Identification of Anti-Idiotype Antibodies using Digital SPR

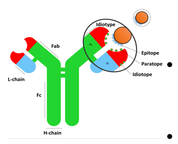
Forrest Epstein[1], Jun Zhang[1], Ming Lu[1], Ke Wei Gong[1], Linjiang Li[1], Weiping Jia[1], Michael Piazza[2], Ryan Denomme[2], Dennis Slamon[1], Benjamin Hoffstrom[1]

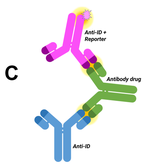
[1]Dept of Medicine, Hematology-Oncology, David Geffen School of Medicine, UCLA. [2]Nicoya Lifesciences, Kitchener, Canada

Introduction

Anti-idiotypic antibodies (anti-IDs) recognize the unique variable region of another antibody called the idiotype (defined below). Anti-IDs are commonly used in bioanalytical assays to monitor pharmacokinetics (PK) and the immunogenicity of therapeutic antibodies in animal models and human clinical trials. A typical bioanalytical assay requires a *complementary pair of anti-IDs* that bind to unique epitopes on the therapeutic antibody. For example, in a total antibody serum assay, the first anti-ID functions to "capture" the therapeutic antibody from a dilute serum sample and the second, reporter-conjugated anti-ID, "detects" the amount of therapeutic antibody that has been captured. Traditional workflows for discovering anti-IDs have relied on multiple rounds of ELISA assays for kinetics and epitope binning which can be labor and reagent intensive, thereby limiting the number of candidates that can be investigated. This study aims to streamline the discovery of anti-IDs by combining digital surface plasmon resonance (SPR) and high throughput flow cytometry to efficiently screen thousands of anti-ID candidates, ultimately identifying high-performance pairs for bioanalytical assays.

Anti-Idiotype Antibodies





- **Idiotype** – The unique set of antigenic determinants found in the variable region of an antibody; including the antigen-binding site.
- **Paratope** – The specific region on the variable domain of an antibody that recognizes and binds to an antigen, forming the antigen-binding site.
- **Epitope** – The specific part of an antigen that is recognized and bound by an antibody, also the region to which the paratope of the antibody binds.
- **Idiotope** – Specifically refers to the combination of the idiotypic determinants that make an antibody's antigen-binding site distinct from other antibodies.
- **Non-blocking Anti-ID Antibody (A)** – Anti-idiotype antibody that binds specifically to the paratope region and does not inhibit antigen binding of the target (therapeutic) antibody, making them suitable for detection, quantification, and monitoring of antibody levels.
- **Blocking Anti-ID Antibody (B)** – Anti-idiotype antibody that binds specifically to the paratope region and interferes with antigen-binding site of the target (therapeutic) antibody.
- **Anti-ID Pair Complex (C)** – Pharmacokinetic assays typically utilize complementary pairs of anti-ID antibodies. The therapeutic antibody (**Green**) is "captured" from dilute serum by anti-ID (**Blue**). The second anti-ID (**Purple**) is conjugated with a reporter molecule (R) and detection reagent to quantify the amount of captured therapeutic antibody in the assay.

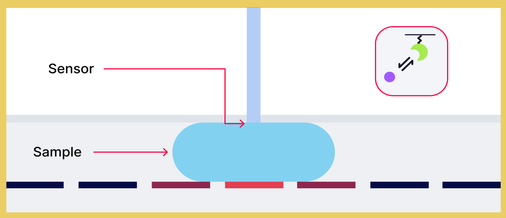
Alto™ Digital SPR: A label-free solution for characterization of antibodies

Nicoya's Alto system uses digital microfluidics (DMF) to deliver automatically diluted sample droplets to SPR sensors for effortless real-time characterization of biomolecular interaction analysis including quantitation, screening, epitope binning and binding kinetics.

- HT analysis with 16 independently addressable channels.
- Full kinetics and epitope characterization from 2 µL of crude or purified sample.
- Automated epitope binning and data visualization.
- Elimination of dilutions, tagging, degassing, cleaning, and manual assay design.

What are Digital Microfluidics (DMF)?

DMF is a liquid-handling technology capable of accurately controlling and manipulating discrete nanoliter-sized droplets across an array of electrodes. The fluidics are contained within a disposable cartridge, allowing Alto to overcome the major limitations associated with increasingly complex fluidic systems present in traditional label-free instruments.

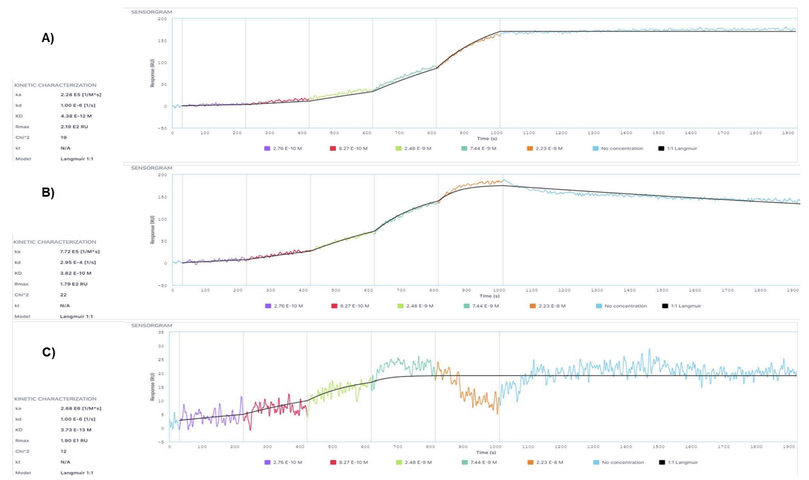


Anti-idiotype Antibody Discovery

CloneID	Drug 2 mAb	Drug 2 ADC	Drug 1 mAb	Drug 1 ADC	Poly hIgG	Confirmed	Inhibition Assay	mIgG Concentration (µg/ml)
1D2	1675	5308	6984	16236	5716	0	Non-Blocker	11.89
1G2	1079	1233	69663	270853	7343	1	Strong Blocker	11.88
1H4	794	1215	73664	162442	6176	1	Strong Blocker	15.17
1A5	1449	2422	85977	254450	5415	1	Medium Blocker	20.15
1A6	4133	1623	104549	400133	10492	1	Strong Blocker	10.10
1A7	1600	1296	240542	522808	5507	1	Non-Blocker	11.71
1F7	1338	2302	118175	413609	7381	1	Non-Blocker	13.97
2D3	1258	1459	119712	429195	5003	1	Weak Blocker	4.49
2G4	1045	1281	49215	147997	4858	1	Medium Blocker	9.735
3G1	2871	6081	97956	298524	6969	1	Strong Blocker	15.60
3B5	1600	1732	130733	469868	5399	1	Strong Blocker	12.87
3B8	1105	2133	81577	300949	6675	1	Non-Blocker	21.40
4A5	795	1400	15345	40568	5253	1	Non-Blocker	10.92
5F7	171996	167708	159989	365936	157177	0	Strong Blocker	3.17
6D1	690	1272	5813	14724	5348	0	Non-Blocker	10.89
6F4	1659	1288	152473	450039	6093	1	Strong Blocker	5.47
6D5	1270	1079	132888	317285	7313	1	Strong Blocker	1.42
6E12	1310	1136	116985	352810	6376	1	Strong Blocker	11.07
6F12	1856	2170	155484	495898	5858	1	Strong Blocker	6.03

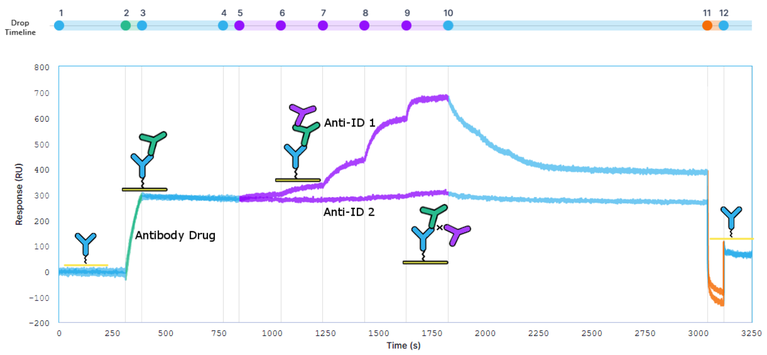
Hybridoma Antibody Discovery: Anti-Idiotype antibodies were generated in mice immunized with antibody Drug 1. The above table shows binding data of a representative subset (19 clones) from a total of 571 Drug 1 positive binders. Non-confirmed (0 vs 1) hits, as well as clones yielding antibody supernatant concentrations below 9 ug/mL, were not advanced to kinetic assays. Additionally, we characterized the clones for their ability to compete for antigen binding (Inhibition Assay) to have a qualitative understanding of Drug 1 epitope recognition.

Alto™ Direct Kinetics using Hybridoma Supernatant



Alto™ Direct Kinetics of Antibodies in Hybridoma Supernatant: Five automated 3-fold serial dilutions of the anti-ID in solution are introduced to the surface-immobilized antibody Drug in increasing concentrations. Direct kinetics was run on 240 anti-idiotype hybridomas using culture supernatant normalized to common concentrations (15, 10, and 9 µg/mL). A range of binding profiles were identified including **A)** picomolar KD with minimal off-rate; **B)** sub-nanomolar KD with a faster off-rate; **C)** poor antigen binding.

Alto™ Epitope Binning of anti-Idiotype Antibodies



Epitope Characterization on Alto™: Alto allows users to design an 8x7 or 16x16 assay to characterize the simultaneous binding of mAbs to an antigen, tested in a pairwise manner. In the classical sandwich assay format, the antigen is captured by up to 16 unique surface-coupled antibodies, which is followed by the pairwise binding of solution antibodies. Alto uses only 100ng of each antibody for the entire experiment. An antibody that blocks another antibody from binding to the antigen will be deemed to have the same target epitope and will be 'binned' together. If both antibodies bind to the antigen at the same time, it can be inferred that they have non-overlapping epitopes.

Detection Anti-ID \ Capture Anti-ID	1D1	1C4	1B7	1F7	3G1	3A3	4F5	1G2	1H4	1A6	1F6	1F8	2C4	3B8	1H5	2G4	6D1	6G3	6A11	6A12	6E12	6F12
1D1		-	-	++	-	-	++	-		-					++			-			-	-
1C4	+		-	-	-	-	-															
1B7	-	-		-	-	-	-															
1F7	-	-	-		-	-	-															
3G1	-	-	-	-		+	-															
3A3	-	-	-	-	-		-															
4F5	-	-	-	-	-	-																
1G2									-	++	-	-	-	+								
1H4				-	-			+++		+	+	-	-	-	-			-			-	++
1A6								+	-		-	-	-	-								
1F6								-	-	++		-	-	-								
1F8								-	-	+++	-		-	-								
2C4				-	-			+	-	+++	-	-		-	++			-			-	+
3B8								-	-	+	-	-	-									
1H5				++	+++			+		+++						-	-	+++	-	-	++	+++
2G4				-	-			-		-					+++		-	+	-	-	-	-
6D1				-	-			-		-					+++	-		-	-	-	-	-
6G3				+++	++			-		+++					++	-	-		-	-	++	+++
6A11															++	-	-	-		-	-	
6A12															++	-	-	-	-		-	
6E12															-	-	-	-	-	-		

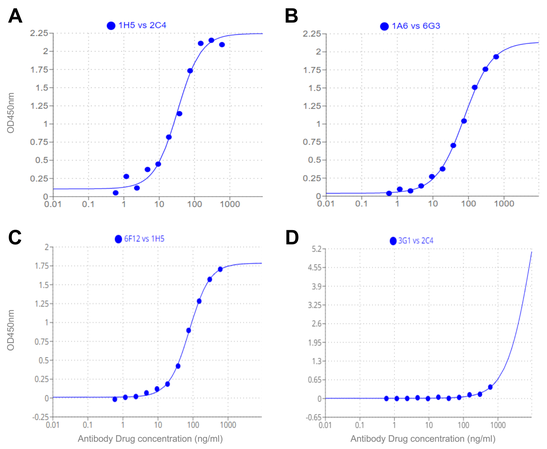
Epitope Binning from Hybridoma Supernatant: Using the combined functional and kinetic data, four 7x7 binning matrixes were designed and run on the Alto. From the 164 interactions explored several good anti-ID pairs were identified (+++), while most were found to compete for the same idiotope (-). Some anti-IDs were shown to work best as only a capture or detection antibody while others show good paring in either format.

Verified Matrix ELISA

Detection Anti-ID \ Capture Anti-ID	6D1	1F7	1D1	1G2	3G1	2C4	2G4	1H4	1A6	1H5	6E12	6G3	1A5	3B5	6F4	6F12
6D1	0.18	0.24	0.25	0.15	0.24	0.10	0.10	0.15	0.34	0.09	0.07	0.07	0.22	0.19	0.19	0.11
1F7	0.12	0.41	0.61	0.38	0.21	0.21	0.11	0.33	1.33	1.24	0.14	0.91	0.18	0.42	0.32	0.13
1D1	0.22	0.45	0.52	0.34	0.27	0.38	0.16	0.34	0.59	1.01	0.25	0.89	0.13	0.49	0.28	0.09
1G2	0.21	0.36	0.48	0.15	0.21	0.22	0.10	0.24	1.18	1.51	0.09	0.98	0.20	0.31	0.15	0.12
3G1	0.12	0.62	0.78	0.51	0.31	0.40	0.45	0.42	0.48	1.64	0.29	1.22	0.10	0.55	0.24	0.11
2C4	0.16	0.83	0.85	0.71	0.52	0.50	0.56	0.63	0.75	1.76	0.43	1.41	0.10	0.61	0.36	0.16
2G4	0.28	0.37	0.72	0.25	0.23	0.30	0.28	0.26	0.34	1.75	0.15	1.28	0.42	0.33	0.28	0.06
1H4	0.29	0.34	0.11	0.16	0.19	0.22	0.29	0.21	0.46	1.31	0.11	0.93	0.34	0.38	0.37	0.12
1A6	0.20	0.44	0.71	0.57	0.26	0.29	0.14	0.47	0.35	1.85	0.20	1.42	0.21	0.49	0.36	0.19
1H5	0.10	1.34	1.19	0.81	1.61	1.68	1.67	0.80	1.39	0.29	1.54	0.11	0.18	1.75	1.88	1.89
6E12	0.11	0.81	1.02	0.73	0.56	0.61	0.30	0.54	0.66	1.78	0.42	1.52	0.18	0.69	0.26	0.21
6G3	0.09	1.43	1.34	1.18	1.43	1.48	1.42	1.04	1.30	0.51	1.39	0.18	0.23	1.47	1.72	1.78
1A5	0.26	0.10	0.10	0.08	0.13	0.10	0.06	0.06	0.05	0.09	0.14	0.14	0.11	0.08	0.10	0.23
3B5	0.12	0.53	0.57	0.36	0.31	0.34	0.33	0.32	0.41	1.63	0.25	1.08	0.18	0.39	0.38	0.13
6F4	0.14	0.40	0.43	0.29	0.29	0.31	0.22	0.30	0.47	1.70	0.28	0.92	0.18	0.39	0.39	0.18
6F12	0.10	0.57	0.58	0.41	0.39	0.47	0.37	0.36	0.47	1.87	0.33	1.30	0.37	0.48	0.46	0.24

Validation Sandwich ELISA: The sixteen most promising anti-IDs, determined by epitope binning were purified and screened in a sandwich ELISA. This format closely mimics the final PK assay and was used to determine the final pairs that were advanced to sequencing, expression, and validation. The top pairs showed good pairing in both the SPR and ELISA formats.

Titration ELISA Results in Human Serum



EC50: The EC50 was calculated by a titration ELISA for 48 potential anti-ID pairings. An EC50 was successfully calculated for 20 of the pairings including **A)** 33.04ng/mL, **B)** 76.198ng/mL, **C)** 78.68ng/mL, **D)** 10,571.29ng/mL
The completed data set together with the yield and stability profiles for each anti-ID was used to select the final pairs for PK assay validation.

Conclusions

We screened a library of approximately 70,000 hybridomas and identified 571 anti-idiotype binders by flow cytometry. We characterized the binding kinetics of 240 idiotype-specific clones by SPR using 2µl (~50-100ng) of normalized hybridoma supernatant per Alto assay. All 240 anti-IDs were ranked by their KD, k_{off}, and antigen blocking activity in order to design four epitope-binning matrixes. From the resulting binning matrixes, 16 anti-IDs were purified and tested by ELISA in the presence of human serum to replicate methods used in the PK assay. These 16 anti-IDs were also run in a titration ELISA to generate EC50s. Ultimately, seven candidates were sequenced, expressed, and validated by SPR and ELISA. This sequence of methods allowed us to quickly gather direct kinetics on over 200 anti-IDs and epitope binning on 164 different pairings without the need to sequence, express, or purify large numbers of antibodies.

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