

# Identification and Repair of Positive Binding Antibodies Containing Randomly Generated Amber Codons from Synthetic Phage Display Libraries

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Phage display technology allows for the rapid isolation and characterization of monoclonal antibodies that have vast potential for therapeutic and diagnostic applications. However, the panning process, which utilizes a host strain that suppresses termination by the amber codon, has an inherent bias toward clones containing randomly generated amber stop codons, complicating identification of positive binding antibodies when the antibody genes are finally expressed in a nonsuppressor host. Here, we perform biopanning against a Histone 2A peptide using streptavidin- or anti-biotin-coated beads. After four rounds, a dominant clone is characterized but contains a spurious amber stop codon. A protocol is given that readily corrects the amber codon, allowing for soluble antibody production once the phagemid is transformed into a nonsuppressor bacterial strain. This work also highlights the ability to isolate antibodies against a protein antigen by using only a small peptide (15 amino acids) representing a portion of the antigen.

## Introduction

Phage display antibody libraries represent a powerful, readily available tool for selection and isolation of monoclonal antibodies. The relative ease by which one can obtain a specific antibody against an antigen makes phage display a very attractive technology, and it is currently being utilized by scientists from a wide variety of disciplines for a host of therapeutic and diagnostic applications (1–3). Most antibodies or antibody fragments isolated from phage display libraries to date have been generated against protein antigens, with only limited reports of antibodies isolated against small peptides, suggesting potential difficulties may exist when panning against peptides. Studies in our laboratory suggest these difficulties are due to the presence of randomly generated amber stop codons (TAG) within the antibody genes. The amber codon does not interfere with the initial panning and selection process, which utilizes the suppressor E strain, *E. coli* TG1, where the TAG codon is translated as glutamine (4). However, when the phagemid containing this sequence is transformed into the nonsuppressor strain, *E. coli* HB2151, used for soluble antibody selection and secretion, the amber codon is translated as a stop signal, production of the full-length antibody does not occur, and no binding is detected. Although we have isolated clones against full-length proteins from synthetic libraries that did not contain any amber codons (5–7), when panning against a number of small peptide antigens (10–15 amino acids), we isolate a disproportionately large fraction of clones containing an amber codon. Although isolation of clones with amber codons should be a relatively common occurrence, there are only very limited reports in the literature (8).

Here we describe the results obtained when panning against a small biotinylated peptide representing an exposed segment of the chromatin-regulating protein Histone H2A (9). We suggest a modified screening protocol using phage ELISA to identify positive binding clones, as opposed to the soluble antibody screening performed in some previous studies (10, 11), and also a simple method to correct the amber codon using site-directed mutagenesis.

## Materials and Methods

**Materials.** A sample of the phage antibody libraries Tomlinson (I + J: <http://www.geneservice.co.uk/products/proteomic/datasheets/tomlinsonIJ.pdf>) and *E. coli* strains TG1: K12,  $\Delta(\text{lac-pro})$ , *supE*, *thi*, *hsdD5*, *F'traD36*, *proA*<sup>+</sup>*B*<sup>+</sup>, *lacI*<sup>q</sup> $\Delta$ M15 and HB2151: K12, *ara*,  $\Delta(\text{lac-pro})$ , *thi*, *F'proA*<sup>+</sup>*B*<sup>+</sup>, *lacI*<sup>q</sup> $\Delta$ M15 were obtained from the Medical Research Council (MRC), Cambridge, England. The biotinylated peptide corresponding to residues 2–16 of histone H2A (SGRGKQGGKARAKAK-Biotin) was synthesized by the Protein Analysis and Synthesis Lab at Arizona State University. Anti-biotin-coated magnetic beads, streptavidin-coated magnetic beads, and MACS separation columns were purchased from Miltenyi Biotec Inc. (USA). Anti-myc-tag and goat-anti-mouse IgG (HRP conjugated) antibodies were purchased from Santa Cruz Biotechnology (USA). 3,3'-Diaminobenzidine (DAB) substrate system was purchased from Sigma-Aldrich (USA). DpnI and NEBuffer 4 were purchased from New England Biolabs (USA).

**Biopanning.** The biopanning procedure is essentially as described previously (12). Briefly, 10<sup>12</sup> phage units were incubated with the biotinylated H2A peptide in solution. Streptavidin- or anti-biotin-coated magnetic beads (used alternately each round) were then added to the solution and used to extract the peptide/phage by running the solution through the MACS separation column attached to a magnet. The column is washed extensively, the magnet is removed, and the bound beads were expelled by inserting a plunger into the top of the

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Table 1. Phage ELISA Values

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.111	<b>0.160</b>	0.119	0.114	<b>0.169</b>	0.107	0.106	<b>0.371</b>	<b>0.184</b>	0.111	0.104	0.099
B	0.121	<b>0.142</b>	<b>0.264</b>	0.117	0.107	0.106	<b>0.138</b>	<b>0.283</b>	0.105	0.103	0.097	0.114
C	0.119	<b>0.141</b>	0.106	0.108	0.116	0.113	0.121	0.091	0.094	<b>0.167</b>	0.094	0.116
D	0.113	<b>0.254</b>	0.123	0.104	<b>0.125</b>	0.106	0.104	0.095	0.100	0.103	<b>0.134</b>	<b>0.166</b>
E	0.116	0.123	<b>0.129</b>	0.114	0.111	<b>0.314</b>	<b>0.207</b>	0.107	0.104	<b>0.145</b>	0.100	<b>0.203</b>
F	0.117	<b>0.127</b>	<b>0.131</b>	0.122	<b>0.214</b>	<b>0.149</b>	<b>0.148</b>	<b>0.212</b>	<b>0.192</b>	<b>0.138</b>	0.109	0.109
G	<b>0.308</b>	<b>0.138</b>	0.116	0.106	0.110	0.115	<b>0.209</b>	0.101	0.104	<b>0.125</b>	<b>0.151</b>	0.110
H	<b>0.135</b>	0.102	0.101	0.105	0.121	0.109	<b>0.245</b>	<b>0.176</b>	0.118	0.112	0.111	<b>0.184</b>

column. The eluted solution was treated with trypsin at a final concentration of 1 mg/mL for 30 min. The eluted phage is then amplified as instructed in the Tomlinson (I + J) protocol and used for further rounds of panning.

**Monoclonal Phage/scFv ELISA.** ELISAs were performed as described on the MRC website (<http://www.geneservice.co.uk/products/proteomic/datasheets/tomlinsonIJ.pdf>). Briefly, after four rounds of panning, individual clones in *E. coli* TG1 were grown in microtiter wells and phage were produced by addition of KM13 helper phage. Antibody-displayed phage were then added to corresponding wells of an avidin (5  $\mu$ g)/H2A peptide-coated (10  $\mu$ g) plate and detected by an anti-M13 (HRP) antibody. For scFv ELISAs, the periplasmic fraction of induced *E. coli* HB2151 clones was added to the wells instead of phage. Control wells were coated with 2% milk powder in PBS (MPBS). The myc-tag of the scFv was detected by the anti-myc antibody and a secondary goat-anti-mouse IgG (HRP) antibody was also used. The periplasmic fraction of an induced, overnight culture is prepared by resuspending the bacterial pellet in a 1:20 (v/v) amount of TSE (50 mM Tris, 20% sucrose, 1 mM EDTA, pH 7.5) for 30 min at 4 °C. This mixture is then spun down for 30 min at 10,000  $\times$  g, and the supernatant contains the periplasmic fraction.

**Phagemid DNA sequencing.** Isolation of the pIT2 phagemid vector was performed using the QIAGEN Plasmid Isolation Kit (QIAGEN Inc., Valencia, CA). The phagemids were sequenced by the DNA Laboratory at Arizona State University using the pHEN seq (5' CTA TGC GGC CCC ATT CA 3') or LMB3 (5' CAG GAA ACA GCT ATG AC 3') sequence primers.

**Site-Directed Mutagenesis.** Primers were designed to change the randomly generated amber codon (TAG) to CAG (glutamine) by using the 21 nucleotides flanking the amber codon: forward (5' TCG ACG AAG GGT ACT AGG ACA CAG TAC GCA GAC TCC GTG AAG GGC 3') and reverse (GCC CTT CAC GGA GTC TGC GTA CTG TGT CCT AGT ACC CTT CGT CGA 3'). The following mix was constructed in a PCR tube on ice: 33  $\mu$ L of water, 2  $\mu$ L of 50 mM MgSO<sub>4</sub>, 5  $\mu$ L of 10x Pfx Amplification Buffer, 4  $\mu$ L of dNTPs, 1  $\mu$ L of each mutagenesis primer, 5  $\mu$ L of isolated phagemid, 2  $\mu$ L of Platinum Pfx Polymerase. The following thermocycler conditions were used: initial cycle for 60 s and 94 °C, then 12 cycles of 30 s at 94 °C, 30 s at 55 °C, and an extension time of 10 min at 68 °C (extension time is based on 2 min/kbp of phagemid). A 1  $\mu$ L aliquot of DpnI and 5.7  $\mu$ L of NEBuffer 4 were added to the PCR mixture and incubated at 37 °C for 1 h. A 200  $\mu$ L aliquot of chemically competent *E. coli* HB2151 cells was added to the mixture. The mixture was placed on ice for 30 min, then heat shocked for 3 min at 42 °C, and then iced for 3 min. A 100  $\mu$ L aliquot of mixture was plated onto LB agar plates (supplemented with 100  $\mu$ g/mL of ampicillin) and grown overnight at 37 °C. Individual colonies were picked, grown, and induced with 1 mM IPTG. Dot blots were performed on supernatants and periplasmic fractions of each clone to check for expression.

**Dot Blot.** A 2  $\mu$ L sample was placed on a nitrocellulose membrane. The membrane was blocked with 4% milk in PBS, pH 7.4 for 2 h at room temperature. The membrane was washed once with PBS and then stained with anti-myc-tag mouse antibody at 1:500 dilution in PBS overnight at 4 °C. The membrane was washed 3 times with PBS and then stained with a goat anti-mouse IgG HRP conjugate antibody at 1:1000 dilution in 2% MPBS for 1 h. The membrane was then washed 3 times with PBS and developed using the DAB substrate system.

## Results and Discussion

Aliquots of the Tomlinson I and J scFv libraries were combined and used for biopanning. After four rounds of biopanning against the H2A peptide, a soluble scFv ELISA was performed on 96 individual clones, and no positive clones were detected (data not shown). A phage ELISA of 96 individual clones showed that 36 (highlighted in bold) out of 96 clones exhibited strong binding (Table 1), as determined by OD readings greater than 2 standard deviations (95% confidence interval) from a background average represented by the lowest 25 readings (0.108  $\pm$  0.008). DNA sequencing indicated that the 10 strongest binding clones all had the same protein sequence (Figure 1) containing an amber codon in one of the randomized positions of the heavy chain.

During the biopanning selection process, a suppressor E strain, *E. coli* bacteria, TG1, was used for amplification of phage, which translates the TAG codon as a glutamine, so the amber codon does not interfere with the selection process. However, to obtain soluble scFv expression, a nonsuppressor strain, *E. coli* HB2151, is used that recognizes TAG as an amber stop codon, and expression is terminated. The phage ELISA results demonstrate that a high percentage of scFv/phage complex produced by *E. coli* TG1 binds to the H2A peptide even though no positive results were obtained by the soluble scFv ELISA. We selected the dominant strong positive sequence and mutated the amber codon (TAG) to a glutamine codon (CAG) to test if full protein expression and function could be obtained in a nonsuppressor host.

The production of scFv from the periplasmic fraction and supernatant of the clones were compared before (+ amber) and after (– amber) mutagenesis to remove the amber codon (Figure 2). After replacing the amber codon with the CAG codon, encoded for glutamine residue, soluble scFv production was readily detected in both supernatant and periplasmic fractions. The corrected scFv also retains its binding specificity against the H2A peptide based on the soluble scFv ELISA (Figure 3).

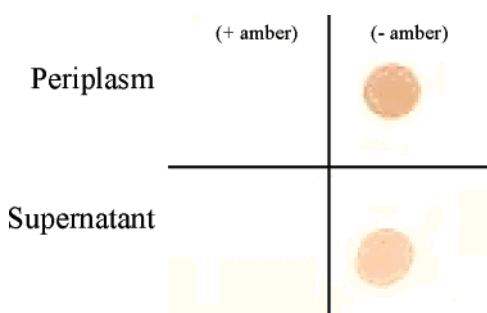
Finally, we ensured that the anti-H2A peptide scFv could bind the full length H2A protein. The peptide antigen is located on the tail of H2A and is expected to remain exposed after protein folding. The scFv did bind full length H2A based on the soluble scFv ELISA (Figure 4).

This work has outlined a method for obtaining monoclonal antibodies against peptide antigens or full proteins using a small

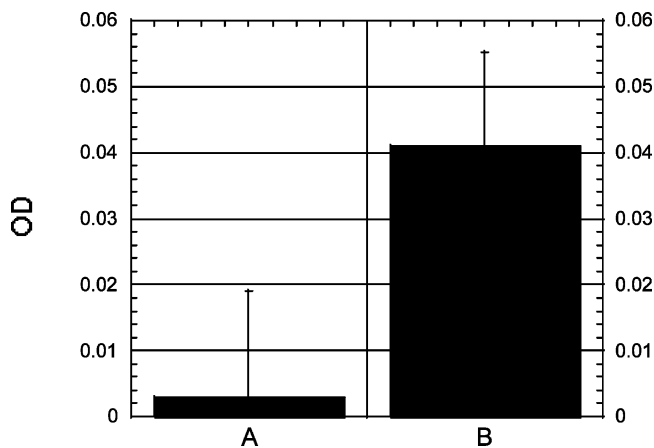
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MAEVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWV      50
                                CDR1
STISTKGTRT*YADSVKGRFTISRDNKNTLYLQMNSLRAEDTAVYYCAK      100
                                CDR2
RSKHFDYWGQGLVTVSSGGGGSGGGGSGGGGSTDIQMTQSPSSLSASVG      150
                                CDR3          linker
DRVVITCRASQSISSYLNWYQQKPGKAPKLLIYSASSLQSGVPSRFRSGSG      200
                                CDR1          CDR2
SGTDFTLTITSSLPEDFATYYCQQTTAISPPTFGQGTKVEIKRAAAHHHHH      250
                                CDR3          HIS-tag
HGAAEQKLISEEDLNAA      268
                                myc-tag
    
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**Figure 1.** Amino acid sequence of anti-H2A scFv heavy and light chain, respectively, connected by a flexible (GGGGS)<sub>3</sub> linker. The 18 randomized amino acids in the library are shown in bold. The spurious amber codon, one of the randomized sequences, is represented by an asterisk (\*). The CDRs were determined by the Kabat antibody sequence database (13).

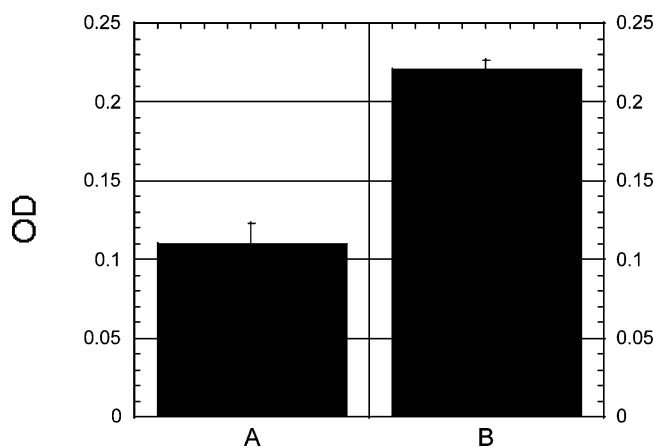


**Figure 2.** Characterization of soluble expression of uncorrected (+ amber) and corrected (– amber) clones. Expression of soluble antibody fractions in the periplasmic fractions (top) and supernatants (bottom) of bacterial clones induced by 1 mM IPTG containing uncorrected (left) and corrected (right) anti-H2A phagemids.



**Figure 3.** Functional characterization of uncorrected (+ amber) and corrected (– amber) clones. Functionality was determined by monoclonal scFv ELISA using the periplasmic fraction obtained from the (A) uncorrected and (B) corrected clones. OD values are the absorbance values measured at 650 nm subtracted from the values measured at 450 nm. The values are the averages of five samples with background values from control wells subtracted from the sample wells, and the error bars are standard deviations.

peptide segment of the protein. The protocol described circumvents problems associated with a selection bias for amber codons. Amber codons in the randomized region of synthetic phage display libraries occur with a predictable frequency. The synthetic scFv libraries used for the panning studies reported here (<http://www.geneservice.co.uk/products/proteomic/datasheets/>



**Figure 4.** Monoclonal ELISA of anti-H2A scFv against full H2A protein. The ELISA was performed using the periplasmic fraction of the corrected anti-H2A clone. The left bar (A) is the average value of five control samples. The right bar (B) is the average of five samples with full H2A protein fixed to the wells. OD values are the absorbance values measured at 650 nm subtracted from the values measured at 450 nm. Error bars are standard deviations.

tomlinsonIJ.pdf) contain 18 randomized residues with diversities of  $1.47 \times 10^8$  and  $1.37 \times 10^8$  for the I and J libraries, respectively. Assuming a binomial distribution, there is a 25% chance that at least one amber codon will occur in any given clone in the I library, which utilizes random codons, and a 43.59% chance in the J library, which utilizes an NNK codon bias (N=A, T, G or C; K=T or G). Clones containing amber codons are readily eliminated when the genes are expressed in a nonsuppressor host. However, when panning against short peptides representing different regions of proteins such as  $\alpha$ -synuclein,  $\beta$ -amyloid, histone 2A, and Brahma-related gene 1 (BRG1) and using several synthetic libraries (Nissim (14), Griffin.1 (15), Tomlinson I, and Tomlinson J libraries), in our lab the overwhelming majority of the strongest binding clones identified from phage samples produced from *E. coli* TG1 by ELISAs contain an amber codon. When panning against small peptides we typically find that 10–50% of positive clones assayed by phage ELISA are positive (37.5% in this work), whereas less than 1% of clones assayed by soluble scFv ELISA are positive, indicating the preponderance of clones selected during panning against small peptides contain amber codons.

Statistically, the chance of selecting a clone with an amber codon is less than 50%; however, the panning results we obtain

indicate a very high probability, so there must be a driving force toward amber codon selection, likely the toxicity of the scFv to bacteria. Although expression of the scFv is under the control of a lacZ promoter, leaky expression occurs (16). For a given scFv containing a glutamine in a randomized position, three different codon usages are possible: CAG, CAA, or TAG. Expression of the scFv containing the CAG and CAA codons will produce a full length scFv that may be toxic to the cells. However, when the TAG codon is expressed in the *E. coli* TG1 suppressor strain, it is translated as glutamine about 20% of the time but as a stop signal the other 80% of the time. Therefore, less of the full protein is produced, resulting in lower cytotoxicity. Thus, amber codon containing clones are preferentially amplified over the other glutamine containing clones.

Although randomly generated amber codons occur frequently, this phenomenon is only rarely mentioned (8) and is not problematic unless positive clones cannot be identified by soluble ELISA studies. However, with various antigens, only clones with amber codons may be isolated and for even more cases the strongest binding clones may contain an amber codon and are discarded. Therefore, given that increasing number of researchers are utilizing this technology for an increasing variety of applications, the issues outlined in this work may be very beneficial.

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