

Immunogenicity Assays Using a Novel ELISA Format: Comparison of Sensitivity and Drug Tolerance to Electrochemiluminescence (Meso-Scale Discovery)

AUTHORS

Robert J. Kubiak, Joseph F. Bower, Russell Hensel, Renuka C. Pillutla, and Ira S. DuBey

INTRODUCTION

Immunogenicity assays utilizing electrochemiluminescence (ECL) technology based on Meso-Scale Discovery (MSD) platform are characterized by high sensitivity and high tolerance to circulating antigen. At the present time, ECL is supplied by a single vendor thus raising concerns about long-term viability of ECL-based immunoanalytical methods. A need arises to develop and explore alternative assay platforms that could:

- Δ Replace or substitute MSD electrochemiluminescence
- Δ Be available from multiple vendors
- Δ Challenge the performance characteristics of MSD-ECL (sensitivity, dynamic range, matrix effects, and drug tolerance among others).

This work presents a novel ELISA format (3D-ELISA) that is comparable in performance characteristics to MSD-ECL and at the same time utilizes critical reagents from multiple vendors.

COMPARISON OF BRIDGING FORMATS USED FOR IMMUNOGENICITY ASSAYS

GENERAL ADVANTAGES

- Δ Bridging formats are not specific for species or antibody subclass.

GENERAL LIMITATIONS

- Δ Any bivalent drug receptor can form a bridge resulting in false positives.
- Δ Some antibody subclasses like IgG4 are functionally monovalent and are not detected in bridging assays resulting in false negatives.

TRADITIONAL 2D-ELISA FORMAT FOR IM ASSAYS

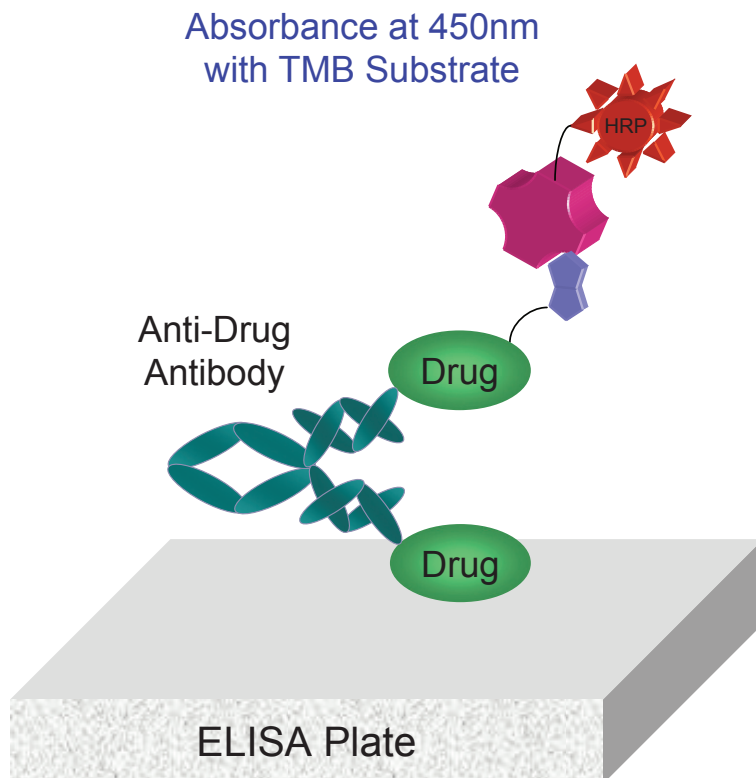
- Δ Bivalent antibodies bind to drug passively absorbed on solid surface and biotinylated drug.
- Δ Antibody-drug complex is built stepwise by subsequent additions of immuno-complex components.

ADVANTAGES

- Δ Easy to perform.
- Δ All components available from multiple commercial sources.

LIMITATIONS

- Δ Poor sensitivity and narrow dynamic range.
- Δ Poor tolerance to free drug.
- Δ Drug/Anti-drug interactions take place on two dimensional surface (hence 2D-ELISA) eliminating access to some potentially reactive epitopes.



ECL FORMAT FOR IM ASSAYS

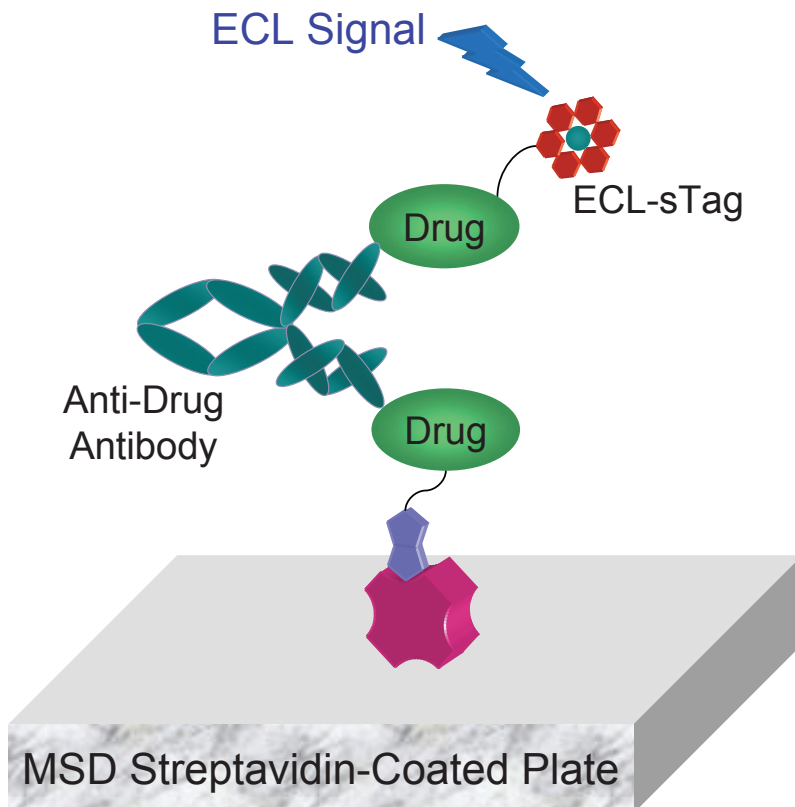
- Δ Bivalent antibodies bind to both ruthenylated and biotinylated drug.
- Δ Antibody-drug complex is captured on streptavidin-coated surface and read on MSD instrument.

ADVANTAGES

- Δ Antibody-drug interactions take place in solution ("3D-format") and not on the solid/liquid interface.
- Δ High capacity surface of MSD plates is a main factor contributing to high drug tolerance.
- Δ Broad dynamic range and good sensitivity.

LIMITATIONS

- Δ All reagents and instrumentation supplied by a single vendor.



3D-ELISA FORMAT FOR IM ASSAYS

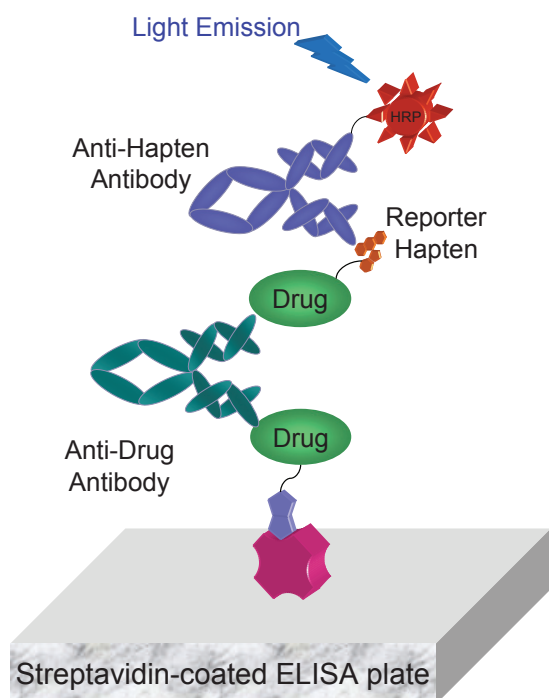
- Δ Drug is labeled with biotin and a small hapten molecule (e.g. fluorescein or digoxigenin).
- Δ Antibody forms a bridge in solution between drug molecules labeled with biotin and hapten.
- Δ The immuno-complex is captured on streptavidin-coated plate.
- Δ Hapten is detected with anti-hapten antibody conjugated with HRP.
- Δ Signal is generated upon addition of a luminescent substrate.

ADVANTAGES

- Δ Antibody-drug interactions take place in solution (hence 3D-ELISA) and not on the solid/liquid interface.
- Δ High capacity surface allows for high drug tolerance.
- Δ Broad dynamic range and good sensitivity.
- Δ Instrumentation and variety of reporting systems available from multiple sources.

LIMITATIONS

- Δ More complicated detection with an additional wash step.



METHODS

CASE STUDY: HUMAN ANTI-MOUSE ANTIBODIES (HAMA) ASSAY

Development of an immunogenicity assay for Human Anti-Mouse Antibodies (HAMA) served as a case study for evaluation of 3D-ELISA format as an alternative to ECL.

REAGENTS

- Δ Drug: Polyclonal mouse IgG (Jackson ImmunoResearch Cat# 015-000-003)
- Δ Drug-Biotin: Mouse IgG-Biotin (Jackson ImmunoResearch Cat# 015-060-003)
- Δ Drug-Hapten: Mouse IgG-Fluorescein (Jackson ImmunoResearch Cat# 015-090-003)
- Δ Drug-sTag: Mouse IgG labeled with MSD sulfo-Tag (MSD Cat# R91AN-1) prepared in house
- Δ Positive Control: Donkey anti-mouse pAb (Jackson ImmunoResearch Cat# 715-005-150)
- Δ Anti-Hapten: Rabbit anti-fluorescein pAb-HRP conjugate (Abcam Cat# ab19492-500)

COMPARISON OF MSD AND ELISA FORMATS: EXPERIMENTAL CONDITIONS

2D-ELISA

Day 1

- Δ Coat ELISA plate with 1 µg/µL mouse IgG solution in carbonate buffer overnight

Day 2

- Δ ELISA plate (Nunc Maxisorp) washed and blocked with 3% BSA/PBST
- Δ Serum samples diluted 1:10 Assay Buffer (1% BSA/PBST) and incubated on ELISA plate for approximately 2 hrs
- Δ After wash step, 1 µg/µL solution of biotinylated mouse IgG added followed by incubation for approximately 2 hours
- Δ Plate washed and incubated with 1:50,000 SA-HRP (Invitrogen Cat# 43-4323) conjugate for approximately 1 hour

ECL

Day 1

- Δ Serum samples diluted with 1:10 Assay Buffer (1% BSA/PBS)
- Δ 100 μL of diluted sample treated with 50 μg/μL of 3 μg/μL mouse IgG labeled with biotin and sTag
- Δ Incubated overnight in darkness in polypropylene plate

Day 2

- Δ Streptavidin-coated plate (MSD Cat# L15SA-1) blocked with 150 μL/well of Assay Buffer for 1-2 hrs
- Δ Samples transferred into blocked and washed plate (50 μL/well) and incubated for 2 hrs
- Δ Plate washed
- Δ 2xT Read buffer (MSD Cat# R49TC) is added (150 μL/well) and plate is read on MSD Sector Imager 2400

3D-ELISA

Day 1

- Δ Serum samples diluted 1:10 Assay Buffer (5% Casein/PBS)
- Δ 100 μL of diluted sample treated with 50 μg/μL of 3 μg/μL mouse IgG labeled with biotin or fluorescein
- Δ Incubated overnight in darkness in polypropylene plate

DAY 2

- Δ Neutravidin-coated plate (Pierce Cat# 15510) blocked with 150 μL/well Assay Buffer for 1-2 hrs.
- Δ Samples transferred into blocked and washed plate (100 μL/well) and incubated for 2 hrs.
- Δ Plate washed
- Δ 1:1000 dilution of anti-fluorescein-HRP conjugate is added and plate incubated for 90 minutes.
- Δ Plate washed and luminol substrate (Pierce Cat# 37069) added (100 μL/well)
- Δ Plate read on Molecular Devices SpectraMax M5

RESULTS

CUT POINT COMPARISON ACROSS FORMATS

- Δ 30 Individual human serum samples (Bioreclamation) were tested on two separate days using 2D-ELISA, 3D-ELISA and ECL
- Δ Cut point calculations assume that population under investigation is naive. No such assumption could be made in case of human anti-mouse antibodies.
- Δ Confidence limit of 95% was used in calculations: Cut Point = Mean + 1.645*SD
- Δ ELISA and ECL assays gave different individuals as potential positives (S12 for ECL and S9 and S15 for ELISA). This was probably due to different nature of the labels used for detection (fluorescein vs. ECL-Tag) and capture (streptavidin-biotin vs. passive absorption)

Table 1. Cut Point Determination

Serum #	2D-ELISA		3D-ELISA		ECL	
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
	Mean OD	Mean OD	Mean RLU	Mean RLU	Mean ECL	Mean ECL
S1	0.179	0.220	495	484	267	318
S2	0.288	0.345	453	506	259	298
S3	0.171	0.195	469	494	189	202
S4	0.290	0.360	459	596	470	553
S5	0.235	0.294	475	659	430	457
S6	0.179	0.262	494	796	513	583
S7	0.212	0.283	497	633	557	637
S8	0.215	0.251	503	571	463	392
S9	0.349	0.384	1004	1301	559	703
S10	0.162	0.184	440	497	143	181
S11	0.218	0.268	475	470	187	204
S12	0.356	0.565	592	907	1255	1876
S13	0.234	0.282	560	617	464	500
S14	0.243	0.336	660	893	120	202
S15	0.449	0.648	1118	2965	523	471
S16	0.207	0.171	1056	989	356	410
S17	0.341	0.485	466	543	729	1167
S18	0.176	0.241	471	512	433	459
S19	0.168	0.185	730	789	777	851
S20	0.143	0.163	704	1054	165	219
S21	0.165	0.185	565	907	564	790
S22	0.177	0.268	542	588	193	232
S23	0.110	0.210	576	502	172	204
S24	0.190	0.247	514	566	266	348
S25	0.189	0.219	572	608	566	639
S26	0.198	0.234	464	513	194	246
S27	0.197	0.231	969	1262	211	232
S28	0.267	0.337	478	584	257	302
S29	0.184	0.229	542	670	350	426
S30	0.604	0.804	588	636	212	273

Table 2. Cut Point Summary Comparison

Run #	Mean Signal	Cut Point	Cut Point Factor	Statistical Outliers	Potential Positives
2D-ELISA Day 1	0.228 OD	0.347	1.524	S30	S9, S15
2D ELISA Day 2	0.282 OD	0.472	1.673	S30	S12, S15, S17
3D-ELISA Day 1	598 RLU	911	1.524	None	S9, S15, S16, S27
3D-ELISA Day 2	695 RLU	1073	1.544	S15	S9, S27
ECL Day 1	374 ECL	670	1.794	S12	S17, S19
ECL Day 2	431 ECL	819	1.902	S12	S17, S19

2D-ELISA Mean Cut Point Factor = 1.598

3D-ELISA Mean Cut Point Factor = 1.534

ECL Mean Cut Point Factor = 1.848

ASSAY SENSITIVITY COMPARISON

- Δ Titrations of Positive Control (donkey anti-mouse Ab) were performed on three separate occasions (two for 2D-ELISA).
- Δ End point titer was calculated as a linear interpolation at the dilution giving signal equal to the run cut point.
- Δ Assay sensitivity was determined as an average sensitivity for each titration.

Sensitivity: 2D-ELISA						
Positive Control [ng/mL]	Run 1		Run 2			
	Mean OD	S/N	Mean OD	S/N		
1000	3.614	16.4	3.618	26.8		
500	3.349	15.2	3.336	24.7		
250	2.388	10.8	2.255	16.7		
125	1.424	6.45	1.254	9.30		
62.5	0.855	3.87	0.723	5.36		
31.3	0.507	2.30	0.434	3.22		
15.6	0.366	1.66	0.292	2.16		
7.81	0.286	1.29	0.212	1.57		
Sensitivity	14 ng/mL		8.7 ng/mL			
Average	11 ng/mL					
Sensitivity: 3D-ELISA						
Positive Control [ng/mL]	Run 1		Run 2		Run 3	
	Mean RLU	S/N	Mean RLU	S/N	Mean RLU	S/N
1000	41271	88.8	24710	50.4	38530	73.6
200	7783	16.7	4894	10.0	9114	17.4
40.0	1989	4.28	1518	3.10	2432	4.65
8.00	911	1.96	857	1.75	1177	2.25
1.60	564	1.21	643	1.31	744	1.42
0.320	505	1.09	546	1.11	612	1.17
0.0640	482	1.04	501	1.02	555	1.06
0.0128	416	0.895	497	1.01	526	1.00
Sensitivity	4.4 ng/mL		4.9 ng/mL		2.5 ng/mL	
Average	3.9 ng/mL					
Sensitivity: ECL						
Positive Control [ng/mL]	Run 1		Run 2		Run 3	
	Mean ECL	S/N	Mean ECL	S/N	Mean ECL	S/N
1000	201107	832	128348	503	154078	536
200	37691	156	25663	100	31159	108
40.0	8272	34.2	6591	25.8	7031	24.5
8.00	1918	7.94	2155	8.44	2067	7.19
1.60	714	2.96	889	3.48	861	3.00
0.320	368	1.52	511	2.00	570	1.98
0.0640	316	1.31	401	1.57	386	1.34
0.0128	275.5	1.14	308	1.21	338	1.17
Sensitivity	0.61 ng/mL		0.23 ng/mL		0.27 ng/mL	
Average	0.37 ng/mL					

DRUG TOLERANCE

- Δ Titrations of Positive Control (donkey anti-mouse Ab) were performed in pooled human serum containing 0, 2, 5, 10 and 20 µg/µL of mouse IgG ("Drug") for 3-D ELISA and ECL or 0, 0.5, 1, 2 and 5 µg/µL of mouse IgG for 2-D ELISA.
- Δ Drug tolerance across different formats is expressed and compared as assay sensitivity in the presence of unlabeled mouse IgG ("Drug").

Table 4. Drug Tolerance

Drug Tolerance: 2D-ELISA										
Positive Control [ng/mL]	No Drug		0.5 µg/mL Drug		1 µg/mL Drug		2 µg/mL Drug		5 µg/mL Drug	
	Mean OD	S/N	Mean OD	S/N	Mean OD	S/N	Mean OD	S/N	Mean OD	S/N
1000	3.618	26.8	2.137	15.9	1.107	8.21	0.558	4.14	0.384	2.85
500	3.336	24.7	0.888	6.59	0.562	4.17	0.378	2.81	0.246	1.82
250	2.255	16.7	0.578	4.29	0.348	2.58	0.265	1.96	0.203	1.50
125	1.254	9.30	0.361	2.68	0.230	1.70	0.200	1.49	0.172	1.28
62.5	0.723	5.36	0.249	1.84	0.181	1.34	0.163	1.21	0.153	1.14
31.3	0.434	3.22	0.195	1.44	0.161	1.20	0.146	1.09	0.146	1.08
15.6	0.292	2.16	0.165	1.23	0.147	1.09	0.145	1.07	0.142	1.05
7.81	0.212	1.57	0.150	1.11	0.144	1.07	0.151	1.12	0.140	1.04
Sensitivity	8.2 ng/mL		43 ng/mL		107 ng/mL		154 ng/mL		324 ng/mL	
Drug Tolerance: 3D-ELISA										
Positive Control [ng/mL]	No Drug		2 µg/mL Drug		5 µg/mL Drug		10 µg/mL Drug		20 µg/mL Drug	
	Mean RLU	S/N	Mean RLU	S/N	Mean RLU	S/N	Mean RLU	S/N	Mean RLU	S/N
1000	41271	88.8	4381	9.42	3211	6.90	1743	3.75	1136	2.44
200	7783	16.7	1170	2.52	1001	2.15	786	1.69	633	1.36
40.0	1989	4.28	709	1.52	694	1.49	650	1.40	585	1.26
8.00	911	1.96	551	1.19	596	1.28	534	1.15	598	1.29
1.60	564	1.21	550	1.18	545	1.17	527	1.13	516	1.11
0.320	505	1.09	514	1.11	506	1.09	520	1.12	507	1.09
0.0640	482	1.04	488	1.05	496	1.07	590	1.27	538	1.16
0.0128	416	0.895	516	1.11	486	1.05	492	1.06	526	1.13
Sensitivity	4.4 ng/mL		42 ng/mL		50 ng/mL		114 ng/mL		327 ng/mL	
Drug Tolerance: ECL										
Positive Control [ng/mL]	No Drug		2 µg/mL Drug		5 µg/mL Drug		10 µg/mL Drug		20 µg/mL Drug	
	Mean ECL	S/N	Mean ECL	S/N	Mean ECL	S/N	Mean ECL	S/N	Mean ECL	S/N
1000	201107	832	29556	122	19982	82.7	10582	43.8	6712	27.8
200	37691	156	6368	26.4	3147	13.0	2470	10.2	1299	5.37
40.0	8272	34.2	1979	8.19	887	3.67	824	3.41	526	2.18
8.00	1918	7.94	763	3.16	394	1.63	396	1.64	311	1.29
1.60	714	2.96	398	1.65	289	1.19	293	1.21	266	1.10
0.320	368	1.52	302	1.25	257	1.06	259	1.07	244	1.01
0.0640	316	1.31	284	1.18	253	1.05	253	1.05	243	1.00
0.0128	276	1.140	257	1.06	255	1.06	247	1.02	247	1.02
Sensitivity	0.61 ng/mL		2.5 ng/mL		11 ng/mL		12 ng/mL		28 ng/mL	

CONCLUSION

Table 5. Format Comparison Summary

Assay Format	Cut Point Factor	Sensitivity [ng/mL]	Sensitivity in Presence of Free Drug			
			2 µg/mL	5 µg/mL	10 µg/mL	20 µg/mL
2D-ELISA	1.598	11	154 ng/mL	324 ng/mL	>400 ng/mL	>400 ng/mL
3D-ELISA	1.534	3.9	42 ng/mL	50 ng/mL	114 ng/mL	327 ng/mL
MSD-ECL	1.848	0.37	2.5 ng/mL	11 ng/mL	12 ng/mL	28 ng/mL

- Δ The 3D-ELISA format presented here is generic and can be easily applied to other immunogenicity assays with all reagents and supplies available from multiple vendors.
- Δ 3D-ELISA allows for a large flexibility in design by using different haptens for labeling (e.g. fluorescein, digoxigenin, dinitrophenol) and different detection modes (colorimetry, luminescence or fluorescence).
- Δ 3D-ELISA format can be easily implemented in any immunoanalytical lab with standard equipment.
- Δ Although the MSD-ECL is highly sensitive and more tolerant to circulating antigen, the 3D-ELISA displays higher sensitivity and drug tolerance than typical ELISA assays commonly used for immunogenicity evaluation. 3-D ELISA assay performance meets industry guidelines delineated in the 2005 white paper by Mire-Sluis et al.

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Tandem Labs-Utah

1121 East 3900 South
Building C, Suite 105
Salt Lake City, UT 84124
(801) 293-2400

Tandem Labs-New Jersey

115 Silvia Street
West Trenton, NJ 08628
(609) 434-0044

Tandem Labs-New England

35R Cabot Road
Woburn, MA 01801
(781) 933-2769 x 123