

Application of the TNT[®] T7 Quick System to Selection and Evolution of Antibody Combining Sites



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Editor's Note: This feature highlights experiments abstracted from published, peer-reviewed journal articles that feature contributions by Promega's reagents and systems to novel observations in the life sciences. The journal's publisher and authors have granted Promega permission to reproduce the figures shown here. Minor changes to the information presented in the original article have been reviewed by M. Taussig.

He, M. and Taussig, M.J. (1997) Antibody-ribosome-mRNA (ARM) complexes as efficient selection particles for in vitro display and evolution of antibody combining sites. *Nucleic Acids Research* **25**, 5132.

INTRODUCTION

The display of peptide and protein libraries and the ability to search these libraries by affinity selection have important implications for the rapid generation of antibodies with specific characteristics. Display of proteins on the surfaces of phage (1,2), bacteria (3), viruses (4) and prokaryotic polysomes (5,6) has been previously described. These techniques offer *in vitro* routes for the generation of antibodies that bypass using the immune system. In the article cited here, He and Taussig introduce a new *in vitro* eukaryotic method for selection and evolution of antibody combining sites using antibody-ribosome-mRNA (ARM) complexes as the display selection particles. The ARM method uses Promega's TNT[®] T7 Quick Coupled Transcription/Translation System^{*(a)} to generate antibodies *in vitro*. An important feature of this ARM method is the preservation of the link between the peptide or protein of interest and the gene that encodes that molecule.

^{*}U.S. Pat. Nos. 5,324,637, 5,492,817 and 5,665,563, and European Pat. No. 0 566 714 B1, have been issued to Promega Corporation for coupled transcription/translation systems that use RNA polymerases and eukaryotic lysates.

^(a)U.S. Pat. No. 5,552,302 has been issued to Promega Corporation for the methods and composition of human recombinant placental ribonuclease inhibitor (PRI). Inhibitors of Angiogenin, which comprises a segment of human PRI, is the subject of U.S. Pat. Nos. 4,966,964, 5,019,556 and 5,266,687 assigned to the President and Fellows of Harvard College and exclusively licensed to Promega Corporation.

The ARM strategy is based upon two important findings. First, rabbit reticulocyte lysates, which are a cell-free system used for generation of proteins, produce functional single-chain antibodies (7). Second, in cell-free systems, nascent proteins and their corresponding mRNAs form stable ternary polypeptide-ribosome-mRNA complexes in the absence of a stop codon (8,9). These results allow for the production of antibodies in rabbit reticulocyte lysate systems, along with capture of the corresponding mRNA. Using antigen-coupled magnetic beads, ARMs with a specific combining site were selected, and the associated mRNA was used to generate cDNA by one-step reverse transcription and coupled polymerase chain reaction^{**} (RT-PCR) on the ribosome-bound mRNA (Figure 1).

^{**}The PCR process is covered by patents issued and applicable in certain countries. Promega does not encourage or support the unauthorized or unlicensed use of the PCR process. Use of this product is recommended for persons that either have a license to perform PCR or are not required to obtain a license.

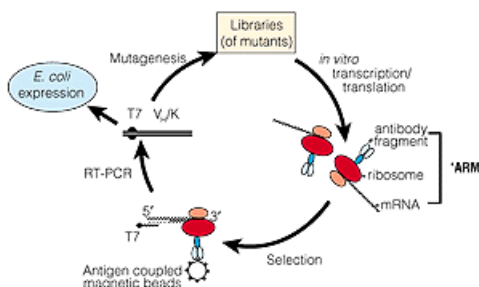


Figure 1. The ARM display cycle. The generation of an ARM library begins with mutagenesis of a V_H/K template, followed by antigen-selection of a specific binding ARM and then recovery of the genetic information by RT-PCR.

ARM DISPLAY AND SELECTION

The ARM library was produced by mutagenesis of the starting template, a single-chain fragment ($V_{H/K}$) from the anti-progesterone antibody DB3. The DB3^R and DB3^{H35} $V_{H/K}$ mutants, which were used to test the principle of ARM selection, were produced from the $V_{H/K}$ template using the 'megaprimer' PCR method (10). In the megaprimer method, two rounds of PCR are conducted: The 'megaprimer' is the product of the first reaction; it is used as one of the primers in the second reaction. The DB3^R mutants bind progesterone strongly ($K_a \sim 10^9/M$) when expressed from *E. coli* but have much lower affinity for testosterone and none detectable for BSA; the DB3^{H35} mutants bind progesterone weakly or not at all.

DNA fragments for *in vitro* transcription/translation were produced from the DB3 library by PCR using a downstream primer lacking a stop codon and an upstream primer that contains the T7 promoter, protein initiation sequence and degenerate, complementary mouse antibody 5' sequence. The DNA fragments were expressed in Promega's TNT[®] T7 Quick Coupled Transcription/Translation System, a rabbit reticulocyte lysate system used for coupled transcription/translation directly from a DNA template. The ARMs produced in the TNT[®] Quick System were subjected to selection by mixing the translation mix with magnetic beads coupled to BSA, progesterone-11alpha-BSA, testosterone-3-BSA or purified rat anti-mouse kappa antibody. After incubation, the beads were collected and washed, and RT-PCR was performed on the mRNA of antigen-selected ARMs using Promega's Access RT-PCR System^{**} with the T7 primer and a nested downstream primer.

In additional cycles of ARM generation, PCR products were added directly to TNT[®] reactions, and the resulting mRNAs were used to generate cDNAs. For each cycle, nested downstream primers, relative to those used in the previous cycle, were used. Thus, the recovered DNA fragment was shorter in each progressive cycle, but the full-length $V_{H/K}$ could be regenerated by recombinational PCR at any cycle.

The specificity of DB3^R for progesterone-11alpha-BSA was demonstrated by RT-PCR of mRNA from ARMs selected with magnetic beads coupled to BSA, progesterone-11alpha-BSA or testosterone-3-BSA. After RT-PCR a DNA fragment was seen only from the ARMs selected with progesterone-11alpha-BSA coupled beads (Figure 2A, lane 2). The binding of the DB3^R to progesterone-11alpha-BSA could be inhibited by the progesterone analogue, progesterone-11alpha-hemisuccinate, confirming the antibody specificity. No bands were obtained with nontranslated DB3^R mRNA or with PCR alone (Figure 2A, lanes 1 and 3). Therefore, the bands produced by RT-PCR are due to mRNA selected by the ARM method and not due to DNA contamination or mRNA carryover.

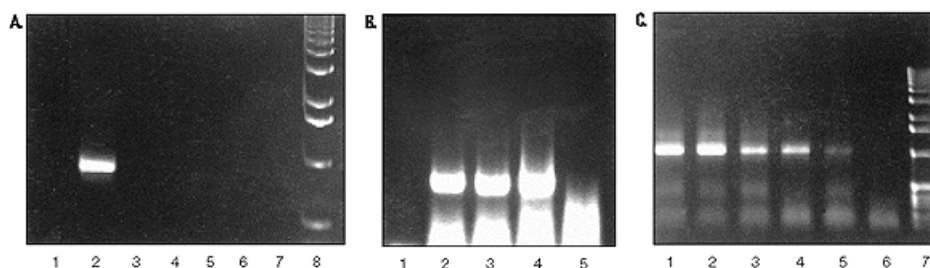


Figure 2. Selection of DB3 mutants. **Panel A:** Specific selection of DB3^R ARMs by progesterone-11alpha-BSA. Lane 1, RT-PCR of nontranslated DB3^R selected by progesterone-11alpha-BSA; lane 2, RT-PCR of DB3^R ARMs selected by progesterone-11alpha-BSA; lane 3, PCR of DB3^R ARMs selected by progesterone-11alpha-BSA; lane 4, RT-PCR of DB3^R ARMs selected by testosterone-3-BSA; lane 5, PCR of DB3^R ARMs selected by testosterone-3-BSA; lane 6, RT-PCR of DB3^R ARMs selected by BSA; lane 7, PCR of DB3^R ARMs selected by BSA. **Panel B:** DB3^{H35} ARM library does not bind progesterone-11alpha-BSA. Lane 1, RT-PCR of solution control; lane 2, RT-PCR of DB3^R ARMs selected by rat anti-kappa-coupled beads; lane 3, RT-PCR of DB3^R ARMs selected by progesterone-11alpha-BSA; lane 4, RT-PCR of DB3^{H35} ARMs selected by rat anti-kappa-coupled beads; lane 5, RT-PCR of DB3^{H35} ARMs selected by progesterone-11alpha-BSA. **Panel C:** Selection of DB3^R with progesterone-11alpha-BSA from ARM libraries containing different ratios of DB3^R and DB3^{H35}. Lane 1, ratio of DB3^R and DB3^{H35} of 1:10; lane 2, 1:10²; lane 3, 1:10³; lane 4, 1:10⁴; lane 5, 1:10⁵; lane 6, DB3^{H35} mutant library alone; lane 7, 1kb DNA marker.

ENRICHMENT OF A SPECIFIC COMBINING SITE

Specific selection of DB3^R versus DB3^{H35} was demonstrated by mixing the two mutants and performing the ARM technique. No DNA band was seen after selection with progesterone-11alpha-BSA in RT-PCR when the DB3^{H35} library was translated as ARMs (Figure 2B, lane 5); however, translation of the DB3^{H35} mutant antibody was demonstrated by selection with rat anti-kappa antibody (Figure 2B, lane 4). When the DB3^R DNA and DB3^{H35} DNA were mixed together in ratios from 1:10 to 1:10⁵ to form an ARM library and selected with progesterone-11alpha-BSA, a $V_{H/K}$ band was recovered in all cases (Figure 2C, lanes 1-5). Before selection, the DB3^R

mutant was not seen by sequencing in 1:10² to 1:10⁵ ratio libraries; however, after one cycle of selection, DB3^R cDNA was the dominant molecule in the 1:10³ ratio library and a major component of the PCR product from the 1:10⁴ and 1:10⁵ ratio libraries, based on codon detection by DNA sequencing. In 1:10⁶ ratio libraries, there was no RT-PCR band visible after one cycle, but after two additional cycles of ARM generation and selection, a V_H/K fragment was recovered (Figure 3).

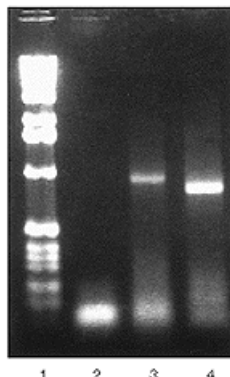


Figure 3. Enrichment of DB3^R from a 1:10⁶ (DB3^R:DB3^{H35}) library by repeated ARM cycles. Selection was performed with progesterone-11alpha-BSA-coupled beads. Lane 1, 1kb DNA marker; lane 2, RT-PCR after first cycle of selection; lane 3, RT-PCR after second cycle; lane 4, RT-PCR after third cycle. The shortening of the bands between cycles 2 and 3 is due to the use of nested primers in progressive rounds of the ARM technique.

SUMMARY

The method of eukaryotic ARM display and selection is a rapid and powerful technique for selection of antibodies and has potential applications for studying receptor and protein:protein interactions. The technique takes approximately eight hours to complete per cycle without the need for mRNA elution and purification and allows for mutations to be introduced in repeated cycles without DNA cloning. The method uses the TNT[®] T7 Quick Coupled Transcription/Translation System for expression of mutants at each cycle, providing the advantages of expression in the rabbit reticulocyte lysate system in a fast and simple format.

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Ordering Information		
Product	Size	Cat.#
TNT [®] T7 Quick Coupled Transcription/Translation System	each	L1170
TNT [®] T7 Quick Coupled Transcription/Translation System Trial Size	each	L1171
Access RT-PCR System	100 reactions	A1250
	500 reactions	A1280
Access RT-PCR Introductory System	20 reactions	A1260

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