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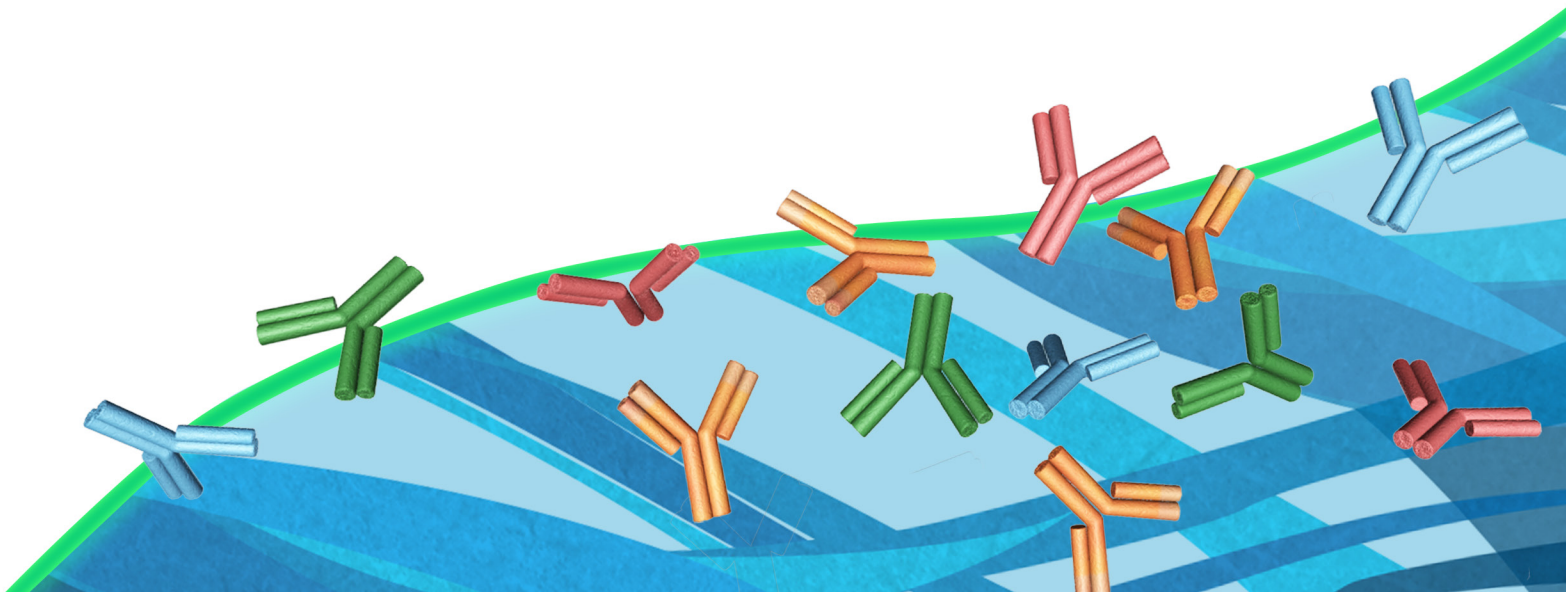
Detection and Isotyping of Anti-Infliximab Antibodies using a Multiplex Photonic Ring Immunoassay

The present study shows the performance characteristics of a human anti-Infliximab Multi-Tier Anti-Drug Antibody (hMT-ADA) assay. This is a multiplex assay that determines the isotype and IgG subclass of ADA in a single run, including IgG4. The sensitivity and free drug tolerance exceed FDA guidelines.



Introduction

There is currently exceptional growth in the development of biologic therapeutics. Along with their impressive clinical utility has come the realization that patients produce an antibody response against many protein drugs. Regulatory agencies are now requiring evaluationof all new protein drugs for their immunogenicity, including isotype and subclass of ADA in some cases [1-3]. There is growing concern that methods used to detect ADA include the ability to measure IgG4. Since IgG4 has a unique structure caused by switching Fab domains to generate a bispecific antibody, some techniques like the bridging assay do not detect IgG4 ADA at the same sensitivity as other subclasses. The Genalyte hMT-ADA detects all isotypes and IgG subclasses with high sensitivity.

Materials and Methods

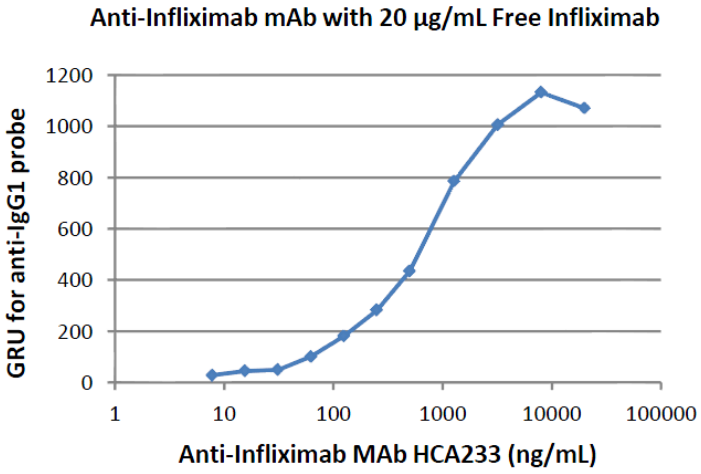
All experiments were conducted using the Genalyte hMT-ADA assay and run on the Maverick™ M24 instrument, which is designed to measure binding of macro-molecules to ring sensors on small (3.5 by 5.6mm) chips. In the hMT-ADA PRI (Photonic Ring Immunoassay), seperate sensors are spotted with an anti-isotype capture probe to the Fc portion of human IgA, IgM, IgE, IgG1, IgG2, IgG3 or IgG4 and a streptavidin control. Binding of ADA is detected by a shift in the wavelength of ring resonance associated with that probe. The shift is proportional to the mass that has bound above the ring and is measured in Genalyte Response Units (GRU).
All steps of the assay from running the samples over the chip, wash steps, and amplification of bound analyte are automatically performed by the Maverick™ M24.

Specifically, Infliximab is biotinylated with a commercially available reagent. After off-line affinity capture and elution (ACE) of ADA performed in a 96 well plate, the ADA are flowed over the chip, and any ADA present are captured by the specific probe. In the next step, biotinylated Infliximab is flowed over the chip, followed by amplification with streptavidin coated nanonospheres. The amount of mass bound by each isotype and IgG subclass capture probe is determined by the shift in the ring resonance of that probe.

Results

The sensitivity of the assay in serum in the absence of free drug is 16 ng/ml, which is greater than 3 times the background (not shown). Monoclonal anti-infliximab (HCA233, IgG1) was spiked into human serum, treated by the ACE procedure and run on the hMT-ADA assay on the Maverick™.

The sensitivity of the assay in serum in the presence of 20 ug/ml of free Infliximab is 62 ng/ml of ADA. Note that the graph below is in log scale and the initial concentration is 20,000 ng/ml of ADA.



The free drug tolerance of monoclonal anti-Infliximab HCA233 at 250 ng/ml in the MT-ADA was tested in serum in the presence of free drug at 40, 20, 10, 5 and 0 ug/ml of Infliximab. The ACE procedure was performed at each concentration and the antibodies run in the hMT-ADA PRI. Within run precision at various concentrations of ADA was determined by running samples at the concentrations noted in Table 1 below 6 times each on the same array. For all

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positive samples the %CV was less than 6%.

TABLE 1. *Within run precision N=6*

1000 ng/ml		250 ng/ml		62.5 ng/ml		0 ng/ml	
GRU	970	GRU	546	GRU	254	GRU	39
S.D.	59	S.D.	24	S.D.	13	S.D.	9
%CV	6%	%CV	4%	%CV	5%	%CV	23%

Between day precision at various concentrations of ADA was determined by running the same samples on 8 different days as shown in Table 2 below. For all positive samples the %CV for between day precision was less than 20%.

TABLE 2. *Between day precision N=8*

1000 ng/ml		250 ng/ml		62.5 ng/ml		0 ng/ml	
GRU	958	GRU	533	GRU	214	GRU	28
S.D.	64	S.D.	54	S.D.	34	S.D.	14
%CV	7%	%CV	10%	%CV	16%	%CV	50%

Sera from thirty five patients with rheumatoid arthritis (RA), Crohn's disease or ulcerative colitis (UC) who had taken Infliximab were purchased from Bioreclamation. Their ADA status was not known. They were tested on the human MT-ADA assay and the results are shown in Table 3 below. None of the samples were positive for IgE antibodies, so those results are not shown.

TABLE 3. *Patients taking Infliximab*

Disease	Lot #	Anti-IgG4	Anti-IgG3	Anti-IgG2	Anti-IgG1	Anti-IgM	Anti-IgA
RA	1	<5	9	<5	14	6	<5
	2	<5	6	<5	19	<5	84
	3	763	280	30	218	9	29
	4	55	658	36	456	<5	33
	5	<5	9	<5	20	6	<5
Crohn's	6	74	21	<5	49	<5	<5
	7	51	8	<5	10	5	<5
	8	19	<5	<5	<5	9	355
	9	119	48	5	108	9	43
	10	705	107	22	118	9	70
	11	<5	<5	<5	<5	<5	<5
	12	<5	<5	<5	<5	60	32
	13	526	646	48	534	13	34
	14	<5	38	<5	444	6	35
	15	1203	586	408	606	53	178
	16	61	5	<5	16	<5	9
	17	7	3	<5	5	35	<5
	18	<5	<5	<5	<5	7	<5
	19	6	8	<5	<5	25	17
	20	<5	3	<5	15	18	18
	21	<5	<5	<5	1	<5	<5
U.C.	22	<5	2	<5	13	<5	
	23	160	51	9	27	8	
	24	30	20	34	15	22	
	25	1045	649	352	482	18	
	26	264	22	8	21	16	
	27	934	345	70	204	18	
	28	258	93	6	64	35	
	29	<5	10	<5	<5	10	
	30	6	11	<5	<5	9	
	31	158	78	<5	118	<5	
	32	6	9	<5	<5	<5	<5
	33	<5	<5	<5	<5	<5	<5
	34	<5	<5	<5	<5	<5	<5
	35	16	5	<5	30	<5	<5
*Cuttoff		40	40	40	40	100	100

*For the purpose of this study, the cutoff was calculated by testing 23 normal samples and then using the average GRU + 2 standard deviations for each isotype.

Seventeen of the 35 (48%) patients taking Infliximab were ADA positive on the MT-ADA PRI (Table 3). This is a higher percentage positive than early studies but is in line with more recent findings[2].

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Interestingly, in samples 7 and 16, IgG 4 is the only subclass of ADA detected, while in 6 and 23, it is the predominant subclass.

In sample 8, IgA is the only isotype of ADA detected.

Twenty one samples from patients taking Infliximab who were tested on another technology to measure anti-Infliximab antibodies were also tested on the Maverick™ hMT-ADA PRI. Nineteen of them tested positive on the LISA Tracker Theradiag ADA ELISA, while 1 was reported as positive and 1 negative by Prometheus Laboratory. Seventeen of the 19 samples that were positive by Theradiag ELISA were also positive on the hMT-ADA. The reason for the discrepancy with the other 2 samples is unknown except that as seen in Table 4 below, the samples were completely negative on the hMT-ADA. The sample that was positive by the Prometheus test was also positive on the hMT-ADA, as was the negative sample. A possible explanation for the positive results on the hMT-ADA assay is that the negative sample by Prometheus Laboratory could have to do with free drug tolerance. The sample in question was reported as having 10 ug/ml of free drug.

TABLE 4. *hMT-ADA Results for Patients tested by other technologies*

Sample ID	Anti-IgG4	Anti-IgG3	Anti-IgG2	Anti-IgG1	Anti-IgM	Anti-IgA	Anti-IgE
1	769	979	288	880	22	91	30
2	570	295	40	459	18	25	10
3	1249	833	159	791	22	148	18
4	<5	3	<5	<5	47	<5	<5
5	1444	898	922	708	263	434	167
6	<5	7	<5	<5	<5	<5	8
7	1314	1041	360	745	51	124	47
8	1447	1251	823	1072	127	771	80
9	1174	1137	255	1060	20	107	23
10	1042	733	397	971	62	44	19
11	84	1205	248	1269	62	901	46
12	16	15	<5	45	11	<5	8
13	33	32	<5	100	<5	31	7
14	26	92	<5	160	5	5	14
15	914	175	49	132	149	20	<5
16	804	497	43	519	7	53	5
17	743	199	88	479	<5	30	<5
18	1309	1030	349	1154	27	267	26
19	1249	665	807	474	275	572	71
20	519	274	143	821	20	37	23
21	55	15	<5	155	23	106	16
Cutoff	40	40	40	40	100	100	100

As seen in Table 5, in the 35 patients who are positive for ADA to Infliximab, the most common ADA are found in IgG subclasses IgG4 and IgG1 at 89% each. IgG3 is also common at 77%, while IgG2 and IgA are less common at 54% and 34%, respectively. IgM and IgE are rare, with only 1 sample having IgE ADA.

TABLE 5. *Frequency of Isotypes and subclasses of ADA*

N=35	Anti-IgG4	Anti-IgG3	Anti-IgG2	Anti-IgG1	Anti-IgM	Anti-IgA	Anti-IgE
Number	31	27	19	31	4	12	1
Frequency	89%	77%	54%	89%	11%	34%	3%

Conclusions

The sensitivity and free drug tolerance of the Maverick human MT-ADA PRI as applied to Infliximab exceed the FDA guidelines of 250 ng/ml of ADA in the presence of 20 ug/ml of free drug. The percent positive samples in a commercially available group of patients taking Infliximab is in accordance with other

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recent studies. There is very high concordance between the results obtained with the Maverick MT-ADA PRI and other technologies.

There is increased pressure from regulatory agencies to ensure that ADA testing detects all isotypes and IgG subclasses. The ability to measure the isotypes and IgG subclasses of ADA in a single multiplex assay is an important improvement over other technologies.

Interestingly, there were 5 samples in the group of patient taking Infliximab who were not tested by other technologies who were predominantly IgG4 or IgA positive. However, none of the samples that were chosen because they were positive on other technologies showed that ADA profile. Further studies need to be done to follow up on this observation.

References

1. Guidance for Industry: Immunogenicity Assessment for Therapeutic Protein Products. www.fda.gov.
2. Chamberlain PD. Multidisciplinary approach to evaluating immunogenicity of biosimilars: lessons learnt and open questions based on 10 years' experience of the European Union regulatory pathway. Biosimilars 2014:4 23–43.
3. Guidance for Industry: Scientific Considerations in Demonstrating Biosimilarity to a Reference Product. www.fda.gov.

Part No.	Description
85000	Maverick M24 Detection System
85023	Maverick hMT-ADA PRI Kit

Please inquire for other species

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