Preface

The discovery of catalytic RNAs in the mid-1980s marked the beginning of a new era in RNA biology and an ever-increasing appreciation of the diverse and critical roles played by this fascinating molecule. RNA structure—from primary sequence to complex tertiary folds with a rich landscape of posttranscriptional modifications—is intimately connected to its biological function. As a result, detailed molecular analyses of RNA molecules, their structures, modifications, conformational dynamics, stabilities, and interactions, are important challenges in the contemporary biological and biomedical sciences.

The essential foundation to such research is our ability to produce functional RNA molecules suitable for study in the laboratory. Many distinct approaches exist to overcome the various hurdles one might encounter, from initial construct design and synthesis through to the purification and other procedures that are critical to producing RNA samples that are chemically pure, conformationally homogeneous, and modified as required for the intended application. This book aims to provide a collection of approaches that guide the reader from the inception of a new RNA project to the final sample ready for analysis. These experimental protocols combine established work-horse approaches, developed over several decades in many labs around the world, with some of the latest innovative methods for RNA synthesis and purification.

Working with RNA presents significant challenges unique to this macromolecule; many of the necessary precautions and steps that should be taken when working with RNA are described in the individual chapters of this book. However, for the true RNA novice, the opening chapter of another recent volume of the MiMB series, "Working with RNA" (H. Neilson, in H. Neilsen Ed., *RNA*, Methods in Molecular Biology 703), is an excellent starting point.

Content and Organization of This Volume

The most common approaches to RNA synthesis for biochemical, biophysical, and structural studies are solid-phase automated chemical synthesis and enzymatic in vitro transcription. While each approach has its own set of capabilities, strengths, and limitations, today the former is largely the purview of commercial synthesis services and is therefore not a topic covered in depth in this book (though it is worth noting that many protocols, such as those for conjugation of fluorescent dyes to the RNA termini, are equally applicable, or could be readily adapted, for use with chemically synthesized RNA). Here, the focus will be largely on RNA in vitro transcription and the various ways this method can be adapted to produce highly purified, homogeneous, and, where required, specifically modified or functionalized RNAs for downstream experiments. We begin, however, with a new and quite different *recombinant* approach to RNA production in bacteria.

In the first two chapters, Ponchon and colleagues describe their recently developed "tRNA scaffold" approach for overexpression of defined RNA molecules in *Escherichia coli*. Target RNAs are embedded within a plasmid-encoded tRNA sequence so that they are expressed in a disguised form that the bacteria recognize and modify identically to endogenous tRNAs and do not target for degradation. This allows these engineered chimeric RNAs to accumulate in the cell, often in very large quantities. Chapter 1 introduces the tRNA scaffold system and describes protocols for pilot and large-scale RNA expression experiments and native purification of the chimeric RNAs by liquid chromatography. Next, Chapter 2 develops the approach through protocols that allow the target RNA to be accurately excised from its tRNA disguise using DNA oligonucleotides to direct RNase H cleavage of the chimeric RNA. Together these protocols provide a cost-effective means of producing large quantities of defined RNA sequences with the potential, for example, to directly incorporate isotopic labels simply by culturing the bacteria in medium containing appropriately labeled components.

In vitro transcription remains the predominant approach for RNA synthesis in the laboratory. All that is required is a DNA template encoding the RNA downstream of a suitable promoter sequence, the polymerase—typically T7 RNA polymerase (T7 RNAP)—and each ribonucleotide triphosphate (rNTP) building block. Polymerase and NTPs are both widely available from commercial sources and the enzyme can also be expressed and purified "in-house" (indeed, it is recommended that a T7 RNAP overexpression strain is obtained from one of the several sources cited in this book, particularly where large quantities of RNA are desired). This leaves only the template DNA to be obtained. For very short RNA fragments, chemically synthesized DNA oligonucleotides may be used directly for in vitro transcription. Alternatively, where a DNA template already exists, the promoter may be added via the primers in a polymerase chain reaction (PCR) and the product used directly for transcription or cloned into a suitable plasmid. The later approach has the advantage that new constructs can be sequence-verified, plasmid template can be simply and cheaply propagated in *E. coli* in large quantities, and additional sequences, such as ribozymes for transcript 3′-end processing, can be preloaded onto the plasmid to reduce the complexity of generating subsequent transcription constructs. But what if no template is available and the target is beyond the scope of a standard DNA oligonucleotide synthesis? While custom gene synthesis is an option, albeit an expensive one particularly if many variants of the sequence are needed, a versatile alternative is described in Chapter 3 by Bowman et al. that uses a "recursive PCR" protocol for long double-stranded DNA template synthesis. This ligation-free approach uses a series of tiled, partially complementary oligonucleotides of 50–70 nucleotides (nt) in length and can be simply adapted to produce sequence variants including mutations, insertions, or deletions, by substitution of just one oligonucleotide. The chapter covers all aspects of the approach: initial DNA design considerations including the approaches to create sequence variations, the recursive PCR reaction itself, product purification, and, finally, cloning into a destination vector for RNA in vitro transcription.

The next three chapters set out protocols for RNA in vitro transcription and established approaches to RNA purification by denaturing and native methods. These protocols should each serve as an excellent starting point for new RNA projects. While there is some overlap in the topics discussed in these three chapters, this serves to emphasize an important point: there is no single correct approach to follow—the best option for any given RNA may need to be teased from the details and additional suggestions provided in these chapters. Fortunately, the protocols described here should be readily adaptable to tackle any new RNA synthesis challenge.

Chapter 4 describes the in vitro transcription reaction in detail with basic protocols for plasmid template preparation, RNA synthesis, and denaturing purification. The chapter begins with a robust and scalable protocol for plasmid DNA purification, which removes the need for expensive commercial plasmid purification kits (which contain RNases!) or for CsCl gradients and their attendant large quantities of ethidium bromide. Next, a broadly applicable set of conditions for RNA in vitro transcription is described, followed by a protocol for RNA purification by denaturing polyacrylamide gel electrophoresis. In Chapter 5, Lu and Li continue this theme but with an emphasis on short RNA targets transcribed directly from chemically synthesized DNA oligonucleotides. This chapter also introduces native RNA purification by gel filtration chromatography and its application to studying RNA–protein interactions. Next, Chapter 6 by Booy et al. provides further exploration of the RNA in vitro transcription reaction coupled with detailed protocols for subsequent desalting/buffer exchange of synthesized RNA and its purification using Superdex 75 or 200 gel filtration chromatography columns on an FPLC system. This chapter closes with essential protocols and considerations for assessing RNA purity, concentration, and storage.

The next two chapters describe the use of ribozymes (RNA enzymes) to process RNA in vitro transcripts. Like other polymerases, T7 RNAP often adds additional nontemplated nucleotides to the 3′-end of its RNA transcripts and such heterogeneity can be a significant issue for some downstream applications. Heterogeneity can also occur at the 5′-end but, more commonly, issues here center on sequence limitations imposed by the promoter or difficulties encountered removing the 5′-end triphosphate for labeling or modification. Each of these problems can be circumvented using ribozymes to process the nascent transcript. First, in Chapter 7, Avis et al. describe the use of tandem 5′-hammerhead and 3′-hepatitis delta virus (HDV) *cis*-acting ribozymes. Detailed protocols guide the user from initial generation of new double ribozyme constructs—only the 3′-HDV can be preloaded on the plasmid—for their own target RNAs through to optimizing the balance between overall RNA yield and dual ribozyme cleavage efficiency. Chapter 8, by Szafraniec et al., describes the use of an antigenomic HDV ribozyme for *trans*-processing of target RNAs transcribed with a short (7 nt) sequence appended to their 3′-end. Subsequent precise cleavage of this tag by the *trans*-HDV ribozyme yields target RNAs with homogeneous 3′-ends. Unlike *cis*-acting ribozymes that are produced as a single transcript with the target RNA, here the ribozyme and target RNA are transcribed independently and the latter processed post-synthesis. This approach is therefore likely to be preferable where precious reagents are being used, such as selenium-derived (*see* Chapter 16) or isotopically labeled NTPs (*see* Chapters 17 and 18), since they are not incorporated into co-transcribed ribozyme sequences that are discarded.

In Chapter 9 Cheong et al. provide an alternative approach to transcript 3′-end processing using engineered DNAzymes (DNA enzymes). Protocols are provided detailing sequence specific DNA-affinity purification, transcript cleavage using the DNAzyme and removal of the cleaved tag and DNA to produce the final purified RNA. Affinity-based approaches for RNA purification are further developed in the next two chapters by DiTomasso et al. who describe a new system that exploits immobilization via an optimized λN-GST fusion bound to glutathione-sepharose. In Chapter 10, the expression and purification of the λN-GST fusion protein are described along with key quality control steps before use with RNA samples. Next, Chapter 11 outlines the synthesis of RNAs containing the "ARiBo" tag, comprising the activatable *glmS* ribozyme and λbo*xB* RNA, at their 3′-end. The chapter covers all procedures from template preparation to small- and

large-scale affinity purification and optimization of ribozyme cleavage. This scalable approach could potentially be adapted to be performed in a high-throughput manner, greatly simplifying the process of producing RNA construct libraries for biochemical/functional screening prior to more demanding structural or other biophysical experiments.

The remaining chapters deal with RNA modifications that facilitate specific downstream experiments including chemical or enzymatic probing of RNA structure and dynamics; analysis and quantification of RNA interactions; functional assays where specific modifications such as the 5′-cap, poly(A) tail or methylated nucleotides are critical; and, finally, for high-resolution structural studies by X-ray crystallography or NMR.

Chapter 12 describes a new plasmid system for synthesis of short RNAs within a "structure cassette" for enzymatic and chemical RNA structure probing experiments that use reverse transcription with a labeled DNA primer for readout. 5′- and 3′-flanking hairpin structures and an invariant 3′-end reverse transcription primer sequence are preloaded on the plasmid, thus simplifying the process of inserting new RNA-encoding sequences. Additional steps for analysis and quality control of RNAs transcribed within the structure cassette, which should be performed prior to probing experiments, are also described.

Co-transcriptional incorporation of modified nucleotides, at the RNA 5′-end and internally, are described by Moon and Wilusz in Chapter 13. A procedure is also detailed that allows the precise addition of a poly-A tail to transcribed RNAs. Collectively, these versatile protocols allow preparation of transcripts with a wide variety of modifications to facilitate downstream experiments that address diverse biological questions where such modifications play a critical role in the function or activity of the RNA. Many useful modifications can also be incorporated into the RNA post-synthesis. In Chapter 14, Zearfoss and Ryder describe their approaches for conjugating chemical tags to the 5′- and 3′-ends of RNA molecules (the approaches for 5′-end modification are also applicable to DNA oligonucleotides), including biotin and a wide variety of fluorescent dyes. In addition to detailed protocols for the labeling reactions, this chapter also provides a comprehensive survey of reagents available for RNA 5′- and 3′-end labeling, including the excitation and emission properties for each dye.

Chemical modifications of RNA, such as methylation of base or ribose, are common but their roles are often not well characterized. Essential to systematic and rigorous analyses of RNA modification functions are methods to purify modified RNAs away from unmodified transcripts. Chapter 15 describes a novel approach to isolate site-specifically modified RNAs using the N1-methyl-guanosine (m^1G) tRNA methylation as an example. Using selective DNA oligonucleotide hybridization, where the DNA binds only to unmodified RNA due to the disruption of normal base pairing by the base modification, unmodified transcripts are specifically targeted for degradation by RNase H. This novel approach should prove broadly applicable to other modifications and RNA types where suitably purified modification enzyme is available and Watson–Crick base pairing is disrupted in the modified RNA strand.

High-resolution structural studies of RNAs and RNA–protein complexes, by X-ray crystallography or NMR, are central to our understanding of RNA biology but remain a highly challenging undertaking. Many of the preceding chapters describe approaches that can produce RNA suitable for structural studies. However, for both X-ray crystallography and NMR specific hurdles exist in the process of structure determination that can be overcome through application of the approaches described in the next three chapters.

After obtaining suitably diffracting crystals, the major hurdle remaining in X-ray crystallographic structure determination is likely to be the "Phase Problem." This relates to the fact that in an X-ray diffraction experiment only the intensities of the diffracted X-rays are

measured whereas the phase information is lost. Where a similar structure already exists, this information can be "recovered" by a process known as molecular replacement; otherwise, initial estimates must be experimentally obtained through some form of specific incorporation of electron-rich (or "heavy") atoms into the sample. For proteins, for example, structurally or functionally obligatory ions (e.g., Zn^{2+}) or those incorporated via trial-and-error soaking experiments (e.g., Hg^{2+}) can be used; most commonly today, however, direct protein derivatization is accomplished through selenomethionine addition to the medium for expression in an auxotrophic bacterial strain. Although some parallel approaches exist for nucleic acids, such as incorporation of halogen (bromide or iodide) derivatives of the nucleoside bases during solid-phase chemical synthesis, the options available for RNA are generally more limited. In Chapter 16, Lin and Huang address this deficiency with a description of the synthesis, purification, and use of selenium-derived rNTPs (Se-NTPs). These novel reagents allow the production, via in vitro transcription, of RNA molecules with selenium specifically incorporated into the RNA backbone that can be used for phasing of RNA or RNA–protein complex crystal structures using well-established single- or multiple-wavelength anomalous diffraction experiments.

NMR spectroscopy is a powerful approach for studying the dynamics, interactions, and structures of macromolecules, including RNA. However, multidimensional experiments using isotopically labeled samples are a prerequisite for NMR studies of all but the simplest of systems. Approaches for synthesis of isotopically labeled RNA samples are therefore essential and selective labeling of positions on the ribose or base moieties can greatly simplify NMR spectra and their analysis. Chemical synthesis has the advantage of allowing label incorporation at any desired nucleotide positions within an RNA chain but in vitro transcription provides a significantly more economical route to labeled RNA samples. In Chapter 17, Martino and Conte provide a detailed description of current approaches for producing isotopically labeled rNTPs for this purpose. As described in this chapter, through careful combination of the labeled medium component, the type of expression medium and the bacterial strain, a wide variety of uniform and different selective labeling regimes can be achieved. The protocols of this chapter carefully guide the user through the critical steps of RNA extraction and digestion, purification of 5′-ribonucleoside monophosphates (rNMPs) and their enzymatic phosphorylation to rNTPs for use in in vitro transcription reactions. The approaches described by Martino and Conte provide the user with a mixture of labeled rNTPs but NMR experiments may be further refined if the individual RNA building blocks can be isolated such that labels are incorporated only for selected nucleotides. A new method for preparative isolation of individual labeled rNMPs by ion-pair reversed-phase high performance liquid chromatography (HPLC) is provided by Dagenais and Legault in Chapter 18.

Finally, an approach known as RNA "splint ligation" is described by Kershaw and O'Keefe in Chapter 19. Here, T4 DNA ligase is used to efficiently join two RNA molecules using a DNA oligonucleotide bridge as a guide. Splint ligation can be used to incorporate a short isotopically labeled segment into a larger RNA for structural studies, or to produce RNAs with internal labels or cross-linking groups for analyses of RNA–RNA or RNA–protein interactions. The method thus adds a new dimension of versatility to many of the labeling and modification strategies described in the preceding chapters, as well as a cost-effective means to incorporate specific modifications, available only via chemical synthesis of a short RNA fragment, into RNA molecules of greater size, complexity, and functional utility.

In closing, I would like to express my sincere gratitude to all the authors who have contributed to this book for their outstanding efforts and patience throughout the long process of its preparation. Each deserves great credit for their willingness to invest the time to share these protocols and, in keeping with the tradition of the *MiMB* series, the detailed "tips and tricks" that can be crucial for their successful implementation in the laboratory. I hope that this new volume will live up to this tradition and, in doing so, help bring new investigators with fresh ideas to the fascinating world of RNA.

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