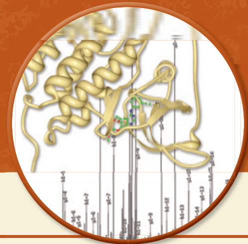

A Proteomics Approach for the Identification and Cloning of Monoclonal Antibodies from Serum

Wan Cheung (Gabriel) Cheung*, **Sean A. Beausoleil***, Xiaowu Zhang, Shuji Sato, Sandra M. Schieferl, James S. Wieler, Jason G. Beaudet, Ravi K. Ramenani, Lana Popova, Michael J. Comb, John Rush, and Roberto D. Polakiewicz. *These authors contributed equally to this work

Cell Signaling Technology, Inc., Danvers MA 01923

Abstract

We describe a proteomics approach, NG-XMT™, that identifies antigen-specific antibody sequences directly from circulating polyclonal antibodies in the serum of an immunized animal. The approach involves affinity purification of antibodies with high specific activity and then analyzing digested antibody fractions by nano-flow liquid chromatography coupled to tandem mass spectrometry. High-confidence peptide spectral matches of antibody variable regions are obtained by searching a reference database created by next-generation DNA sequencing of the B cell immunoglobulin repertoire of the immunized animal. Finally, heavy and light chain sequences are paired and expressed as recombinant monoclonal antibodies. Using this technology, we isolated monoclonal antibodies for five antigens from the sera of immunized rabbits and mice. The antigen-specific activities of the monoclonal antibodies recapitulate or surpass those of the original affinity-purified polyclonal antibodies. This technology may aid the discovery and development of vaccines and antibody therapeutics, and help us gain a deeper understanding of the humoral response.

Nature Biotechnology, In Press 2012

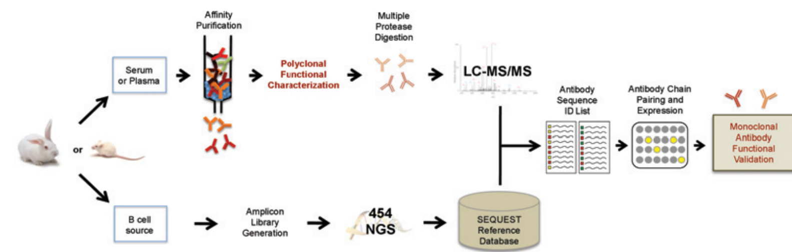

Figure 1: Overview of proteomics approach for identifying functionally relevant monoclonal antibodies from an immunized animal. Serum or plasma from an immunized animal was first purified by protein A or G and subsequently subjected to antigen affinity purification. Purified polyclonal antibodies were then functionally characterized to ensure specific activity enrichment. Validated purified antibodies were digested with various proteases to prepare peptide fragments to be analyzed by high mass accuracy LC-MS/MS. In order to identify peptide sequences corresponding to antibody fragments by SEQUEST, a reference database of Ig V-region sequences was generated by Next Generation Sequencing (NGS) of the immunized animal's B cell repertoire. High confidence V-region sequences that correspond to antibodies purified from the serum were identified using in-house software. These high confidence heavy and light chain sequences were then synthesized and cloned into a single-open-reading-frame antibody expression platform. Recombinant monoclonal antibodies were expressed combinatorially in a matrix of heavy and light chains and screened for precise function and compared to the specificity and activity of the original polyclonal antibody mixture.

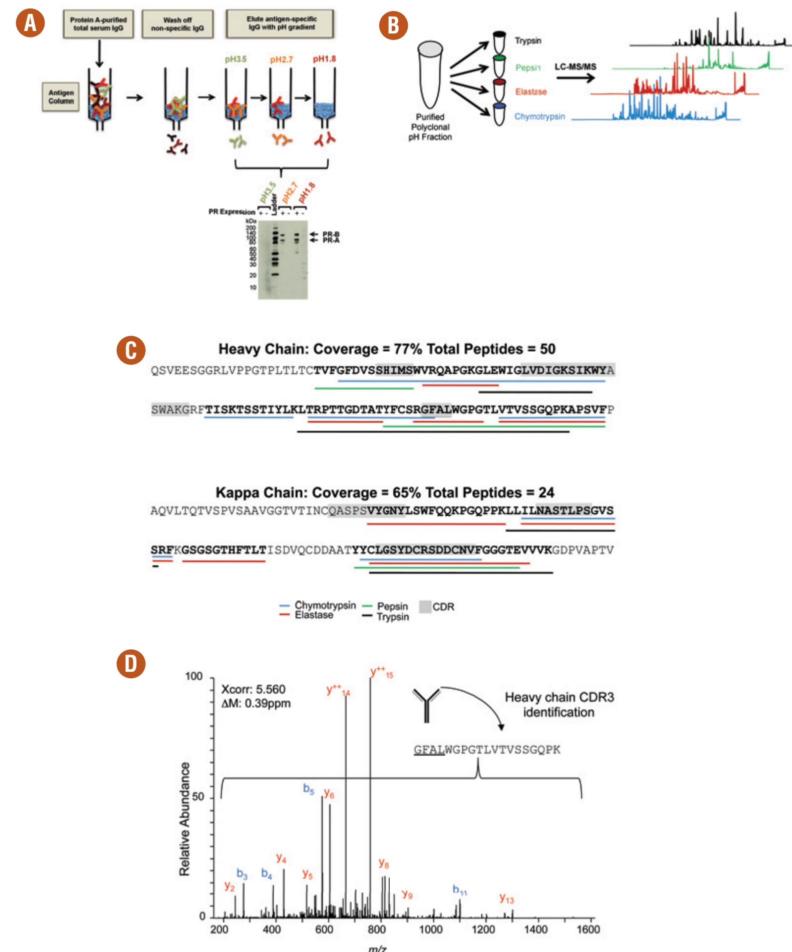

Figure 2: Affinity purification of progesterone receptor-specific polyclonal rabbit IgG. (**A**) Total IgG from the serum of the immunized rabbit was isolated with Protein A and further affinity purified on immobilized antigen peptides by gravity flow. After extensive washing to reduce non-specific IgG, a sequential elution with progressively acidic pH was used to fractionate the antigen-specific polyclonal IgG. Each fraction was tested for specific activity by western blotting at matched antibody concentration (21.5 ng/ml) to detect PR A/B in lysates from T47D cells (+). Negative control lysates from HT1080 (-) were also tested. (**B**) The fraction with the highest specific activity, pH1.8, was processed with four proteases for LC-MS/MS analysis. (**C**) An MS/MS spectrum matched by SEQUEST to the V-region full tryptic peptide GFALWGPGTLVTVSSGQPK containing CDRH3 (underlined) with an XCorr of 5.560 and a DM (observed m/z - expected m/z) of 0.39 ppm. (**D**) Rabbit heavy and light chain sequence identification coverage of clone F9. The depicted V-region sequences, when paired, specifically bind human PR A/B (See figure 3). Amino acids marked by one or more peptides are shown in bold. To maximize V-region coverage and account for highly variable amino acid composition, complementary proteases were used. Blue, Chymotrypsin; Red, Elastase; Green, Pepsin; Black, Trypsin.

100% CDR3 Coverage and ≥ 65% V-region Coverage

Chain	NGS Ref. #	Total Peptide Count	% Variable Region Coverage	CDR3 Sequence	NGS rank by CDR3 frequency	Germline V(D)J
γ chain	G2JXQJ001A2Q81	101	95.69	KLGL	212	IGHV1S45, D4-2, J4
γ chain	G2JXQJ001AGJSJ	91	92.04	GFSL	76	IGHV1S69, * ,J4
γ chain	G2JXQJ001BJE8R	78	98.26	DLGDL	423	IGHV1S45, D3-1, J4
γ chain	G2JXQJ001BT2NA	70	86.21	DLGNL	461	IGHV1S45, D4-1, J4
γ chain	G2JXQJ001AFBNC	61	87.27	GNL	58	IGHV1S44, D4-1, J4
γ chain	G2JXQJ001AL49Y	59	87.72	DFHL	237	IGHV1S45, * ,J4
γ chain	G2JXQJ001BWR23	56	89.17	GSLGTLPL	103	IGHV1S45, D8-1, J2
γ chain	G2JXQJ001BN8MH	50	82.14	GFAL	109	IGHV1S69, * ,J4
γ chain	G2JXQJ001BPNUG	48	81.51	GHDDGYNYVYKL	123	IGHV1S69, D6-1, J4
γ chain	G2JXQJ001BZA42	35	95.54	GFTL	1417	IGHV1S69, * ,J4
κ chain	G2JXQJ001BJ8KJ	93	87.27	LAGYDCTTGDCFA	2769	IGKV1S15, J1-2
κ chain	G2JXQJ001BQM6D	47	95.5	LGGYDCDNGDCFT	85	IGKV1S15, J1-2
κ chain	G2JXQJ001A9VP3	33	92.79	LGTYDCRRADCNT	5654	IGKV1S19, J1-2
κ chain	G2JXQJ001BQJFD	28	98.15	QSTLYSSTDEIV	86	IGKV1S10, J1-2
κ chain	G2JXQJ001BJCLS	28	96.23	QCSYVNSNT	4518	IGKV1S44, J1-2
κ chain	G2JXQJ001AG4TB	24	65.45	LGSYDCRSDDCNV	179	IGKV1S2, J1-2
κ chain	G2JXQJ001AIZ32	17	86.11	LGAYDDAADNS	252	IGKV1S19, J1-2
κ chain	G2JXQJ001BJYR5	15	72.07	LGTYDCNSADCNV	1128	IGKV1S15, J1-2

Table 1: Identification of high confidence heavy and light chains. Heavy and light chains with 100% CDR3 spectrum coverage and overall ≥65% variable region coverage were identified and ranked in order of confidence as measured by total peptide count. CDR3 sequence identity and rabbit germline determination are also indicated. Heavy and light chains were chosen for gene synthesis, cloning, and expression of combinatorial antibodies for characterization. NGS rank indicates the frequency ranking of the given CDR3 sequence identified in the NGS database for each chain. * indicates that no possible D gene can be identified.

Antigen	Immunized species	High confidence heavy + light chains	Unique ELISA+ clones	Unique WB+ clones
PR A/B	Rabbit	8 + 10	12	6
p-MET	Rabbit	11 + 10	6	4
Lin28A	Rabbit	7 + 4	5	5
Sox1	Rabbit	9 + 5	12	1
p-p44/42	Mouse	12 + 13	15	3

Table 2: Functionally relevant monoclonal antibodies against multiple targets identified by the NG-XMT™ platform tested by ELISA and western blot (WB).

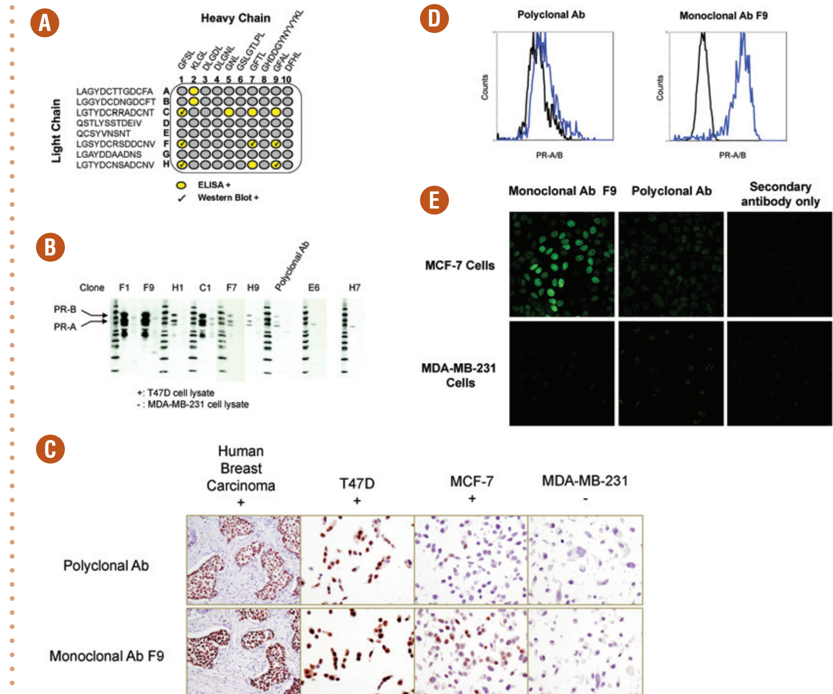

Figure 3: Identification and characterization of functional monoclonal antibodies against progesterone receptor A/B. (**A**) Combinatorial pairing of heavy and light chains yielded 12 antigen-specific ELISA-reactive clones indicated in yellow. CDR3 sequence is used as an identifier. ✓ indicates western blot-positive clones (See Fig. 3b). (**B**) Six clones (F1, F9, H1, C1, F7, and H9) were specific for progesterone receptor A/B detection by western blotting. Clones E6 (ELISA-negative, western-negative) and H7 (ELISA-positive, western-negative) are shown as controls. +, T47D (PR A/B-positive); -, MDA-MB-231 (PR A/B-negative). All antibodies tested at 21.5 ng/mL. (**C**) Comparison of specific activity of clone F9 to the affinity-purified polyclonal mixture by immunohistochemistry. 0.4 µg/mL of F9 specifically stained PR A/B-positive tissue or cell lines (T47D and MCF-7), but not a PR A/B-negative cell line (MDA-MB-231). 0.2 µg/mL of polyclonal antibody was used as positive control. (**D**) Flow cytometry analysis. Blue, T47D cells (progesterone receptor A/B-positive cell line); Black, MDA-MB-231 (progesterone receptor A/B-negative cell line). Polyclonal antibody signal/noise ratio, 1.69; concentration, 3.7µg/mL. Monoclonal antibody F9 signal/noise ratio = 36.4; concentration 0.5 µg/mL. (**E**) Confocal immunofluorescence microscopy analysis showed specific nuclear staining pattern on progesterone receptor A/B-positive cell line MCF-7 but not on MDA-MB-231 cells at 0.46 mg/mL. No primary antibody was included as background staining control. Polyclonal antibodies were also used as comparison at a concentration of 1.85 mg/mL.

Summary

Our NG-XMT™ technology platform for direct identification of circulating antibodies in animals has applications in basic immunology and therapeutics. For example, our platform can provide a basis for understanding central questions in the field of immunology including serum antibody diversity, dynamics, kinetics, clonality, and migration of antibody secreting B cells following antigen exposure. Furthermore, our approach can be readily applied to vaccine research and used to pursue therapeutically relevant human monoclonal antibodies from immunized, naturally infected, or diseased individuals.

Cell Signaling TECHNOLOGY®

Cell Signaling Technology® and NG-XMT™ are trademarks of Cell Signaling Technology, Inc.

Contact Information

Sean A. Beausoleil, Cell Signaling Technology, Inc. **Email:** sean.beausoleil@cellsignal.com

PTMScan Services Department Email: ptmscan@cellsignal.com • **web:** www.cellsignal.com/services