

# Antibody-Scanning and Epitope-Tagging Methods; Molecular Mapping of Proteins Using Antibodies

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**Abstract:** Because synthetic short peptides bearing critical binding residues, can chemically mimic the folded antigenic determinants on proteins, short synthetic peptides can generate antibodies that react with cognate sequences in intact folded proteins. According to this mimotope theory, we produced site-specific antibodies by immunization with short peptides which overlapped each other and covered the entire protein, and used them for domain mapping of influenza virus RNA polymerase (antibody-scanning method). We also used a tagged-epitope and its monoclonal antibodies for topology mapping of clathrin light chains in clathrin triskelions by electron microscopy. Both methods using specific epitopes in combination with their antibodies enable us to determine the domains of interesting proteins systematically without the need to generate monoclonal antibodies or mutant proteins.

## INTRODUCTION

Short synthetic peptides can generate antibodies that react with cognate sequences in intact folded proteins with a surprising degree of success [1,2]. Ever since the structure of an antigenic determinant in proteins was elucidated, antibodies which recognize specific sequences have been widely used in molecular biology [3]. Many of them are produced by immunization with synthetic peptides or short peptide fragments from native proteins. Short peptide fragments of 50-100 amino acids expressed in bacteria are economical and can be used for immunization. Peptides of these lengths usually mimic the epitopes in native proteins.

Small peptides corresponding to sequences from the hemagglutinin of the influenza virus (HA1) were used to raise antibodies that reacted with the HA1 protein itself [4]. Synthetic peptides were also used to map the epitope of monoclonal antibodies and their use is now widely

accepted [2]. Using synthetic peptides as immunogen, antibodies can be raised against epitopes which are not exposed in the native molecules.

Geysen *et al.* also independently established the concept that the synthetic short peptides bearing critical binding residues, mimotopes, can chemically mimic the folded antigenic determinants, epitopes, on proteins [5]. At the same time, a bacteriophage expression vector displaying foreign epitopes on its surface was developed [6]. Then, the idea of using fusion phage (fd or M13 phage) to develop an epitope library was introduced [7]. In this system two viral proteins were used to display epitopes; the minor coat protein pIII and the major coat protein pVIII [8,9]. This phage system could also be utilized not only for screening antibody-specific epitopes but also for producing site-specific antibodies.

Since phage peptide library was introduced, epitope mapping became much easier than before [10]. However, libraries of short linear peptides generally do not contain mimics of large molecular interfaces including 'conformational' epitopes [11]. We have to pay attention to the fact that the ability of an antibody to bind a given linear epitope does not exclude other, non-sequential resi-

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dues, contributing to binding of the native epitopes, *i.e.* a linear epitopes may be just one part of a larger conformational epitope. Two peptides derived from separate regions of the antigen's linear sequences could be found to bind to a single monoclonal antibody [12]. Besides epitope-mapping, the phage displaying peptide library is advantageous for determination of 'binding' sites of the proteins (receptor-ligand, DNA-protein, and protein-protein interaction sites, etc.) [13,14].

Polyclonal antibodies can be raised against epitopes which monoclonal antibodies sometimes fail to recognize. Moreover, polyclonal antibodies can be raised with less effort than monoclonal antibodies. From the reasons mentioned above, epitope mimicking peptides and the antibodies raised against them are of great use for domain-mapping.

Epitope-tagging or sequence-tagging method was widely accepted immediately after it was introduced in order to identify the expressed proteins and their cellular localization signals [15-17]. Tagged sequences can be also used for the pull-down or affinity-purification of proteins, and the complexes which contain the epitope-tagged proteins. Therefore, once we cloned the cDNAs, many information about them could be obtained in a short period by expressing them as epitope-tagged proteins.

Retention of resident proteins in the lumen of the endoplasmic reticulum was achieved in both yeast and animal cells by their continual retrieval from the cis-Golgi, or a pre-Golgi compartment. Pelham and his group demonstrated that sorting of these proteins was dependent on a C-terminal tetrapeptide signal, usually KDEL in animal cells, and HDEL in *Saccharomyces cerevisiae*, by addition or deletion of the sequence [17]. At the same time he introduced the substance P epitope to monitor the expressed proteins [15].

Some epitopes commonly used for tagging and their sequences are listed in table 1 [15,16,18,19]. In these days, many N-or C-terminal epitope-tagged expression vectors are commercially available, and the tagged epitopes can be introduced into any position of the protein unless they disturb the entire structure of the epitope-tagged proteins.

We applied two methods to map the functional and structural domains of target proteins, 'antibody-scanning' which is sometimes utilized to map functional domains of proteins [20], and topology mapping by electron microscope (EM) using tagged epitope and its specific monoclonal antibodies [19].

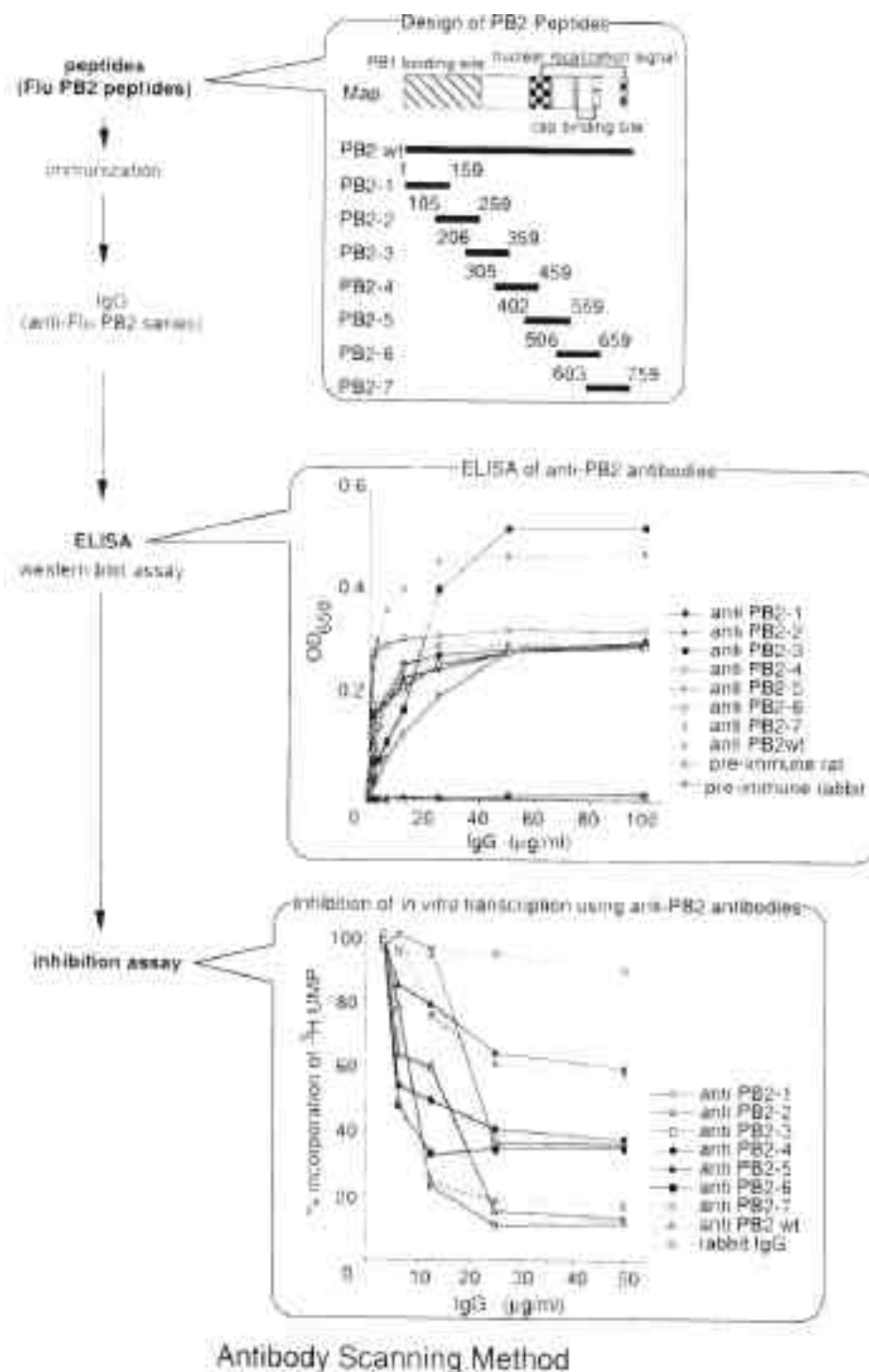
## ANTIBODY SCANNING METHOD

Polyclonal antibodies are systematically raised against overlapping synthetic peptides which cover the entire molecule. These antibodies recognize linear amino acid sequences and sometimes the tertiary structure formed by short peptides which mimic the structure of native proteins. Using these antibodies in biochemical or binding assays, domains sensitive to inhibition can be mapped. With this method, steric effects of antibody have to be considered.

As an example, we demonstrate the functional mapping of influenza virus RNA polymerase [20]. Monoclonal antibodies can also be raised against synthetic or short peptides for this purpose, but sometimes they do not bind the native proteins nor inhibit the activities. For economical reasons, we generated polyclonal antibodies by immunizing rats with bacterially expressed peptides, although rat IgG had a disadvantage that it poorly bound to protein A or protein G.

Influenza virus RNA polymerase with the subunit structure PB1-PB2-PA is involved in both transcription and replication of the RNA genome including the unique cap-I dependent RNase activity. In order to map the domains important for RNA polymerizing, cap-I dependent RNase and cap-I binding activity, we generated site-specific antibodies against overlapping 150 amino-acid peptides which cover each subunit. The strategy is briefly summarized in Fig. (1).

We found that the IgG fractions purified from protein G column chromatography (Amersham Pharmacia Biotech) still contain RNase activities although we did not test for any protease or DNase activities in this fraction. Because these purified antibodies were used for the inhibition of transcription activities, the contaminated RNase activities had to be diminished. To prevent the RNase activity from contaminating the affinity-purified IgG fractions, we used Prime RNase Inhi-



**Fig. (1).** Antibody scanning method; Molecular mapping with site-specific antibodies. The overall steps of molecular mapping with site-specific antibodies are shown. First, peptides are designed, used for immunization of the animals. Next, the produced antibodies are examined for their affinity, specificity. Finally, they are used for inhibition assay (from top to bottom).

bitor (5 prime 3 prime Inc.) because it works without DTT, which may destroy the disulfide bondage between heavy and light chains of immunoglobulin.

We first confirmed that the polyclonal antibodies against each entire subunit inhibited RNA synthesis by adding them to the *in vitro* transcription system. Those against PB1, PB2 and PA inhibited the RNA polymerizing activities.

Site-specific antibodies were tested for binding the native proteins by ELISA using the influenza virus RNA polymerase-RNA genome-nucleocapsid protein (NP) complex (RNP) for antigens because these antibodies were generated by immunizing rats with urea-denatured peptides. These anti-bodies were also tested for specificity by western blot assay.

Influenza virus RNP was incubated for 30 min at room temperature with a serial dilution (50, 25, 12.5, 6.25 and 3.125 µg/ml, as indicated) of the antibodies. Then the RNP was further incubated after substrate and [<sup>3</sup>H]-UTP were added to the reaction mixture. The amount of [<sup>3</sup>H]-UMP incorporated was compared with that in a control experiment with pre-immune antibodies.

Results indicated that the antibodies against PB2-1 and PB2-2 reduced the polymerase activity to less than 20 % of the control value. Those against PB2-3, PB2-4 and PB2-6 reduced it to less than 40 %; and those against PB2-5 and PB2-7 reduced it only to around 60 % of the control value. From these results, we concluded that antibodies against the N-terminal amino acids 1-159 of PB2, which overlap the PB1 binding site on PB2, inhibited RNA polymerase activity as well as did the antibodies for the entire PB2 subunit (anti PB2 wt).

Mapping of ATPase domain of clathrin light chain by rabbit site-specific polyclonal antibodies produced by synthetic peptides was another example of success [21].

## ROTARY SHADOWING USING EPITOPE-TAGGING

Tagged epitopes in combination with specific monoclonal antibodies are also widely used to detect and/ or purify expressed proteins (Table 1). We developed the unique technique by application of this method for structural mapping of proteins by EM. With this technique, single tagged-sequences are inserted at a certain position in the protein and visualized with bound specific monoclonal antibodies by low angle rotary shadowing.

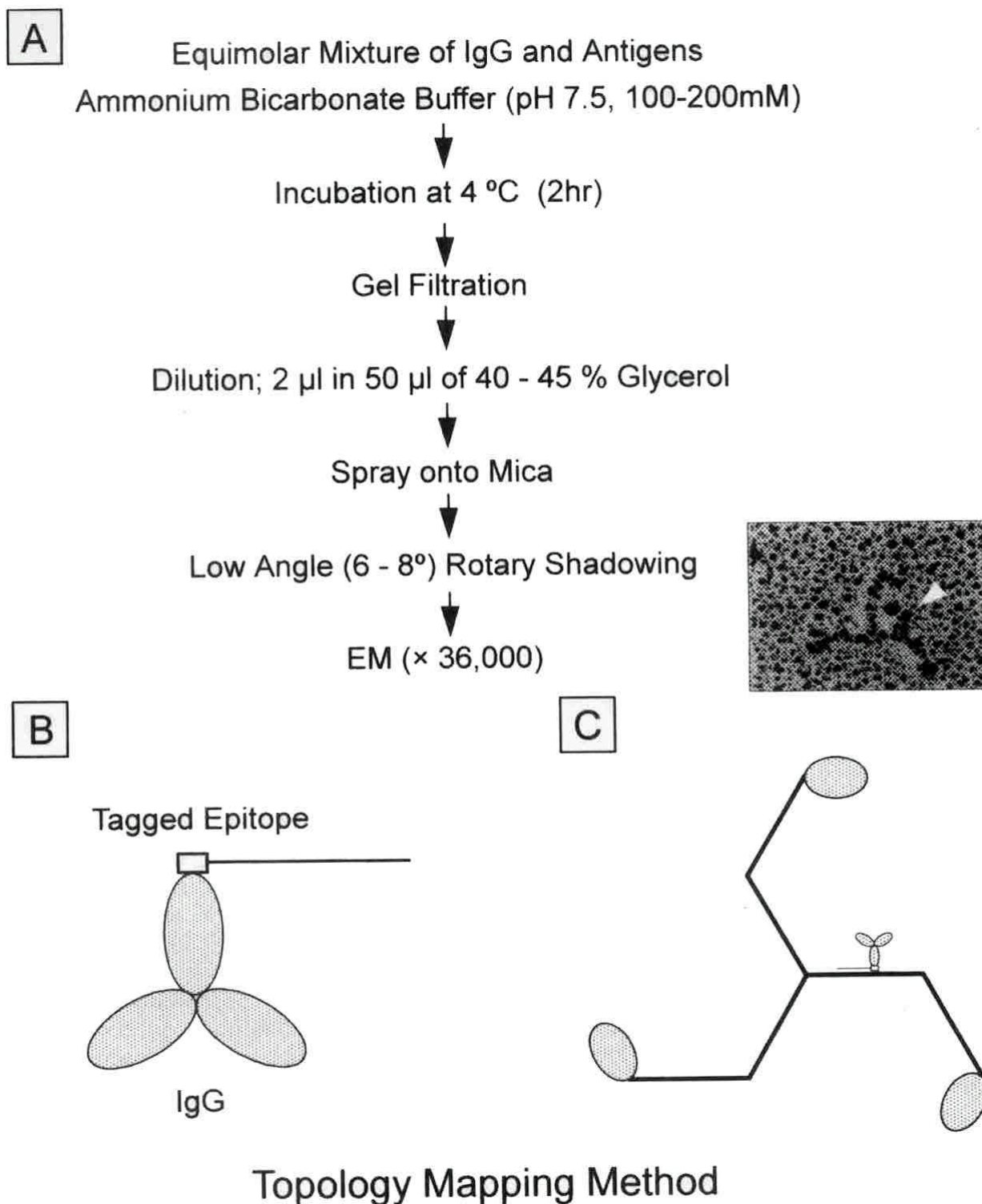
We determined the topology of the clathrin light chain and heavy chain molecules from electron micrographs of the epitope tagged-clathrin trimer obtained with monoclonal antibodies against tagged

sequences [19]. Fig. (2) gives an overview of the topology mapping by EM.

**Table 1. Tagged Epitope Sequences**

epitope	sequences
cMYC	EQKLISEEDLN
collagen XII	FPEPYVPESGPY
FLAG	DYKDDDDK
HA	YPYDVPDYA
substance P	QFFGLM
T7	ASMTGGQQMGR
biotin (Strep-tag)	SAWRHPQFGG
6 x His	HHHHHH
phosphorylation	RRASV

Clathrin, which is a three-legged structure (triskelion) comprising three identical heavy chains and three different light chains, is a major component of coated pits and coated vesicles. Clathrin light chains contain a central domain of 10 heptad repeats necessary for interaction with the heavy chain. Chicken collagen type XII epitope, FPEPYVPESGPY, was tagged to the N- or C-terminus of the clathrin light chain. The epitope-tagged clathrin light chain was expressed in *E. coli* and purified. The clathrin heavy chain trimer, and the epitope-tagged light chains were assembled into triskelions *in vitro*. The clathrin triskelions containing epitope-tagged light chains were incubated at 4 °C for 2 h with an equimolar amount (0.5-1 mg/ml) of purified monoclonal antibody, 75d7 [22], which was raised against the collagen XII epitope, in 100-200 mM ammonium bicarbonate buffer, pH 7.5. The unbound IgG were removed by gel filtration on Sephacryl S-300 equilibrated in the same buffer. The clathrin triskelions bound with monoclonal antibodies were eluted in void volume and immediately processed for rotary shadowing. The samples were diluted in ice-cold 40-45 % glycerol, sprayed on to mica, and platinum rotary shadowed at a 6-8 ° glancing angle. Pictures were taken at a magnification of 36,000. The bound IgG was easily identified because it appeared as heart shaped. The gel filtration step may be omitted. One or two µl of the samples incubated with IgG can be immediately diluted in 50 µl of ice-chilled 40-45 % glycerol, and processed as mentioned above.



**Fig. (2).** Topology mapping by electron microscope using tagged epitope. The overall steps of topology mapping are shown in A. A white arrowhead in the electron microgram indicates the heart-shaped IgG bound to the clathrin triskelion (see also C). The IgG bound to the tagged epitope of the clathrin light chain, which is not identified in the photo (A), is illustrated (B).

The bound IgG appeared as heart-shaped in the electron microgram (Fig. (2) A and C). The topology from the clathrin light chain to the heavy chain was determined by measurement of the

distance between the IgG and the center of the pin-wheel (triskelion) of both N- and C-terminal tagged light chains.

## CONCLUSION

Using antibody-scanning and epitope-tagging methods, one can determine the domains of interesting proteins systematically without generating monoclonal antibodies or mutant proteins.

By molecular biological techniques, any sequence can be introduced into the target proteins. Besides for detection of expressed proteins or topology mapping, tagged epitopes can be used for affinity-purification of the tagged proteins using specific antibodies. 6 x His- and Strep-tag are used for protein purification [23,24]. [<sup>32</sup>P]-labeled protein is made by introducing phosphorylation sequences (RRASV) and by *in vitro* kination with commercial protein kinase A and -[<sup>32</sup>P]ATP [25] (Table 1). The topology of membrane proteins can be determined by introducing a N-glycosylation site (NXT or NXS) into the proteins [26].

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