

Development and Validation of an Electrochemiluminescent (ECL) Immunoassay for the Detection of Anti-Human TPO Antibodies in Human Serum

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Abstract

An electrochemiluminescent (ECL) assay was developed and validated to detect anti-human TPO antibodies in human serum using the Meso-Scale Discovery (MSD) Sector PR™ 100 Plate Reader. Detection of anti-human TPO antibodies is based on the bivalent characteristics of the antibody. Biotinylated-rhTPO (B-rhTPO), rutenylated-rhTPO (Tag-rhTPO), and human serum samples are added to polypropylene tubes and allowed to incubate with shaking. Anti-human TPO antibodies will bind to both B-rhTPO and Tag-rhTPO molecules to form an antibody complex bridge. After incubation, the samples are added to the wells of a MSD-streptavidin plate blocked with a 5% milk solution. The B-rhTPO in the complex will bind to the streptavidin in the wells, allowing unbound material to be washed away. In the presence of tripropylamine-containing read buffer, ruthenium produces a chemiluminescent signal (ECL units) that is triggered when electricity is applied. Only samples that contain antibody bound to both B-rhTPO and Tag-rhTPO will generate an ECL signal.

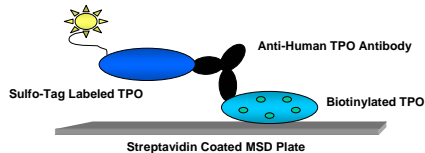
Fifty individual human serum samples were screened in the assay at a 1:50 minimum required dilution. An assay cutpoint response of 240 ECL units was calculated. Three of the fifty samples screened yielded mean responses above the assay cutpoint, corresponding to a false-positive rate of 6.00%. An assay output factor, calculated by dividing the assay cutpoint by the mean response of the fifty individual serum samples was calculated at 1.10. Samples with signal to noise ratios at or above 1.10 are considered potentially reactive. The sensitivity of the assay is 9.83 ng/mL. Inter- and intra-assay precision for high (2400 ng/mL) and low (24.6 ng/mL) anti-human TPO antibody positive controls and a human serum negative control are within 17%. Low and high level positive controls can tolerate up to 100 ng/mL and 10,000 ng/mL TPO, respectively. Room temperature (26 hours) and freeze/thaw (four cycles) stability were established.

Objective

Thrombopoietin (TPO) is a glycoprotein hormone, produced largely by the liver and kidney, that regulates the differentiation of platelets and megakaryocytes. The objective of this work was to develop and validate an electrochemiluminescent (ECL) bridging immunoassay that is both robust and sensitive for the detection of anti-human thrombopoietin (TPO) antibodies in human serum.

Method

- Block MSD Streptavidin Coated Standard Bind MA100 PR Plate with Blocking Buffer (5% Milk in PBS) and incubate overnight at room temperature.
- Prepare Tag-B Reaction Buffer (125 ng/mL) in Assay Diluent (1%BSA in PBS).
- Dilute samples 1:50 in Assay Diluent and add Tag-B Reaction Buffer. Incubate overnight at room temperature with shaking.
- Wash the plate and add Blocking Buffer to all wells. Incubate for 60 minutes at room temperature.
- Wash the plate and transfer the samples to the plate. Incubate at room temperature for 120 minutes with shaking.
- Wash the plate, add MSD Read Buffer T (2X) to all wells, and immediately read the plate on the MSD Sector PR™ 100 Plate Reader.



Assay Cutpoint

- 50 individual human serum samples were tested in the assay, in three separate runs, at the 1:50 assay MRD.
- The mean response of the 50 serum samples, as calculated from the mean responses of each of the individual serum samples, was used to calculate the assay cutpoint and cutpoint factor.

Assay Cutpoint = (Mean Response of 50 Individual Serum Samples) + (1.645 X Standard Deviation)

Assay Cutpoint Factor = Assay Cutpoint / Mean Response of 50 Individual Serum Samples

Mean Serum ECL Response	218
SD	13.4
%CV	6.16
SD X 1.645	22.1
Assay Cutpoint	240
Assay Output Factor	1.10
False Positives	6% (3 of 50)

- Sample S/N (Sample Response/NC Response) ratios at or above 1.10 are considered potentially reactive.

Relative Assay Sensitivity

- To determine the lowest anti-human TPO antibody concentration that can be reproducibly detected above the assay cutpoint, a positive control (PC) sample (6000 ng/mL) was serially diluted and analyzed. Representative data below.

PC Dilution	Theoretical Concentration (ng/mL)	Mean Response	%CV	S/N
1.00	6000	38962	1.54	188
2.50	2400	23025	0.427	111
6.25	960	9838	11.8	47.7
15.6	384	3545	3.71	17.1
39.1	154	1539	1.19	7.43
97.7	61.4	704	12.7	3.40
244	24.6	401	3.53	1.93
610	9.83	274	3.10	1.32
1526	3.93	234	1.81	1.13
3815	1.57	239	4.45	1.15
9537	0.629	207	1.71	1.00
23842	0.252	231	22.0	1.11
59605	0.101	231	7.67	1.11

The lowest anti-human TPO antibody concentration that can be reproducibly detected above the assay cutpoint is 9.83 ng/mL.

Method Validation Data

Assay Interference

- To assess how much rhTPO is required to reduce or eliminate PC detection, low (24.6 ng/mL) and high (2400 ng/mL) PC samples were spiked with rhTPO.
- Samples were pre-incubated for 1 hour at room temperature prior to analysis in duplicate.

rhTPO (ng/mL)	Mean Responses	
	Low PC	High PC
25000	196, 176	219, 195
10000	196, 186	266, 301
5000	193, 202	385, 365
500	259, 235	2721, 2822
100	329, 322	16042, 14915
50.0	342, 323	16301, 15722
10.0	367, 351	17407, 18514
5.00	380, 334	19279, 19308
1.00	353, 357	19125, 18916

Low PC and High PC remain positive in the presence of up to 100 ng/mL and 10,000 ng/mL rhTPO, respectively.

Inter- and Intra-Assay Precision

Inter-Assay Precision

Mean Response High PC (2400 ng/mL)		Mean Response Low PC (24.6 ng/mL)	
22990	404	9	9
22004	448	19308	375
23113	429	16.1	12.2
20483	383		
16989	384		
16590	329		
16346	324		
20193	341		
15070	334		
N	9	9	9
Mean	19308	375	375
%CV	16.1	12.2	12.2

Intra-Assay Precision

(Representative Data from One of Three Days of Analysis)

Mean Response High PC (2400 ng/mL)		Mean Response Low PC (24.6 ng/mL)	
16712	326	4	4
17035	315	16818	326
16567	296	1.29	9.35
16957	368		
N	4	4	4
Mean	16818	326	326
%CV	1.29	9.35	9.35

Stability

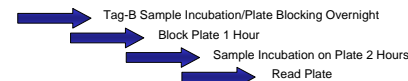
26 hours of stability were established for anti-human TPO antibody in human serum: Low (24.6 ng/mL) and High (2400 ng/mL) PC samples.

	N	Mean Response	%CV
Low PC (Stability)	3	345	13.2
Low PC (Control)	3	351	2.10
High PC (Stability)	3	20773	2.65
High PC (Control)	3	16194	3.38

Four freeze/thaw cycles were established for anti-human TPO antibody in human serum: Low (24.6 ng/mL) and High (2400 ng/mL) PC samples.

	N	Mean Response	%CV
Low PC (Stability)	3	341	5.76
Low PC (Control)	3	313	7.99
High PC (Stability)	3	16870	0.470
High PC (Control)	3	15132	8.24

Conclusion



- A robust, sensitive, and time-efficient assay was developed to detect anti-human TPO antibodies in human serum.
- The assay requires a 1:50 sample MRD and is sensitive up to 9.83 ng/mL.
- Low and high PC samples (24.6 ng/mL and 2400 ng/mL) can be detected in the presence of up to 100 ng/mL and 10,000 ng/mL rhTPO, respectively.
- 26 hours of thawed matrix stability and four freeze/thaw cycles were established.

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