Preface

Over the last 20 years, display technologies have become a very powerful way of generating therapeutic lead molecules and specific reagents for increasing our understanding of biology. Despite first being described shortly after phage display, the use of ribosome display and related methods has been much less widespread. Since this is in part due to the complexity of the methods, it is our hope that the availability of this volume of *Methods in Molecular Biology*™ will allow their extended use. The protocols described range from well-established methods that have been used for a decade to generate high affinity antibodies which are in the clinic to methods that are more at their early stages of application, such as display of peptides incorporating noncanonical amino acids.

At the core of ribosome display and related technologies, such as mRNA display, "in vitro virus," and cDNA display, is the in vitro generation of a library of diverse molecules in which a peptide or protein is associated with the nucleic acid encoding it. From the large libraries of over 10^{12} variants that can be made by these purely in vitro methods, molecules with desired properties can be selected by binding to a partner molecule. In all of these methods, the displayed protein is generated by a transcription/translation process which may use semi-purified extracts, such as the *E. coli* S30 system or purified components of the transcription and translation systems.

In ribosome display, a non-covalent ternary complex is formed between the ribosome, mRNA, and the encoded translated protein, and the complex is selected by binding to the target protein. The complex is stabilized by magnesium ions and can be readily disassociated by the addition of EDTA. In mRNA display, a covalent complex is formed between the mRNA and the encoded protein, and this complex is then selected. In both cases, the mRNA recovered from the selected complexes is reverse transcribed to DNA, followed by amplification to generate DNA that can be assembled and used for subsequent rounds of translation and selection. The non-covalent ribosome display method is most widely used and is described in a number of the methods described (Chapters 3–5, 9–12, 14, 15, 17, and 18), including modifications such as the introduction of a protein–RNA interaction to stabilize ternary complexes (Hara et al., Chapter 4) or in situ DNA recovery (He et al., Chapter 5). An mRNA display protocol is described in Chapter 6 by Wang et al. and applications of mRNA display are described by Cotten et al. in Chapter 16.

The covalent coupling in mRNA display is achieved by the use of puromycin, an antibiotic that mimics the aminoacyl moiety of the tRNA, entering the ribosome A site and accepting the nascent polypeptide forming a peptide bond. Puromycin is attached to the mRNA at its 3' end by a short DNA linker. During in vitro translation, the ribosome reaches the RNA–DNA junction, translation pauses, and covalent coupling of the mRNA to the translated polypeptide takes place. In Chapter 8, Ueno and Nemoto describe an adaptation of mRNA display in which the mRNA–protein fusion, derived using puromycin, is converted into a more stable mRNA/cDNA protein fusion, wherein the cDNA is covalently linked to its encoded protein. The application of disulfide shuffling reactions to a library of disulfide-rich peptides displayed in this format is described by Mochizuki and Nemoto in Chapter 13. A further related technique is SNAP display, described by Kaltenbach and Hollfelder in Chapter 7, in which the translated molecule is covalently coupled to the DNA

encoding it following translation in microdroplets in an emulsion. The complex is subsequently selected after breaking the emulsion. Methods involving stable covalent coupling of the nucleic acid and protein allow chemical modification of displayed molecules or their selection under harsher conditions. They may be most applicable to smaller molecules, such as peptides or proteins that refold readily, as in Chapter 13 (Mochizuki and Nemoto), Chapter 16 (Cotten et al.), and Chapter 21 (Hartman et al.).

In his perspective in Chapter 1, Andreas Plueckthun examines critically the features and advantages of ribosome display and related technologies and gives guidance on how to use methods most effectively, for instance when performing selections to enrich for binding molecules with slower off-rates. We recommend that all readers planning to use the methods described in this book read this chapter to help their overall understanding and experimental design.

At the core of all technologies related to ribosome display are efficient, high quality translation extracts. The "workhorse" for most display technology has been the *E. coli* S30 in vitro transcription/translation extract. Its preparation is described in the chapter by Zawada (Chapter 2), and its application is described in several chapters (e.g., Lewis and Lloyd, Chapter 9, for antibodies and Dreier and Plueckthun, Chapter 15, for Darpins). Use of the S30 extract has been particularly successful for the selection of variant molecules from libraries having fixed frameworks that provide consistent translation yields. In our laboratories at MedImmune, we routinely use an in-house, purified translation system reconstituted from *E. coli* components, which we find gives more consistent results in our generation of antibodies by affinity maturation or selection from naive antibody ribosome display libraries. These purified systems have been used for instance in protocols from Ravn (Chapter 12) and Ohashi (Chapter 14). Purified recombinant *E. coli* systems are now available, for example PURE Express™ from New England Biolabs and PURESYSTEM from Wako. Eukaryotic translation systems, such as rabbit reticulocyte lysate, are also available commercially but require adjustments to the protocols compared to the *E. coli* systems. Display and selection protocols for eukaryotic systems are described in the chapters by Douthwaite (Chapter 3) and He et al. (Chapter 5).

Ribosome display has been most widely used for the generation of high affinity antibodies. This has most usually involved site directed or error prone mutagenesis of lead antibody molecules as described by Lewis and Lloyd in Chapter 9. Ribosome display has also been used to select antibodies with desired characteristics from more diverse populations, either sub-cloned from initial phage display selections (affinity maturation of pools, Chapter 10, Groves and Nickson) or directly from naive antibody ribosome display libraries (Ravn, Chapter 12). The intrinsic error rate of the PCR step between rounds of selection allows the generation of increased diversity and the selection of higher affinity variants. Selection from naive RD libraries generates a diverse range of antibodies with a different bias to those from phage display. Thus, although it is easier to generate a diverse range of antibodies from naive libraries by phage display, ribosome display can be a useful supplement or alternative to widen the diversity of initial lead antibodies. For maturation of antibodies for affinity, ribosome display can offer significant advantages compared to methods involving steps in cells, which arises from its increased library diversity, monovalent nature, and disruption of complexes under mild conditions. To give a fuller picture of how ribosome display has fitted into the development of antibodies, two case studies are given. In Chapter 22, Thom and Minter describe the use of ribosome display to generate an antibody, CAT-354, directed against interleukin-13, that is currently in clinical trial. In Chapter 23, Hufton describes the use of ribosome display to affinity mature a humanized antibody directed against RAGE.

Ribosome display has also been used extensively for selection of molecules from novel scaffold libraries, for example ankyrin repeat domains (Darpins) (Chapter 15) and Sac7d scaffolds (Mouratou et al., Chapter 18). Selection from synthetic peptide libraries has also been performed by ribosome and mRNA display, for the purpose of epitope mapping of antibodies and for derivation of peptides interacting with specific proteins (Chapters 14 and 16). Natural sources of variants have been used to generate libraries for selection in ribosome display or mRNA display format and are described here for isolation of calmodulin-binding proteins from cDNA libraries by Cotten et al. (Chapter 16) and for the identification of vaccine genes from pathogenic bacteria by Lei (Chapter 17).

Ribosome display can not only be used to derive molecules with improved affinity or specificity, but also for derivation of molecules with improved stability by taking advantage of the flexible conditions employable in a pure in vitro method. This is described by Buchanan in Chapter 11, where stress conditions can be used during the translation step (such as the presence of dithiothreitol) or during the selection step (such as high temperature) using binding to a hydrophobic interaction chromatography matrix to discriminate between unfolded and more stable, folded molecules.

A significant advantage of in vitro translation methods is the ability to modify the genetic code to allow the incorporation of non-canonical, unnatural amino acids, to give molecules with novel properties, such as cyclic peptides with increased serum, stability. Three chapters in this book illustrate different methods that are being developed to incorporate unnatural amino acids. Reid et al. (Chapter 19) describe the use of flexizyme, a ribozyme that allows the charging of tRNA, with essentially any amino acid. This technology has been applied to the selection of peptide molecules incorporating thioether linkages. Watts and Forster (Chapter 20) describe pure translational display in which codons are reassigned by chemical synthesis of aminoacyl-tRNAs and unnatural amino acids then incorporated into the chain. In contrast, Ma and Hartman (Chapter 21) use the natural broad substrate specificity of aminoacyl tRNA synthetases to charge tRNAs with unnatural amino acids. This is followed by chemical derivatization to generate cyclic peptide libraries.

We hope that this book will be of value to those with general molecular biology or protein engineering experience who wish to select peptides or proteins by display, those with phage display experience who would benefit from the application of ribosome display, and those with some ribosome display experience who would like to expand the range of applications to which they are applying the technology. We would like to thank all the contributors for sharing their knowledge, the Series Editor, John Walker, for his advice and the invitation to edit the book, and our colleagues at MedImmune for their support.

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