Anti-Biotherapeutic Antibody Development against Ipilimumab with HuCAL PLATINUM®

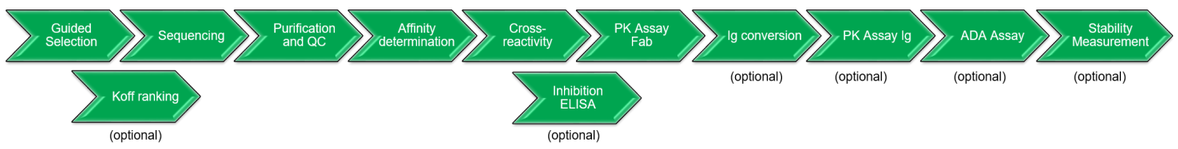
Lena Schmohl, Andre ten Haaf, Christian Frisch, Achim Knappik. Bio-Rad Laboratories, Puchheim, Germany

Abstract

Antibodies specific for biotherapeutic drugs play an important role in preclinical and clinical development, where they are used for pharmacokinetic studies and for the development of immunogenicity assays. By using the fully synthetic Human Combinatorial Antibody Library, HuCAL PLATINUM, in combination with in vitro guided selection protocols, we have generated such antibodies against many marketed drugs, such as adalimumab, trastuzumab, infliximab, ranibizumab as well as against several hundred preclinical antibody drug candidates.

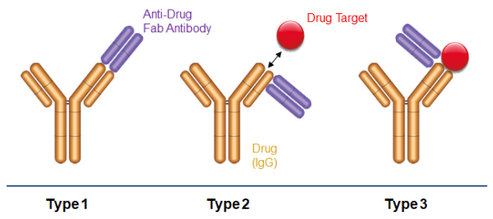
Here, we present a typical workflow of an anti-biotherapeutic antibody project by means of generation and characterization of antibodies against the biotherapeutic drug ipilimumab (YERVOY). Selected antibodies in monovalent Fab format were characterized concerning specificity, affinity, and inhibitory potential and were screened for their ability to work in bridging ELISA for drug quantification. Furthermore, some Fab antibodies were converted into full-length human IgG1 and evaluated in an Anti-Drug-Antibodies (ADA) bridging ELISA. Both inhibitory antibodies that recognize the free drug and antibodies that recognize the drug only when bound to its target were characterized. Several of the antibodies have sub-nanomolar affinities resulting in excellent performance in PK and ADA assays.

1 General Flow Chart of an Anti-Biotherapeutics Antibody Project



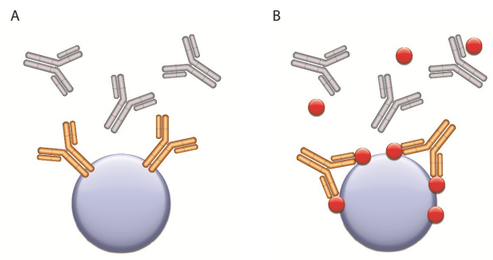
2 Anti-Biotherapeutic Reagent Classification

Binding modes of anti-biotherapeutic antibodies. Type 1 anti-idiotype antibodies bind the paratope of the drug (yellow). They inhibit drug-target binding and detect free drug. Type 2 anti-idiotype antibodies bind outside the drug paratope and do not interfere with target (red) binding. They are used to detect total drug levels. Type 3 antibodies are specific for the drug-target complex. The anti-drug antibodies (purple) are shown as Fab fragments for clarity.



3 Panning

Guided selection strategies for generation of Type 1, 2 and 3 anti-biotherapeutic antibodies by phage display. (a) For Type 1 antibodies, selection on the drug (yellow) coupled to magnetic beads (blue sphere) in the presence of isotype/subclass matched antibodies (gray) avoids enrichment of specificities that bind to the constant regions of the antibody drug. (b) For Type 2 and Type 3 antibodies, selections are performed on the drug-target complex, and blocking is done with isotype/subclass matched control antibodies plus the drug target (red).



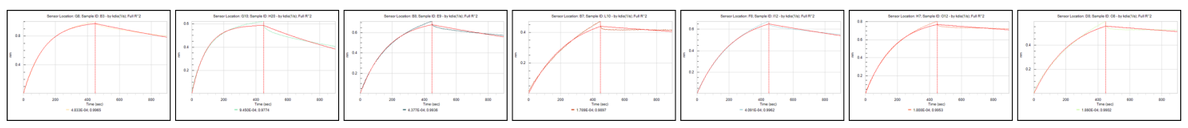
After 3 panning rounds, sub-cloning into an expression vector, *E. coli* transformation and plating, 368 colonies were randomly picked, transferred to a 384 well microtiter plate and grown. Cell lysates were tested for specific antibody binding by primary ELISA screening.

4 Screening and koff Ranking

95 Fab candidates which were specific against the antigen target in primary screening were evaluated in a secondary screening using the off rate k_{off} as selection criterion. The k_{off} values were determined using the ForteBio Octet RED384 instrument with AR2G biosensors. Ipilimumab was immobilized at 10 µg/ml in 10 mM NaAc buffer (pH 6). Crude *E.coli* lysate containing monovalent Fab antibodies were tested to obtain binding curves.

The real time kinetics data were fitted (red lines) using the ForteBio Data Analysis software version 10.0.3.1.

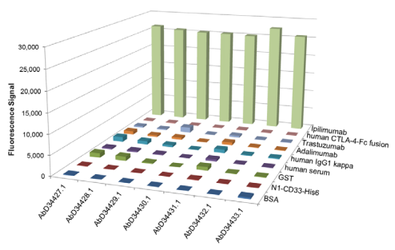
Sample ID (Antibody)	Sensor Location	Response (nm)	k_d (1/s)	Full R^2
AbD34427	G8	1.00	4.83E-04	1.00
AbD34428	G10	0.62	9.45E-04	0.98
AbD34429	B8	0.71	4.38E-04	0.99
AbD34430	B7	0.47	1.79E-04	0.99
AbD34431	F8	0.67	4.09E-04	1.00
AbD34432	H7	0.80	1.81E-04	1.00
AbD34433	D8	0.59	1.86E-04	0.99



5 Hit confirmation

After primary and secondary screening the best hits were sequenced and unique binders were expressed in *E. coli*. Purification was done by an automated one-step chromatography with Strep-Tactin as affinity ligand followed by re-buffering using PD-10 columns into PBS buffer.

Purified Fab antibodies were tested for specific binding to ipilimumab. Various antigens were coated, after washing and blocking with 5% milk in PBST purified Fab (2 µg/ml) was added. Detection was done with HRP-conjugated anti-DYKDDDDK tag antibody followed by QuantaBlu Fluorogenic Peroxidase substrate.

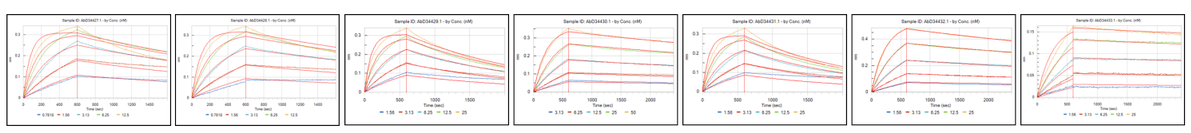


6 Affinity Determination

Affinities were determined for purified monovalent Fab antibodies using the ForteBio Octet RED384 instrument with AR2G biosensors. Ipilimumab was immobilized at 10 µg/ml in 10 mM NaAc buffer (pH 6) to reach an antigen density on the sensor of 2.4 ± 0.8 nm.

Monovalent Fab antibodies were tested at 2.75 µg/ml (50 nM) with a 1:2 dilution series to obtain 5 curves. Sensors were regenerated between measurements with 10 mM glycine pH 2.5.

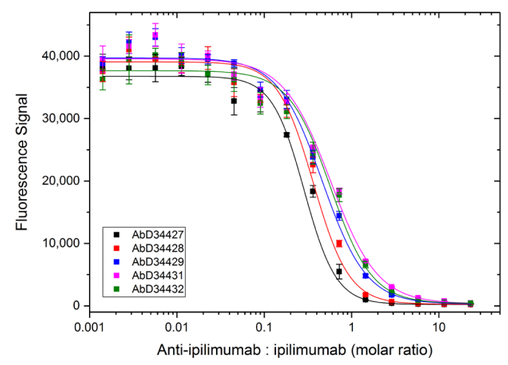
Sample ID (Antibody)	k_a (1/Ms)	k_d (1/s)	K_D (nM)
AbD34427	1.00E+06	3.46E-04	0.3
AbD34428	7.80E+05	2.65E-04	0.3
AbD34429	4.20E+05	5.26E-04	1
AbD34430	9.31E+04	1.11E-04	1
AbD34431	4.23E+05	5.73E-04	1
AbD34432	1.66E+05	1.18E-04	0.7
AbD34433	1.60E+05	4.32E-05	0.3



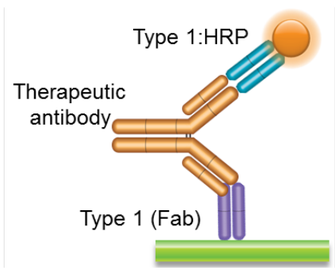
7 Inhibition ELISA (Type 1)

This assay confirms the binding mode of the anti-idiotypic antibody. Type 1 antibodies inhibit drug-target binding.

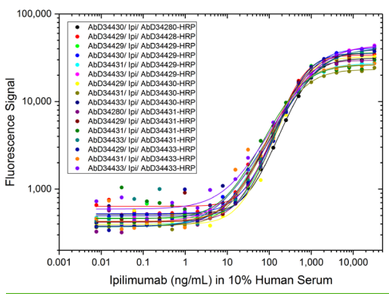
A microtiter plate was coated with human CTLA-4 (1 µg/ml) in PBS. After washing and blocking with PBST +5% BSA, a pre-incubated mixture of HRP-conjugated ipilimumab (0.3 µg/ml) plus increasing concentrations of human anti-ipilimumab antibodies in the monovalent Fab format was added. Free ipilimumab, still capable of binding to the human CTLA-4 coated plate, was detected by adding QuantaBlu fluorogenic peroxidase substrate.



8 PK Assay (Type 1)



For PK bridging ELISA, the anti-idiotypic antibody is immobilized (purple Fab antibody). The therapeutic antibody (gold) forms the bridge and is detected by a conjugated anti-idiotypic antibody (blue Fab with circle representing enzyme label).



Fab antibodies were tested in capture and detection mode to identify the best pairs for bridging ELISA.

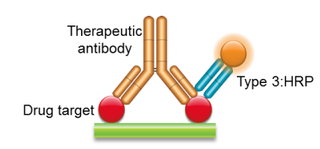
The anti-ipilimumab capture Fabs (1 µg/ml) were coated. After washing and blocking, ipilimumab (2 µg/ml) was added. The HRP-conjugated Fab detection antibodies were added (2 µg/ml) to the plate and detection was performed with QuantaBlu Fluorogenic Peroxidase Substrate.

The signals (x-fold over background) for each pair are color-coded from white to green. Antibody pairs with high signals were further analyzed (yellow borders).

Capture Antibody	Detection Antibody: AbD34280	AbD34282	AbD34287	AbD34391	AbD34427	AbD34428	AbD34429	AbD34430	AbD34431	AbD34432	AbD34433	AbD34403
AbD34280	33	21	4	2	28	52	12	40	50	37	45	3
AbD34282	2	4	2	1	3	6	5	4	6	4	6	3
AbD34287	1	2	2	1	1	2	4	2	2	2	2	1
AbD34391	2	2	1	1	2	3	3	3	3	3	4	2
AbD34427	24	20	6	2	8	33	25	42	42	28	41	6
AbD34428	28	21	5	2	22	33	37	36	40	25	41	9
AbD34429	29	41	6	3	36	61	64	72	64	46	74	4
AbD34430	59	23	8	2	37	46	65	46	54	40	55	16
AbD34431	40	35	9	2	32	54	57	56	55	36	65	16
AbD34432	33	15	4	2	20	31	41	43	49	16	40	14
AbD34433	43	33	5	2	35	43	62	65	66	41	56	15
AbD34403	2	2	1	1	1	2	1	2	2	2	2	1

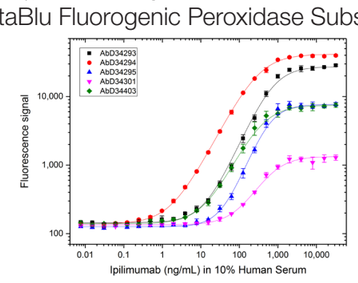
A microtiter plate was coated with the anti-ipilimumab capture antibodies (1 µg/ml). After washing and blocking, ipilimumab spiked in 10% human serum was titrated (23 dilutions). The HRP-conjugated detection antibodies were added (2 µg/ml) and detection was performed by using QuantaBlu Fluorogenic Peroxidase substrate.

9 PK Assay (Type 3)

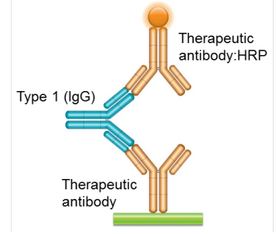


For a PK antigen capture ELISA, the drug target is immobilized (red circle). The therapeutic antibody (gold) forms a complex with the drug target and is detected by a complex-specific, conjugated anti-idiotypic antibody (blue Fab with circle representing enzyme label).

A microtiter plate was coated with human CTLA-4 (5 µg/ml). After washing and blocking with PBST + 5% BSA, 10% human serum spiked with increasing concentrations of ipilimumab was added. Detection was performed using human anti-ipilimumab/CTLA-4 Type 3 Fab antibodies (2 µg/ml), followed by an HRP labeled anti-DYKDDDDK tag antibody and QuantaBlu Fluorogenic Peroxidase Substrate.



10 Ig conversion and ADA Assay Development



The ADA bridging ELISA is performed by immobilizing the therapeutic antibody (gold). An anti-idiotypic antibody (blue) is detected by forming a bridge to the conjugated therapeutic antibody (gold antibody with circle representing enzyme label).

Selected Fab antibodies were converted into full length human IgG1 and expressed in mammalian HKB11 cells. The IgG1 containing cell supernatant was harvested and antibodies were purified by affinity chromatography. Purified antibodies were re-buffered using PD-10 columns into PBS buffer.

A microtiter plate was coated with ipilimumab (1 µg/ml). After washing and blocking with PBST + 5% BSA, PBST with 10% human serum was added spiked with increasing concentrations of human anti-ipilimumab antibodies. Detection was performed using HRP-conjugated ipilimumab.