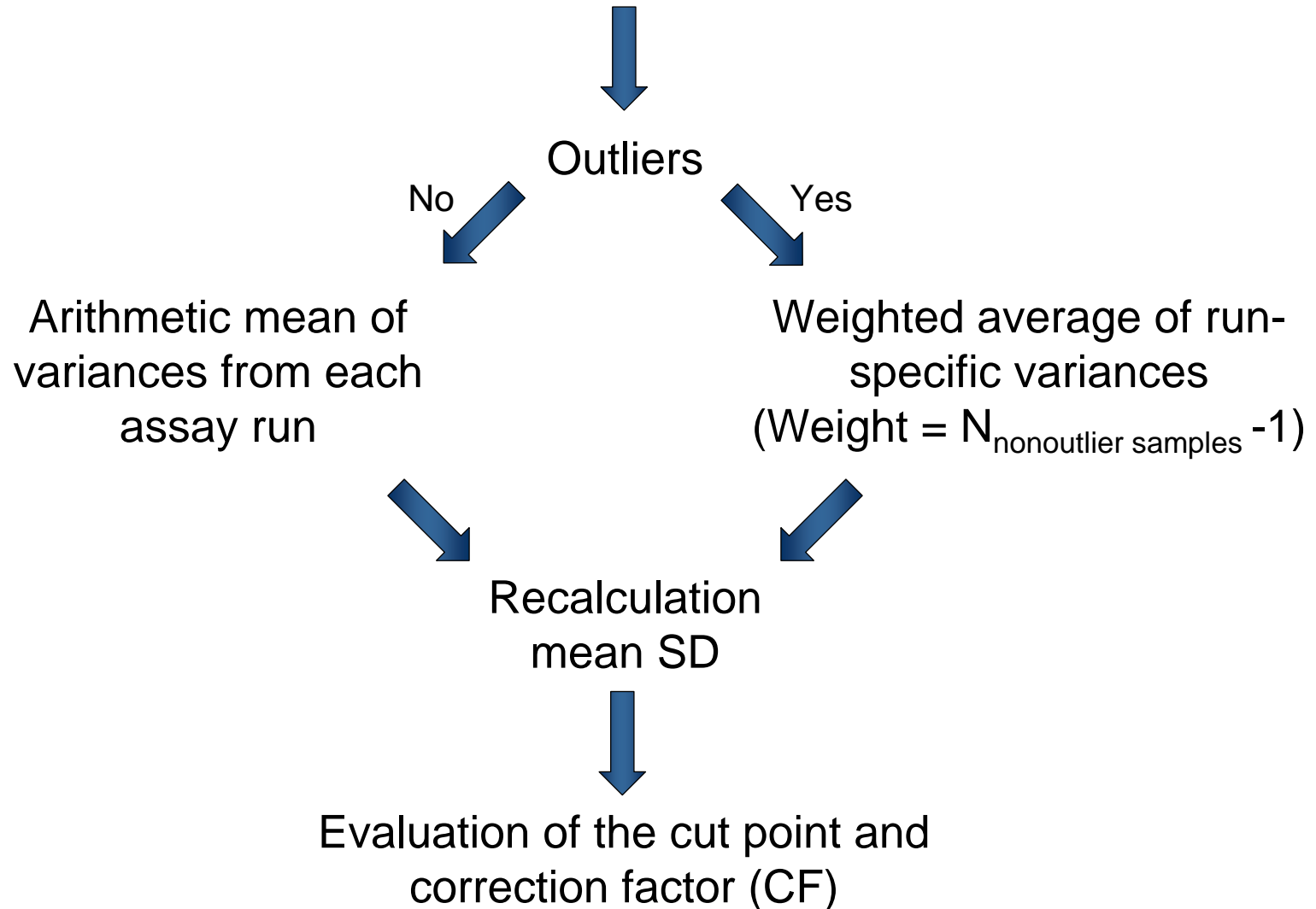


Due to different means and variances the pooled variances approach was used for evaluation of the screening cut point!

Pooled Variances

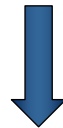
Detection and Quantification of Antibodies to Biopharmaceuticals, First Edition, Edited by Michael G. Tovey, John Wiley and Sons, 2011
Chapter 16, V. Devanarayan and M. G. Tovey pp.: 296-298


Calculation of run-specific variances



Pooled Variances for Determining the Screening Cut Point

Floating Cut Point (CP) with Pooled Variances Approach												
Mean (ECL Signal)	53.057	56.767	53.815	54.178	55.304	56.170	51.022	54.896	55.929	54.122	53.630	54.033
SD	4.167	3.020	3.227	3.477	2.897	2.449	3.518	4.323	4.039	3.084	2.621	3.339
Weighted Var.	1.390	0.747	0.873	0.991	0.703	0.480	1.037	1.636	1.458	0.778	0.576	0.934
Pool. Var.	11.588	= Sum of all weighted variances										
Pool. SD	3.404	= Pooled SD calculated from square root of the pooled variances										
CP	58.657	62.366	59.415	59.778	60.904	61.770	56.622	60.496	61.528	59.721	59.230	59.632
	CP (cut point) = mean signal of the GHD sera + 1.645 * mean pooled SD											
RQC	56.250	66.500	58.500	59.500	67.000	59.750	57.000	64.833	58.167	56.500	56.833	55.333
CP-RQC	2.406	-4.134	0.914	0.277	-6.097	2.019	-0.379	-4.339	3.361	3.221	2.396	4.298
CF	0.329	= Mean Additive Correction Factor (CF) on RQC to Calculate the Cut Point										



 Floating Cut Point with an additive correction factor of
 $+0.329 \text{ ECL}_{\text{Signals}}$

Number of False Positives

Serum sample	Op1/Run 1	Op2/Run 1	Op1/Run 2	Op2/Run 2	Op1/Run 3	Op2/Run 3	Op1/Run 4	Op2/Run 4	Op1/Run 5	Op2/Run 5	Op1/Run 6	Op2/Run 6
1	75	85	68	72	85	85	67	71	66	76	67	72
2	72	86	81	77	74	73	69	65	61	68	75	69
3	55	45	48	50	59	58	54	54	59	59	52	60
4	51	51	56	50	55	55	51	56	61	58	57	57
5	53	62	49	48	57	50	49	52	59	53	55	52
6		58	55	55	57	48	49	52	57	57	54	50
7	52	57	56	53	56	60	49	53	56	58	53	58
8	57	55	56	53	55	59	43	47	53	56	54	54
9	52	58	56	51	59	57	51	49	61	54	57	50
10	70	73	74	74	79	76	65	63	62	71	71	68
11	53	60	52	50	55	62	54	55	59	55	55	54
12	50	59	48	55	51	57	48	51	50	52	53	56
13	57	63	56	60	59	58	53	53	54	51	53	56
14	49	59	52	58	49	57	51	46	55	53	50	53
15	58	57	53	53	55	56	48	51	58	53	60	57
16	54	57	58	56	56	57	48	57	54	55	56	56
17	49	55	54	48	59	60	50	52	50	56	57	58
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32	57	54	54	56	57	53	51	56	50	58	52	56
33	52	58	61	54	51	57	48	56	56	52	56	55
34	52	55	53	61	53	54	47	53	58	49	52	48
35	54	55	55	56	49	56	48	56	52	52	54	53
36	54	53	55	56	56	56	49	54	51	51	52	54
37	63	61	61	57	53	57	53	59	56	53	59	56
38	53	53	55	54	55	54	45	56	53	53	52	52
39	55	61	55	59	57	59	49	57	51	49	54	51
40	51	57	50	57	52	54	49	61	52	52	51	49
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45	58	59	51	56	56	52	48	53	54	53	51	60
46	54	58	52	55	55	57	53	55	54	54	58	57
47	49	57	50	55	54	55	56	56	63	55	53	55
48	53	53	55	55	56	56	52	57	67	56	55	56
49	50	56	55	56	59	58	54	48	58	57	53	55
50	44	53	56	53	54	47	54	53	55	50	54	48
False Positives w/o outliers [%]	20,45	0,00	8,70	11,11	0,00	2,27	8,70	0,00	24,49	24,44	8,70	19,57
Mean False Positives [%]	10,70											
False Positives and outliers [%]	31,25	8,00	15,69	20,00	8,00	10,00	16,00	4,00	26,00	32,00	16,00	26,00
Mean False Positives and outliers [%]	17,74											

Outliers (ECL Signals $>/< 1.5 \times \text{IQR}$)
 False Positives (ECL Signals above run-specific Screening Cut Point)

Confirmatory Cut Point

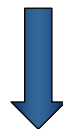
Range of %Inhibition values after outlier evaluation

Serum	OP 1 / Day 1 w/o all outliers	OP 2 / Day 1 w/o all outliers	OP 1 / Day 2 w/o all outliers	OP 2 / Day 2 w/o all outliers	OP 1 / Day 3 w/o all outliers	OP 2 / Day 3 w/o all outliers
1			21.21			
2			9.84			
3	10.19	2.80	3.39	5.93	-7.77	6.67
4	4.95	6.25	9.09	11.30	1.77	1.77
5	-2.06	0.96	12.71	-1.90	-7.34	-1.94
6	1.03	16.35	-1.77	0.00	0.00	-8.08
7	-14.43	1.89	0.00	-1.74	-1.90	-6.90
8	-15.12	-16.13	0.00	3.57	-4.67	-4.63
9	-2.97	-9.28	12.40	-7.48	1.75	-17.00
10			13.82			
11	-6.54	0.00	8.55	-3.67	3.67	-1.85
12	-10.42	0.00	-15.15	5.77	0.00	1.80
13	8.49	-1.89	9.35	2.97	11.32	-1.80
14	9.80	-12.09	16.51	18.87	2.02	-3.77
15	-9.47	3.92	-0.87	4.72	13.33	6.14
16	-2.11	10.53	3.74	11.82	9.82	0.00
17	-16.16	-3.88	-1.00	8.93	8.85	6.09
18	10.89	-14.58	12.96	8.00	5.66	3.48
19	2.97	12.61	-6.25	-0.90	9.91	-4.81
20	6.86	-4.04	2.80	-3.85	8.33	-7.22
21	9.00	-1.80	0.00			-2.75
22	12.73	7.76	-6.31	16.52	5.31	10.81
23	3.06	-5.26	-11.21	17.65	0.86	2.78
24	10.48	5.08	-4.72		0.00	11.76
25	11.43	-2.78	0.00	-7.92	-11.00	-4.67
26	16.52		-13.73	3.70	0.00	-1.82
27	-2.86		5.08	14.66		13.45
28	6.67	14.17	4.88	14.04		-7.84
29						
30	2.86	1.75	9.09	3.03	7.21	6.48
31	-5.26	-7.84	14.81	7.14	-1.01	13.68
32	0.00	6.25	-4.00	5.22	0.97	2.75
33	3.16	-3.57	6.25	9.62	0.89	0.00
34	-7.53	-9.52	4.31	6.19	0.00	0.00
35	5.21	7.14	-14.42	4.85	6.54	7.55
36	7.22	0.93	-9.80	10.89	6.80	8.33
37	1.89	15.38	-0.90	12.26	10.17	14.41
38	-14.44	0.90	-3.81	2.86	10.58	8.74
39	1.03	9.65	-1.98	4.08	-6.54	-1.98
40	3.06		3.85	10.58	-1.96	-1.03
41	11.48	6.84	1.74	8.85	-1.98	
42	10.34	14.16	-2.86	7.69	-5.94	-2.97
43	2.65	-2.88	0.00	-2.00	-12.15	-9.09
44	-5.66	4.27	6.56	6.25	-1.82	12.50
45	-3.16	2.83	0.00	3.81	-12.87	10.92
46	5.71	-2.75	9.35	10.28	0.00	4.42
47	11.71	0.00	17.60	7.34	-2.83	1.82
48	0.97	0.00	16.54	3.57	6.36	1.80
49	1.87	1.04	0.86	-0.88	0.00	7.27
50	0.93	-0.94	13.64	-1.01	1.87	-7.37

Confirmatory Cut Point Evaluation

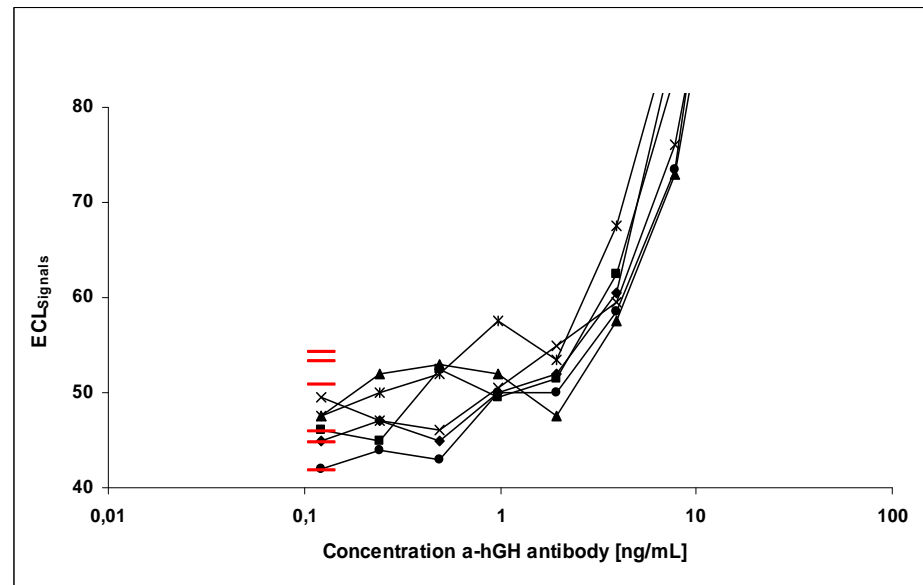
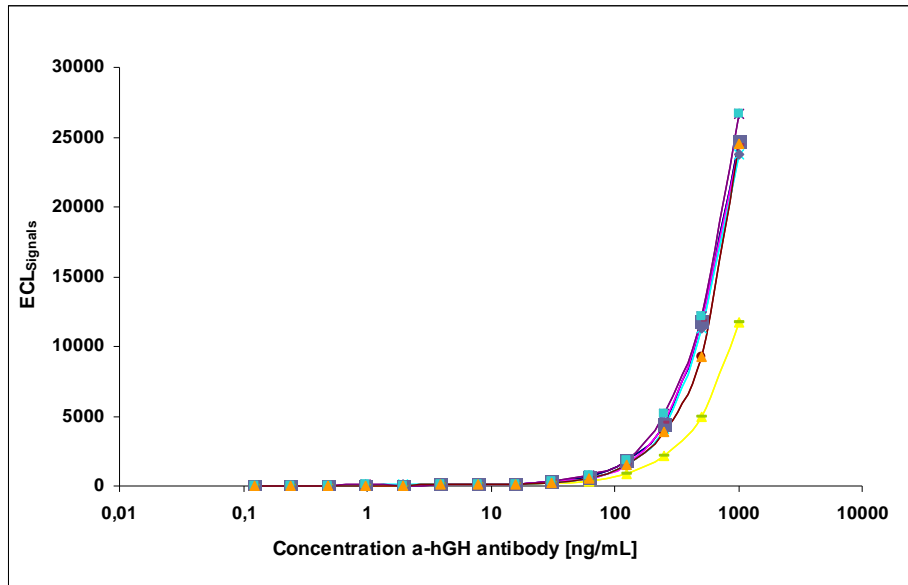
Confirmatory Cut Point	Day 1		Day 2		Day 3	
Operator	1	2	1	2	1	2
	% Inhibition					
	Shapiro-Wilk Normality Test for Data without Outlier					
W	0.957	0.980	0.981	0.987	0.969	0.977
P-Value	0.090	0.650	0.611	0.896	0.300	0.513
Passed	yes	yes	yes	yes	yes	yes
N	46	43	49	44	43	45
Mean	1.67	1.26	3.11	5.49	1.261	1.51
SD	8.15	7.69	8.69	6.35	6.434	7.15
Run confirmatory cut point (% Inhibition)	26.84	25.01	29.95	25.11	21.14	23.60
Mean Confirmatory cut point (% Inhibition)	25.28					

☞ Confirmatory Cut Point of 25.28% inhibition was determined!



Is it possible to confirm low positive samples?

Low Screening Cut Point Exacerbates Accurate Sensitivity Determination



3/6 sensitivity curves did not cross the cut point!

Low Positive Quality Control (LQC)

Plate ID	Operator	a-Genotropin (ng/mL)
45	1	1.79
46	2	
47	1	2.59
48	2	
49	1	1.21
50	2	
Mean		1.86
SD		0.69
%CV		37.16
Calculation of Sensitivity and LQC		
Sensitivity	Mean + $t_{0.05df} * SD$ (Mean + 1.943 * SD)	3.21
LQC	Mean + $t_{0.01df} * SD$ (Mean + 3.143 * SD)	4.04

Sensitivity	Mean	SD	%CV
Operator			
a-hGH (ng/mL) \ NQC	47.6	3.1	6.5
0	47.8	6.0	12.6
0.122	46.3	3.9	8.5
0.244	47.5	4.2	8.9
0.49	48.6	5.0	10.3
0.98	51.6	4.3	8.3
1.95	51.6	3.9	7.6
3.91	61.0	4.9	8.0
7.81	80.4	8.4	10.4
15.63	125.4	15.9	12.7
31.25	251.1	41.5	16.5
62.5	573.5	113.0	19.7
125	1526.4	346.8	22.7
250	4036.2	998.9	24.7
500	10187.3	2684.2	26.3
1000	22645.8	5263.9	23.2

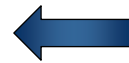
← **LQC**
(reflecting ~39 ng/mL
in undiluted serum)

Suitability of LQC for the Confirmatory Assay

LQC (39.07 ng/mL)			
Plate	LQC mean	LQC + Inh	Inh %
Plate 63	74.5	49.0	34.23
	68.5	53.0	22.63
	57.0	48.5	14.91
Plate 64	79.0	51.5	34.81
	72.0	54.0	25.00
	57.0	50.0	12.28
Mean	68.0	51.0	23.98
SD	9.2	2.2	9.43
%CV	13.5	4.3	39.32

LQC (78.15 ng/ml)			
Plate	LQC mean	LQC + Inh	Inh %
Plate 74	146.0	54.5	62.67
Plate 75	139.0	51.0	63.31
Plate 76	149.0	57.5	61.41
Plate 77	132.0	51.5	60.98
Plate 78	147.5	59.0	60.00
Plate 79	141.0	56.5	59.93
Plate 80	156.5	50.0	68.05
Plate 81	150.0	50.0	66.67
Plate 82	155.0	55.5	64.19
Plate 83	110.0	56.5	48.64
Plate 84	120.0	57.5	52.08
Plate 85	109.0	57.5	47.25
Plate 86	115.0	53.5	53.48
Plate 87	102.5	54.0	47.32
Plate 88	105.5	53.0	49.76
Plate 89	80.0	49.0	38.75
Plate 90	81.0	55.5	31.48
Plate 91	74.5	52.5	29.53
Plate 114	90.5	56.5	37.57
Plate 115	86.0	59.0	31.40
Plate 116	84.0	58.5	30.36
Plate 117	86.5	54.5	36.99
Plate 118	84.0	63.5	24.40
.	.	.	.
.	.	.	.
.	.	.	.
.	.	.	.
Plate 150	131.5	43.5	66.92
Plate 151	124.5	48.5	61.04
Mean	119.54	53.07	53.86
SD	27.41	5.05	12.12
%CV	22.93	9.52	22.51

- Adjustment of LQC concentration enabled confirmation!
- Recommended limits for sensitivity (250-500 ng/mL) were met!

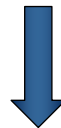


■ % Inhibition value below CCP

Titration of Samples

Mean values are shown as ECL signals

Dilution Factor	1	1:3	1:9	1:27	1:81	1:243	1:729	1:2187	1:6561	1:19683	Cut Point
Operator 1	10284.0	3486.5	1190.0	442.0	204.0	119.5	77.0	63.5	54.0	55.0	51.3
	9764.0	3284.5	1131.0	435.0	192.5	117.5	77.5	68.0	57.0	52.0	55.8
	10918.5	3614.0	1282.5	547.0	220.5	117.5	77.0	66.5	58.0	55.0	51.8
Operator 2	11733.5	3551.5	1323.0	516.0	210.5	123.5	79.0	65.0	60.0	53.0	53.3
	10619.5	3587.0	1158.5	507.5	204.5	117.0	81.5	67.0	60.5	63.5	55.8
	11752.0	4176.0	1608.0	613.0	255.5	135.5	85.5	65.0	59.0	56.5	48.3
Mean	10845.3	3616.6	1282.2	510.1	214.6	121.8	79.6	65.8	58.1	55.8	52.7
SD	793.929	298.418	175.769	66.737	22.033	7.154	3.368	1.633	2.375	4.082	2.888
%CV	7.321	8.251	13.709	13.084	10.268	5.876	4.232	2.480	4.089	7.312	5.479



 Some samples cannot be titrated below the cut point!

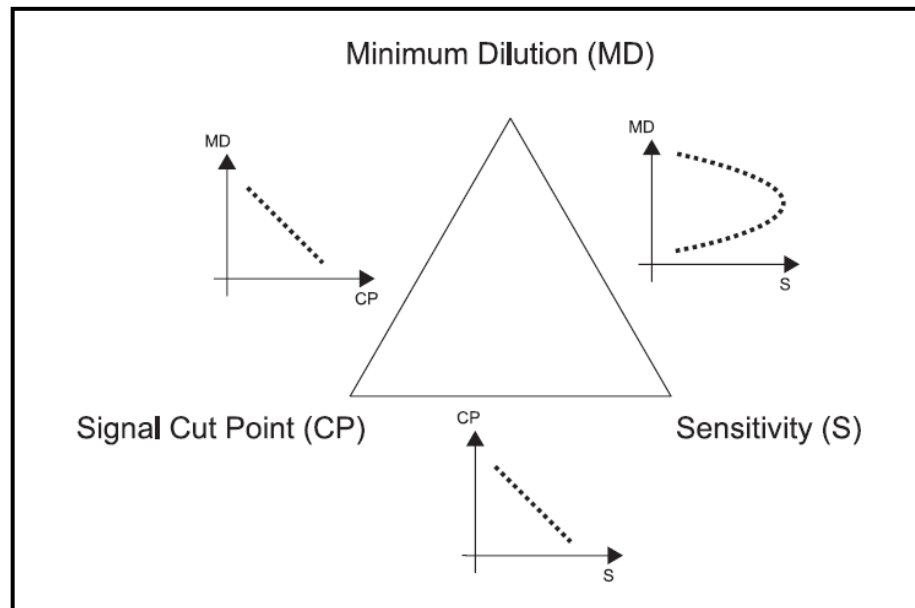
Consequences For Study Sample Analysis

- ☞ High number of false positives > 5%
- ☞ Additional confirmation tests
- ☞ Low positive samples may not be confirmed
- ☞ Accurate titer determination may fail
- ☞ Increase of work and costs

Rationale for the „Validation Trouble“

Minimum Required Dilution

- ECL method allows usage of undiluted samples due to low matrix interference
- As sample volume is limited decided to use 1:5 MRD
- Dualism to reduce unspecific matrix effects but remain a distinct difference between instrument background and sample signal



Mire-Sluis et al. J Immunol Methods. 2004 Jun;289(1-2):1-16

Rationale for the „Validation Trouble“

☞ Low Background and Biological Variability

- Sample cohort for CP determination showed only minor interindividual differences in ECL signal intensity
- ECL_{Signals} of the serum samples were only marginal above the instrument background (40-50 ECL_{Signals})
- Analytical variability exceeds the biological variability



Cut point is strongly influenced by analytical variability and appears not to reflect the biological variability of the patient cohort!

Troubleshooting

Pre-validation

- Obtain serum samples from different vendors for the validation process
- More detailed characterization of samples from the validation cohort
- Ratios of labeled drugs used in the assay (Sulfo-TAG and Biotin-labeled drug)

Validation

- Modified outlier determination (3 x IQR instead of 1.5 X IQR) for sample collectives with low inter-individual differences
- Modified evaluation of LQC with regard to the confirmatory cut point and the recommended sensitivity ranges of the „White Papers“
- Determination of Confirmatory Cut Point using low concentration antibody spiked instead of antibody negative samples