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Development of Non-Human Primate and Human Immunoglobulin Isotyping Assays

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1 Abstract

Purpose: Immunoglobulins play a major role in immune response. Five heavy-chain isotypes (IgA, IgD, IgE, IgG, and IgM) mediate specific biological functions, including responses to pathogens and immunological diseases such as allergies and autoimmune disorders. Quantification of immunoglobulin isotypes can provide useful insight into complex immune responses. Here we describe multiplexed immunoglobulin isotyping assays that quantitatively measure IgA, IgE, IgG, and IgM in both human and non-human primate (NHP) matrices.

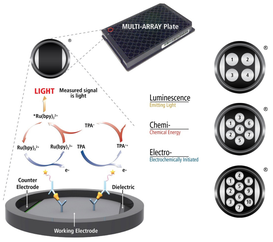
Methods: MSD® developed multiplexed immunoassays according to fit-for-purpose principles. A variety of antibodies to each isotype were screened for sensitivity, specificity, and normal sample values. Multiplexed assays were developed from the best antibody pairs and then optimized for specificity and matrix tolerance.

Results: Normal cynomolgus monkey and human serum samples were tested at multiple dilutions using the MSD immunoglobulin isotyping assays. The 250,000-fold dilution produced optimal analyte detection with the exception of IgE, which required lower dilution due to lower native levels in serum and plasma. Non-specific binding of each isotype was less than 1%. Each assay's limits of detection were either equivalent to or better than those of other available immunoassays.

Conclusions: MSD's multiplexed immunoglobulin isotyping assays can quantitatively measure IgA, IgE, IgG, and IgM in human and NHP samples. They use simple protocols, require very little sample, and may provide a useful tool for quantification of immunoglobulin isotypes when studying various immunological diseases.

2 Methods

MSD's electrochemiluminescence (ECL) detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® and MULTI-SPOT® microplates.



Electrochemiluminescence Technology

- Minimal non-specific background and strong responses to analyte yield high signal-to-background ratios.
- The stimulation mechanism (electricity) is decoupled from the response (light signal), minimizing matrix interference.
- Only labels bound near the electrode surface are excited, enabling non-washed assays.
- Labels are stable, non-radioactive, and directly conjugated to biological molecules.
- Emission at ~620 nm eliminates problems with color quenching.
- Multiple rounds of label excitation and emission enhance light levels and improve sensitivity.
- Carbon electrode surface has 10X greater binding capacity than polystyrene wells.
- Surface coatings can be customized.

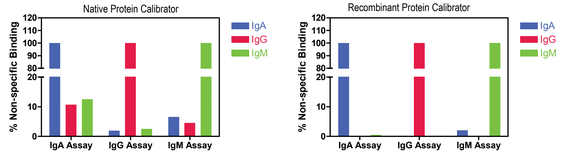
Protocol

1. Add 150 µL/well of MSD Blocker A; incubate for 30 min at room temperature (RT).
2. Wash with PBS-T; add 25 µL/well of calibrator or diluted samples; incubate for 2 hours at RT with shaking.
3. Wash with PBS-T; add 25 µL/well of conjugated detection antibody; incubate for 2 hours at RT with shaking.
4. Wash with PBS-T; add 150 µL/well of Read Buffer T (2X). Read on MSD instrument.

3 Multiplexed Isotyping Panel: Reagent Selection

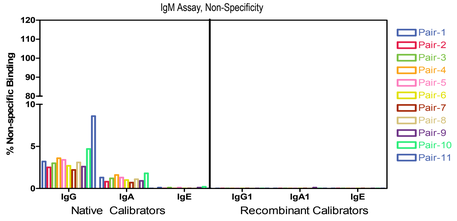
Immunoglobulin isotypes share homology, so researchers often report antibody cross-reactivity or non-specific binding for isotyping multiplexes. Reagents from most vendors are poorly characterized, leading to contamination, lot-to-lot variation, and non-specific binding in multiplexed isotyping assays.

Native proteins are purified for use as calibrators but can still be contaminated with homologous isotypes. In the examples below, the IgA assay using native IgG and IgM calibrators showed 10% and 12% non-specific binding (left), whereas using recombinant IgG and IgM as calibrators in the IgA assay (right) produced minimal non-specific binding.



These IgA, IgG and IgM assays use polyclonal antibodies for capture and detection. Polyclonal IgM antibodies still showed some non-specific binding (2%) even with recombinant calibrators. We addressed this problem by extensive antibody pair screening. The graph below illustrates this process.

MSD screened many pairs of antibodies with a preference for monoclonal antibodies as the capture. The 2% IgM non-specific binding illustrated in the graph above (right) was eliminated by careful selection of antibody pairs. Results from testing 11 antibody pairs for IgM assays are shown below. Each pair was screened using both recombinant and native calibrators.



4 Specificity of MSD Multiplex Assays

Final antibody pairs were selected based on each antibody's specificity, ability to recognize human and NHP protein, and ability to recognize sub-classes of each isotype.

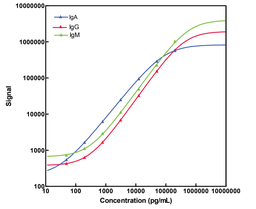
	Native Calibrator					
	Human			NHP (Rhesus)		
Assay	IgA	IgG	IgM	IgA*	IgG	IgM
IgA	632 236	19 105	34 714	-	1608	2 460
IgG	175	137 303	610	-	66 963	350
IgM	9	1 879	97 579	-	26	57 512

*Rhesus IgA native calibrator was not tested

	Human Recombinant Calibrator						
	IgA		IgG				IgM
Assay	IgA1	IgA2	IgG1	IgG2	IgG3	IgG4	
IgA	490 503	671 916	<background	<background	371	<background	<background
IgG	120	38	206 295	37 718	281 866	166 803	34
IgM	50	11	6	92	73	60	73 281

5 Isotyping Panel 1 (human/NHP): Dynamic Range and Sensitivity

Representative calibration curves for MSD's Isotyping Panel 1 (human/NHP) are shown below. The lower limits of detection (LLOD) are calculated concentrations corresponding to a signal 2.5 standard deviations above the background (zero calibrator). Average LLODs across multiple runs are shown.



	Sensitivity
Analyte	Average LLOD (pg/mL)
IgA	2.99
IgG	37.6
IgM	15.2

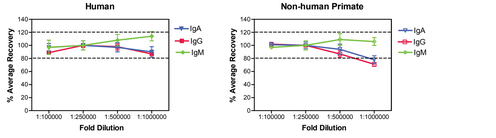
Three NHP serum samples were measured using 4 replicates on multiple runs. Reproducibility across multiple runs is shown in the table to the right. Average intra-run precision is typically <5%; inter-run precision is typically <12%.

	Sample	Runs	Average Conc. (mg/mL)	Average Intra-Run %CV	Inter-Run %CV
IgA	Sample-1	4	0.838	2.9	9.1
	Sample-2	4	0.212	3.9	7.3
	Sample-3	4	0.696	2.6	5.6
IgG	Sample-1	4	5.56	0.9	1.8
	Sample-2	4	4.51	3.5	2.0
	Sample-3	4	8.23	2.5	4.4
IgM	Sample-1	4	0.689	5.4	10.8
	Sample-2	4	0.0528	3.0	N/A*
	Sample-3	4	0.976	2.5	11.5

*Sample ECL signal is close to background

6 Matrix Tolerance: Parallelism

Parallelism was assessed by testing serially diluted serum (n=5 samples) with both human (graph below, left) and NHP (graph below, right) assay kits. The average percent recoveries at each dilution factor are shown. Parallelism was within 20% of the expected concentrations for all samples. The 1,000,000-fold dilution was too high for NHP samples.



7 Human and NHP Sample Quantification

Human and NHP serum samples were obtained from Bioreclamation, Inc., or other sources. They were tested at 250,000-fold dilution. Analyte concentrations are dilution corrected and reported as mg/mL.

		ECL signal at 250,000 dilution			Concentration (mg/mL)		
	Samples	IgA	IgG	IgM	IgA	IgG	IgM
Human	Sample-1	66 018	134 611	12 549	2.11	11.0	0.870
	Sample-2	79 394	185 782	19 343	2.56	14.8	1.31
	Sample-3	47 891	117 272	48 018	1.51	9.68	3.04
	Sample-4	103 008	197 072	30 632	3.40	15.7	2.01
	Sample-5	41 832	303 116	8 750	1.31	23.9	0.614
Non-human primate	Sample-1	42 339	71 514	15 343	1.33	6.20	1.05
	Sample-2	11 846	60 772	2 362	0.370	5.37	0.152
	Sample-3	34 183	98 734	20 416	1.07	8.29	1.38
	Sample-4	41 437	60 063	14 199	1.30	5.31	0.979
	Sample-5	20 115	58 657	1 319	0.628	5.20	0.0660

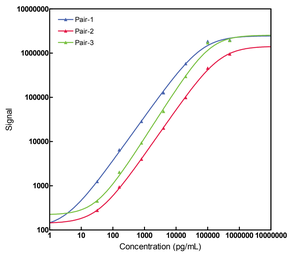
8 Human IgE Assay and Reagents

Antibody Performance

Six hybridoma clones were created by fusing myeloma cells and spleen cells of Balb/c mouse immunized with human IgE protein. The isotype of each hybridoma clone is shown in the table below. The representative standard curves from different antibody pairs are shown on the right. The best mAb combinations for ECL detection technology are shown in the table below.

	Antibody Phenotype	
Antibody	Clone Number	Isotype
Antibody-1	B17-3	IgG2b
Antibody-2	A80-4	IgG1
Antibody-3	A18-1	IgG2b
Antibody-4	A36-2	IgG1
Antibody-5	C06-1	IgG1
Antibody-6	A90-1	IgG2a

	Recommended Antibody Pairs	
	Capture	Detection
Pair-1	B17-3	A36-2
Pair-2	B17-3	C06-1
Pair-3	A80-4	A36-2



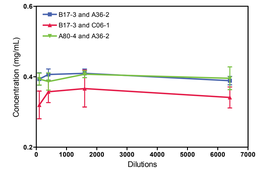
Antibody Specificity

Human IgE antibody specificity was assessed by measuring individual native calibrator signals for IgA, IgE, IgG and IgM. All human IgE antibodies exhibited <0.1% non-specific binding with other isotypes.

		Native Calibrators (20 ng/mL)			
Capture Ab	Detection Ab	IgE	IgA	IgG	IgM
B17-3	A36-2	529 984	<background	583	<background
B17-3	C10-1	337 992	<background	235	<background
A80-4	A36-2	218 810	<background	271	<background
B17-3	C06-1	700 822	<background	722	<background
A18-1	A36-2	224 009	31	218	7
A80-4	A90-1	239 458	177	291	232
A18-1	A90-1	252 220	32	229	51
A18-1	C06-1	291 895	<background	227	<background

Sample Quantification

Human normal serum was obtained from Bioreclamation, Inc. It was tested at multiple dilutions (1:100, 1:400, 1:1,600 and 1:6,400) using multiple IgE antibody pairs. Sample concentrations are reported as the dilution-corrected concentration (mg/mL). Data suggest all IgE antibody pairs have a good parallelism between 100 and 6,400-fold dilution.



9 Conclusions

The specificity of multiplex isotyping assay relies on the purity of its calibrator and its antibody performance. Polyclonal antibodies often have less specificity than monoclonal antibodies. MSD has developed high performance assays to measure antibody isotypes (IgA, IgG and IgM), both human and NHP, that exhibit <1% cross reaction or non-specific binding with other analytes.

Multiple monoclonal human IgE antibodies were developed and tested for specificity, sensitivity, and sample measurement. They provide a useful tool for the study of various immune responses.

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