Methods 65 (2014) 333–341

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Production of pure and functional RNA for *in vitro* reconstitution experiments

Franziska Theresia Edelmann [1], Annika Niedner [1], Dierk Niessing *

Institute of Structural Biology, Helmholtz Zentrum München, German Research Center for Environmental Health, Ingolstädter Landstraße 1, 85764 Neuherberg, Germany

ARTICLE INFO

Article history:
Available online 8 September 2013

Keywords:
RNA
RNA quality
RNA purification
RNP reconstitution
RNA folding
RNA–protein interactions

ABSTRACT

Reconstitution of protein complexes has been a valuable tool to test molecular functions and to interpret *in vivo* observations. In recent years, a large number of RNA–protein complexes has been identified to regulate gene expression and to be important for a range of cellular functions. In contrast to protein complexes, *in vitro* analyses of RNA–protein complexes are hampered by the fact that recombinant expression and purification of RNA molecules is more difficult and less well established than for proteins. Here we review the current state of technology available for *in vitro* experiments with RNAs. We outline the possibilities to produce and purify large amounts of homogenous RNA and to perform the required quality controls. RNA-specific problems such as degradation, 5′ and 3′ end heterogeneity, co-existence of different folding states, and prerequisites for reconstituting RNAs with recombinantly expressed proteins are discussed. Additionally a number of techniques for the characterization of direct and indirect RNA–protein interactions are explained.

1. Introduction

In vitro reconstitution experiments have been used since a long time to obtain mechanistic insights into molecular interactions and functions. An obvious advantage is that molecules are studied under controlled conditions, with no unknown factor modulating the function. To date, most reconstitution experiments have been performed with recombinantly expressed proteins or proteins and DNA. Recombinant protein technology is very mature, allowing for standardized and easy purification of protein sample up to 90–95% homogeneity. Also large amounts of DNA can be produced either by total chemical synthesis of up to 200 bases, by PCR-based gene synthesis of up to several thousand bases, or by the classical recombinant bacterial production and subsequent isolation. The combination of these techniques and the large range of DNA endonucleases allows for the production of well-defined DNA fragments of almost any desired yield. Another advantage of both types of biomolecules is their stability. If treated correctly, most proteins and DNA are stable enough to be used in complex reconstitution and functional analysis. Early examples include the landmark studies of the reconstituted transcriptional activity of DNA polymerase I by Arthur Kornberg [1] and of the bacterial DNA gyrase activity by Nicholas Cozzarelli [2].

A considerable number of reconstitution experiments have also been performed with RNA. One of the most impressive achievements is the full reconstitution of an IRES-dependent translation-initiation complex from recombinant factors by Christopher Hellen and Tatyana Pestova [3]. However, working with RNA is more difficult than with DNA. Reasons include the faster degradation of RNAs by nucleases and the resulting greater requirements for pure and controlled work. In addition, the technical options to produce large amounts of RNA are more limited. In contrast to plasmid DNA, it is not easily possible to produce replicating RNA in bacteria and to subsequently cleave out a desired oligonucleotide fragment with sequence-specific endonucleases. A third problem arises from the greater flexibility and folding heterogeneity of RNAs. Therefore, the prevention of degradation and folding heterogeneity as well as quality control of RNAs is more important than for DNA.

2. Production of RNA molecules

2.1. General considerations for working with RNA

As RNAs are prone to degradation by nuclease contaminations specific precautions have to be taken. A main problem is that

Abbreviations: DEPC, diethylpyrocarbonate; EMSA, electrophoretic mobility shift assay; GST, glutathion S-transferase; HPLC, high pressure liquid chromatography; MBP, maltose-binding protein; PAGE, polyacrylamide gel electrophoresis; RNP, ribonucleoprotein particle.

* Corresponding author.
E-mail address: niessing@helmholtz-muenchen.de (D. Niessing).

[1] Both authors contributed equally to this work.

1046-2023
http://dx.doi.org/10.1016/j.ymeth.2013.08.034

RNases are more difficult to remove than DNases because they can not be completely inactivated by denaturation. Thus, autoclaving is not an effective strategy to inactivate RNases. Therefore, the first rule for protecting your RNA is to have a clean and tidy work place. Many labs have dedicated RNA workbenches with separate pipets and filter tips to prevent RNase contaminations. Additionally a separate set of chemicals dedicated for work with RNA is advisable.

All buffers and solutions should be sterile filtered and treated with DEPC to remove contaminations and inactivate RNases [4]. Glassware should be decontaminated by incubation for at least 1 h at $\geqslant$180 °C. If such a treatment is not applicable (e.g. on surfaces and tools) a decontamination with a solution consisting of 3% (v/v) hydrogen peroxide, 100 mM sodium hydroxide and 0.1% (w/v) SDS removes RNases. Plastic ware can be ordered certified as RNase-free.

When choosing an adequate buffer system, the pH and the content of magnesium ions have to be considered. Since RNAs tend to hydrolyze at room temperature at pH 8 or higher, and below pH 5, buffers with extreme pH range should be avoided. Buffers for RNP reconstitution usually contain low divalent ion concentrations, such as 1–2 mM magnesium, to stabilize the fold of RNA [5]. Usage of higher magnesium concentrations can lead to RNA aggregation [6] and in combination with elevated temperatures to degradation of RNA. For RNP-reconstitution experiments the addition of small amounts of commercially available RNase inhibitors might be used to inactivate minor RNase contaminations, which were possibly co-purified with the recombinant proteins.

When stored over a longer time, even trace amounts of RNases are sufficient to impact RNA integrity. Thus, RNAs have to be stored with particular precaution. Keeping the samples frozen in aqueous environment is a good habit but does not necessarily preserve RNA molecules from degradation. A better method for long-time storage of RNAs is to perform a precipitation [7] and store the nucleic acids in ethanol at −20 °C. Immediately before usage the sample is spun down and the precipitate is dissolved in appropriate buffer. However, RNA sometimes forms aggregates upon precipitation and is therefore difficult to dissolve in buffer again [8]. In these cases RNAs should be lyophilized, and subsequently stored at −80 °C. If only stored for subsequent analysis by PAGE, RNA might also be stored under denaturing conditions in formamide loading dye. A comprehensive description of protocols for RNA biochemistry can be found in references [7,9] and to some extent in [4].

2.2. In vitro transcription of RNA

In the last decades, a main technique to produce milligram amounts of RNA has been *in vitro* transcription with RNA polymerase from the T7 bacteriophage [10]. Depending on the size of the desired transcripts, oligonucleotides (up to 150 nt), PCR products (70–1000 nt), or plasmids (up to several thousand nt) can be used as DNA templates for *in vitro* transcription.

It is necessary to include a T7 promoter sequence of at least 18 bases 5′ to the template and, in case of plasmid DNA as template, a restriction site for a DNA endonuclease at the 3′ end. The endonuclease cleavage at the 3′ end of the template is necessary to ensure that the T7 polymerase terminates transcription at a defined site and produces RNA molecules with defined length. However, during the run-off reaction T7 polymerase sometimes terminates a few bases before the 3′ end or adds bases beyond the template length. This heterogeneity in RNA species might interfere with subsequent applications. For instance co-crystallization of RNPs might require removal of inhomogeneities of transcripts by purification procedures like chromatographic approaches or PAGE (see Section 3 below). Furthermore the quality of every *in vitro* transcription should be assessed to ensure homogeneity of transcripts and folding states (Fig. 1A).

When heterogeneity exceeds an acceptable limit, ribozyme technology can be used to trim both ends (Fig. 2A) and to generate a homogenous end product [11,12]. However, a main disadvantage of this method is the greater complexity of the experimental setup. A closely related approach is the use of DNAzymes for the removal of heterogeneous transcript ends [13]. It should be noted that DNAzymes are less frequently used. Very recently a third approach has been reported, in which a so-called CRISPR stem-loop and the Cse3 endoribonuclease were utilized to generate homogenous 5' ends of stem-loops [14].

To date, ribozymes are considered as working horses for trimming the ends of transcripts with heterogeneous lengths. It will be interesting to see if the DNAzymes or the promising CRISPR approach will be able to supersede this standard technique.

Furthermore, these RNA-cleavage approaches can be combined with RNA-based tags, which are bound by specific proteins. Purification of the desired RNA can easily be achieved by using affinity columns that recognize the specific protein. For instance, such RNA-tags can be located 3′ to a ribozyme-cleavage site. After binding the RNA–protein complex to the affinity column, the RNA is eluted by ribozyme or DNAzyme cleavage. RNA-binding proteins such as the SRP protein from *Tetrahymena thermophile* [15], the bacteriophage MS2-coat protein [16,17], or the λN peptide [14,18] have been used for this approach. Depending on the subsequent experimental demand, such elaborate tagging/cleavage strategies might not be required and careful purification procedures (see Section 3) are sufficient to yield homogenous RNAs.

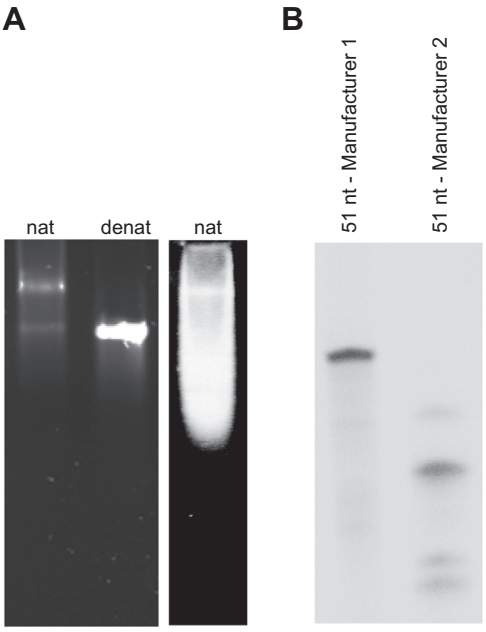

Fig. 1. Gel electrophoresis as a tool for quality control of RNAs. (A) Quality assessment of *in vitro* transcription by using 1% (w/v) agarose gel in 1× TBE buffer. Two *in vitro* transcriptions (left and right) using the same DNA template (2200 nt) from different preparations are depicted. Left gel: In native agarose gel electrophoresis two bands of the *in vitro* transcribed RNA are visible, whereas under denaturing conditions only one band corresponding to the molecular weight of the RNA is present. This indicates the presence of two conformational states or RNA dimerization under native conditions. Right gel: *In vitro* transcription showing a predominant smear and a minor band at the height of the *in vitro* transcribed RNA in the left gel. The poor quality of this *in vitro* transcription might be due to poor template quality or contaminations in the reaction. (B) Assessment of the quality of chemically synthesized RNA by denaturing PAGE. Left lane shows a product of good quality. However, a faint smear is also observed in this sample. The right lane shows the same RNA synthesized by another manufacturer. Here, no full-length RNA was obtained and only a number of byproducts were synthesized. The poor quality had escaped the manufacturers attention. Thus, assessment of the quality of purchased RNA is advised. Shown is a 10% urea PAGE with about 250 ng of RNA loaded in each lane.

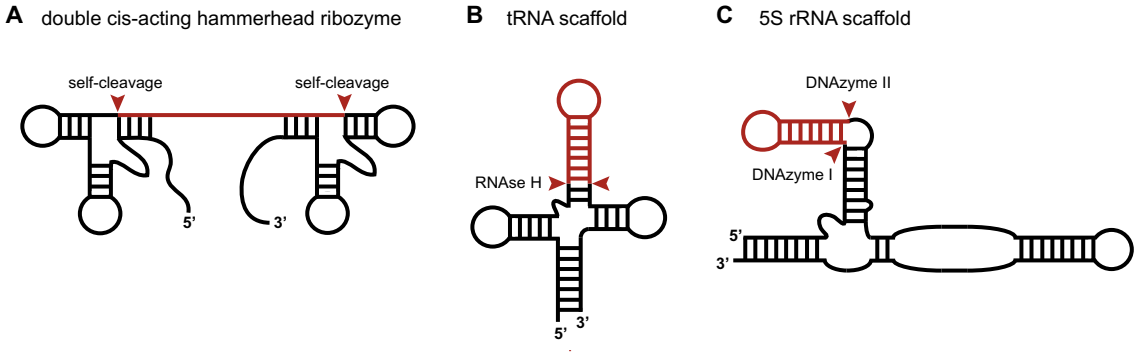

Fig. 2. Schematic drawing of recombinant techniques for the production of homogenous RNA specimen. (A) Production of RNA fragments with defined ends using ribozymes. The RNA fragment of interest (red) is flanked at the 5′ and 3′ site each with a hammerhead ribozyme that cleaves the desired RNA fragment at defined sites (red arrowheads) upon addition of magnesium. (B) The tRNA-scaffold technique takes advantage of the fact that tRNAs are not degraded in bacteria as rapidly as mRNAs. The RNA of interest is fused to the anticodon loop of a tRNA and expressed in bacteria. After purification milligram amounts of RNA can be obtained. The RNA fragment of interest can be cleaved from the scaffold by specific DNA primers and the addition of RNase H, which cleaves DNA-RNA hybrid strands. (C) The 5S rRNA scaffold technique uses a similar strategy as tRNA scaffolds. The RNA of interest is inserted in the helix III-loop C segment of 5S rRNA and expressed in bacteria. Excision of the insert is performed by the addition of biotinylated DNAzymes.

2.3. Total chemical synthesis of RNA

In recent years, the efficiency and accuracy of total chemical synthesis of RNA molecules has increased considerably [19]. It is now possible to synthesize RNA molecules of up to 50–60 bases with acceptable quality. Several commercial suppliers are on the market offering custom RNA synthesis. Since the efficiency of coupling reactions for each nucleotide rarely approaches 100%, for long oligomers incomplete byproducts usually constitute a significant fraction of the synthesized molecule. Depending on the required quality, it is recommended to purify RNA molecules longer than 15–20 bases by PAGE or HPLC. Even if an RNA was purified by the company after synthesis, it is recommended to assess the quality of the RNA product prior using it (for an example see Fig. 1B).

An advantage of a successful RNA synthesis is its high yield of pure RNA suitable for *in vitro* reconstitution experiments and crystallization trials. Another advantage is the possibility to include modified bases and to attach fluorescent dyes or affinity tags. Usually companies offer RNA synthesis in the price range between 5 and 15 € per base, depending on the requested synthesis scale. We noticed great variations between different companies, when comparing the suggested yields and the delivered amounts.

2.4. tRNA-scaffold technology

An interesting alternative for obtaining milligram amounts of RNA is the recently invented so-called tRNA-scaffold technology [20,21]. Similar to what is customary for proteins since decades, this approach allows the expression of large amounts of recombinant RNA in bacteria and their subsequent purification. Ponchon and Dardel took advantage of the fact that tRNAs are not polyadenylated and thus escape the bacterial degradation machinery [22,23]. Expression vectors were generated in which sequences of interest can be cloned into the anticodon loop of a tRNA gene. The result is a gene that expresses a heterologous and stable RNA molecule in bacteria (Fig. 2B). In our hands this technology works well [24], indeed yielding milligram amounts of pure RNA. Although a number of different *Escherichia coli* strains had been suggested to be suitable for this approach [20,21], in our hands only *E. coli* JM101 gave good results [24], indicating that different strains should be tested for expression. Detailed protocols can be found in reference [21].

It should be noted that despite of these advantages, the technology might be limited to RNAs with stem-loop secondary structures. Very recently a strategy has been presented to overcome this limitation (see below) [25]. Depending on the planned experiment, a second disadvantage may be the fact that the heterologous tRNA molecule is fused to the RNA of interest. This potential problem can be overcome by the use of DNA oligonucleotide-guided RNase H cleavage between the tRNA scaffold and RNA fragment of interest (Fig. 2B) [26].

Towards the same goal, the tRNA-scaffold technique was extended by the addition of cis-acing hammerhead ribozymes at the 5′ and 3′ ends of the insert [27]. In this approach the RNA fragment of interest is cleaved from the tRNA upon addition of magnesium ions and subsequently separated by PAGE. A potential problem with this ribozyme-based setup is that a number of RNA products with different lengths are generated that have to be separated from each other. This might be particularly difficult for the purification of RNA products having a length very similar to the cleaved ribozyme.

Very recently, this tRNA-scaffold technology was further improved by the option to co-express the tRNA-fused RNA with an interacting protein [25]. This new feature facilitates the pre-assembly of RNA–protein complexes already before cell lysis and might therefore be a great step forward towards the purification of RNAs that rapidly degrade in absence of its protein binding partner(s) and vice versa. Another approach derived from this methodology is to package fast-degrading RNAs into pseudo-viral particles and thus protect them from degradation during purification. These new features are likely to support the stable expression of single-stranded RNAs. The technology is also suitable for the co-expression with RNA-modifying enzymes and thus production of modified RNAs. A detailed description of these recent advancements is described in reference [25].

An approach related to the tRNA-scaffold strategy is the use of 5S ribosomal RNA instead of tRNA (Fig. 2C). In the position of the helix III-loop C segment of the 5S rRNA of *Vibrio proteoliticum* recombinant RNAs were inserted [28,29]. Instead of using RNase H for removal of heterologous RNA, the authors used biotinylated DNAzymes to cleave the produced RNA from the chimera. It remains to be shown if this approach will be as useful as the tRNA-scaffold technique.

3. RNA purification, quality control, and conformational homogeneity

For all RNAs, the quality should be controlled before initial use. This can either be achieved under native or under denaturing conditions. The first gives an account on the homogeneity of RNA

conformations and folding, and also provides a rough estimate of the molecular weight. The latter allows for an exact assessment of the homogeneity of the molecular weight of the sample.

Regardless of whether *in vitro* transcription, tRNA-scaffold, or ribozyme-cleavage technology is used, the product has to be purified to remove enzymes, nucleic acids and other contaminations. While for smaller RNA species reversed-phase HPLC purifications might be suitable, longer species require purification by native/denaturing PAGE or by chromatography (see Sections 3.2–3.4 below). To obtain highly pure RNAs chromatographic approaches are often used as first choice. They offer a good balance between purity, scalability, and the possibility to concomitantly perform quality control. If quality of the RNA is more important than its yield, PAGE purification is often the method of choice. Small molecular-weight contaminations can be removed by precipitation or extraction (see Section 3.1). In addition to selective binding of RNAs to an anion-exchange column, protein contaminations can be eliminated by a subtractive chromatography step, in which RNAs do not bind to the column (for instance cation-exchange, heparin, or hydrophobic columns).

3.1. RNA purification by precipitation and extraction

A simple and efficient way to remove contaminations such as free nucleotides is the precipitation of RNAs [7]. For instance 2.5–3 volumes of ethanol in presence of 2.5 M ammonium acetate efficiently precipitates RNA without co-precipitating free nucleotides and other small contaminations [4]. A disadvantage of using ammonium acetate is its potential interference with downstream enzymatic reactions such as phosphorylation by the polynucleotide kinase (PNK). As alternative, alcohol precipitation can be performed with sodium acetate (final concentration 0.3 M) or sodium chloride (final concentration 0.2 M), which do not impair enzymatic reactions but have the disadvantage to co-precipitate free nucleotides. When less than 1 μg of RNA or a diluted sample (<1 μg/ml) is used, glycogen (20 μg/ml final concentration) or tRNA (50 μg/ml final concentration) can be added as carrier [30]. These carriers help to precipitate RNA but do usually not interfere with downstream reactions.

RNAs of about 150 nt and longer can also be purified by salting out with lithium chloride (3–4 M final concentration) [31,32]. Since precipitation with this method is selective for larger RNAs, it is well suited to remove nucleotide contaminations as well as smaller RNA species such as tRNAs. However, it should be considered that traces of lithium chloride interfere with downstream applications such as reverse-transcription reactions. When small-molecule contaminations have to be removed rather salt-free, over night RNA extraction with an excess of n-butanol can be performed [9,33]. For the removal of transcription byproducts and other macromolecular contaminations chromatographic approaches are required.

3.2. Purification and quality control by anion-exchange chromatography

Similar to proteins, RNA can be analyzed and purified by chromatographic methods. Since nucleic acids bear a negatively charged phosphate backbone, anion exchange chromatography has become the method of choice for DNA and RNA purification [34,35]. In order to separate products of similar but non-identical length, the choice of column resin is important. Several companies offer pre-packed anion-exchange columns with excellent separation properties, which allow resolving byproducts of only a single base difference in oligonucleotides of dozens of bases in length. Purification by anion-exchange chromatography is usually performed under native conditions (see below), allowing for the separation of RNA byproducts and different folding states. Occasionally also denaturing HPLC purification with a Dionex NucleoPac PA100 anion-exchange column is used [36]. Such purifications under denaturing conditions separate RNAs up to 100 nt with a resolution of about 10 nt [37].

Versatile columns for large-scale, native purification with high separation properties include Mono-Q from GE Healthcare, or BioPro QA from YMC (Table 1) [34,35]. There are also less expensive but still well separating columns such as the Source-Q column (GE Healthcare). Other companies provide anion-exchange columns like DNAPac PA100 or PA200 from Dionex that have been optimized for nucleic-acids purification (Table 1). As an alternative to these strong anion-exchange columns, weak anion-exchange chromatography can be performed for instance with the HighTrap DEAE-sepharose FastFlow column (GE Healthcare) [38]. One advantage of the latter is the use of less salt for elution. Despite that the most successful method has to be determined empirically, in our hands starting with strong anion-exchange chromatography under native conditions usually appears to be the most reasonable approach.

Although these columns can also be used for protein purification, it is a good habit to deny RNA columns the contact with (RNase-contaminated) proteins. In case of RNA degradation during the purification procedure, a good first response is to clean the columns for instance with 0.5 M sodium hydroxide. If this does not solve the problem, potential sources of contamination have to be eliminated with a systematic approach.

3.3. Purification by size-exclusion chromatography

In case a single RNA species is obtained, unwanted byproducts can also be removed by size-exclusion chromatography. For small

Table 1
Chromatographic columns for the analysis and purification of RNA. The assigned purification steps for columns are recommendations based on our own experience. Different purification schemes might alter the stage at which these columns are used.

	Column	Manufacturer	Purification step	Separation quality
Anion exchange	HighTrap DEAE-sepharose FF	GE Healthcare	First/Intermediate	++
	Mono-Q	GE Healthcare	Final	+++
	BioPro QA	YMC	Final	+++
	Source Q	GE Healthcare	First/Intermediate	++
	DNAPac PA100	Dionex	All	+++
	DNAPac PA200	Dionex	All	+++
Size exclusion	NAP-5 (gravity)	GE Healthcare	Crude	+ (removes small molecules)
	NucAway (spin column)	Ambion	Crude	+ (removes small molecules)
	Bio-GelA	BioRad	Final	+++
	TSK-GEL G2000SW & related columns	Tosoh Bioscience	Final	+++
	Superose 6/12 Superdex 75/200	GE Healthcare	Final	+++

contaminations such as nucleotides or protection groups from chemical synthesis, small spin columns are sufficient (Table 1). Larger contaminations such as other RNA species and proteins require more controlled size-exclusion chromatographic approaches [39–42]. For this, a large choice of columns is available with different separation properties and resolutions (Table 1).

3.4. Purification and quality control by native and denaturing PAGE

PAGE is routinely used in most biochemistry labs to analyze the quality of protein expression. PAGE can also be used to assess the quality of RNAs and, equally important, to separate and purify full-length synthesis products from incomplete RNA byproducts. For PAGE separation, it is advisable to supplement each sample with two different dyes. Commonly 0.025% (w/v) of each bromphenol blue and xylene cyanol are mixed with the loading buffer. Since the migration rates of both dyes are known (Table 2), they can be used to estimate the migration of RNAs. There are several commercially available RNA markers that help to estimate the molecular weight. More cost-effective but somewhat less accurate is the use of DNA markers in RNA gels.

Denaturing PAGE is the technique of choice whenever degradation or byproducts of the synthesis have to be identified and removed (Fig. 1) [4]. Urea is a well-established agent to separate DNA double-strands. However, for breaking up longer RNA secondary and tertiary structures often stronger denaturing agents are required. While in some cases an urea loading dye might be harsh enough to unfold a particular RNA, in other cases only the combination of formamide-loading dye and boiling leads to satisfying results.

Native PAGE separates the RNA by size and conformation [43]. We always perform native PAGE after the last purification step to elucidate RNA quality and homogeneity. It is also the recommended purification technique to isolate a distinct RNA species from a heterogeneous folding population. However, if such different folding states are in equilibrium this approach will fail. For the analysis of larger RNAs with hundreds to thousands of bases in length, 0.7–1.0 % agarose gels in 1× TBE buffer should be used.

In general, PAGE purification is unparalleled in its ability to yield extremely high purity [43]. A major obstacle of this technique is the somewhat cumbersome recovery of RNA from the polyacrylamide matrix. A second limitation is the comparably small amount of RNA that can be purified per PAGE [43]. If for instance milligram amounts of RNA have to be obtained, chromatographic approaches are likely to be better suited (see Sections 3.2–3.3). For the purification of a distinct RNA species by PAGE, the first step is to unambiguously identify the correct band. For this, UV-shadowing technique is commonly used because no interfering substances have to be added to the sample [44]. Once identified, single RNA species are isolated by band excision, followed by recovery from the gel matrix by an approach called "crush and soak" and subsequent precipitation of the RNA [9]. An alternative to recover RNA from excised gel fragments is electroelution followed by microdialysis [9,45]. For this, a special gel chamber is required and thus might not be the first choice for a lab starting to work with RNAs. If the RNA is purified under denaturing conditions, controlled refolding is usually recommended (see Section 4 below). It should also be kept in mind that PAGE purification often leaves RNAs contaminated with traces of acrylamide oligomers [39]. However, for most downstream experiments this may not be a major concern.

Table 2
Migration rates of RNA in native and denaturing PAGE. Separation ranges of RNA oligomers are estimates only. In particular in native PAGE, RNA migration might differ from suggested values. Migrations of both dye markers have been adapted from [4].

	Separation range of RNA	Percentage of polyacrylamide	Size of DNA co-migrating with bromphenol blue	Size of DNA co-migrating with xylene cyanol
Native PAGE	10–60	20	12	45
	30–100	10	30	100
	100–500	5	65	260
Denaturing PAGE	5–40	20	8	28
	20–100	8	26	76
	40–150	6	29	106

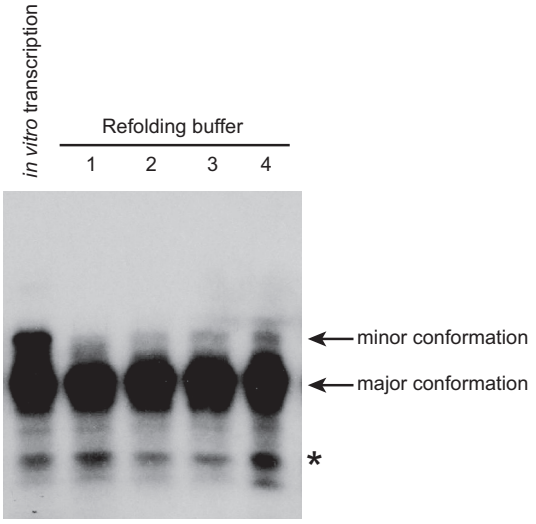

Fig. 3. Assessment of different RNA conformations by native PAGE. Depicted is a 10% native PAGE showing a minor and a major conformation of radioactively-labeled *ASH1*-mRNA fragment E3 118 (left lane). The ratio between the conformations can be changed by refolding the *in vitro* transcribed RNA in different buffers. Lane 1. RNP-reconstitution buffer: 20 mM HEPES pH 7.8, 200 mM sodium chloride, 2 mM magnesium chloride, 2 mM dithiothreitol [24]. Lane 2. Monomer buffer: 50 mM sodium cacodylate pH 7.5, 50 mM potassium chloride, 0.1 mM magnesium chloride [54]. Lane 3. Laemmli-sample buffer: 60 mM Tris–HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue. Lane 4. Water. The RNA was diluted in the indicated buffer and incubated for 2 min at 90 °C before snap cooling on ice. Asterisk marks incomplete RNA byproducts, suggesting the requirement of further purification procedures.

3.5. Quality control by capillary electrophoresis

A major disadvantage of PAGE and most HPLC chromatography systems for quality analysis is the relatively large amounts of required specimen. In mass-spectrometry facilities a range of nanoliter-scale liquid-chromatographic systems (LC–MS) is available as standard equipment for analytical purposes. An alternative is capillary-electrophoresis. Such systems can be used to analyze the homogeneity or degradation of RNAs with excellent resolution. However, as such equipment is not available in most biochemistry labs and since the efficient use of capillary electrophoresis requires experienced users, it may not become a standard tool for quality control of RNAs. The interested reader can find a comprehensive description of capillary electrophoresis with nucleic acids in the following references: [46,47].

4. Conformational homogeneity and RNA dimerization

RNA folding is a dynamic process and sometimes allows more than one stable conformation [48]. RNAs are not expressed as sense and antisense molecules and therefore usually do not fold into fully complementary double-stranded helices. They also lack features like a hydrophobic core that help proteins to fold into globular domains. Instead, the surface of RNAs is fully charged

and can undergo multiple interactions, including a range of non-Watson–Crick base pairs [49] and RNA-specific structural interaction motifs such as kissing loops and A-minor motifs [50,51]. It is therefore not only important to validate the synthesis of RNAs with homogenous length, but also to ensure that an RNA of interest folds into one dominant conformation. A comparably simple way to enforce a uniform conformation is denaturing RNAs by heating and refolding by subsequent cooling [52]. Snap cooling after denaturing is usually an efficient way to enforce the formation of the most stable secondary structures. However, sometimes slowly cooling down an RNA sample gives better results. This and the choice of buffers have to be determined empirically. A good and simple readout is the analysis of RNA folding by native PAGE (see Section 3.4 above, and Fig. 3).

The above-described folding events usually result from intramolecular interactions. However, also inter-molecular RNA interactions and RNA oligomerization do occur. Recent reports on RNA-RNA interactions during the early *Drosophila* development demonstrated that RNA oligomerization does have important functions in an organism [53,54]. Another example is the viral HIV-1 RNA genome. Its dimerization is thought to be required for the production of infectious viral particles [55]. Since it is possible to induce artificial inter-molecular RNA interactions for instance by using wrong buffer conditions, it is important to validate such *in vitro* findings with functional assays.

5. Requirements on proteins for the reconstitution of RNPs

During the purification of RNAs care has to be taken to inactivate or eliminate RNases (see Sections above). For reconstitution experiments, recombinantly produced and potentially RNase contaminated proteins are added to the RNA. Therefore, one should strive for the highest possible purity of the proteins. In our lab, we usually aim to obtain proteins with purities of 95% or higher. We use size-exclusion chromatography with RNase-free buffer as last purification step to remove contaminations and aggregates, and subsequent SDS–PAGE to evaluate protein contaminations.

Since RNA-binding proteins are core factors of every RNP, these factors often contain nucleic-acid contaminations from their bacterial or insect-cell host. In order to perform controlled reconstitution experiments these RNA and DNA contaminations have to be removed. Under no circumstances should RNases be used to remove such contaminations because it is impossible to completely eliminate them afterwards. A well-working alternative is the use of high-salt washes during the protein purification. GST-, MBP- or His_6-tags for affinity purification can be used to bind proteins

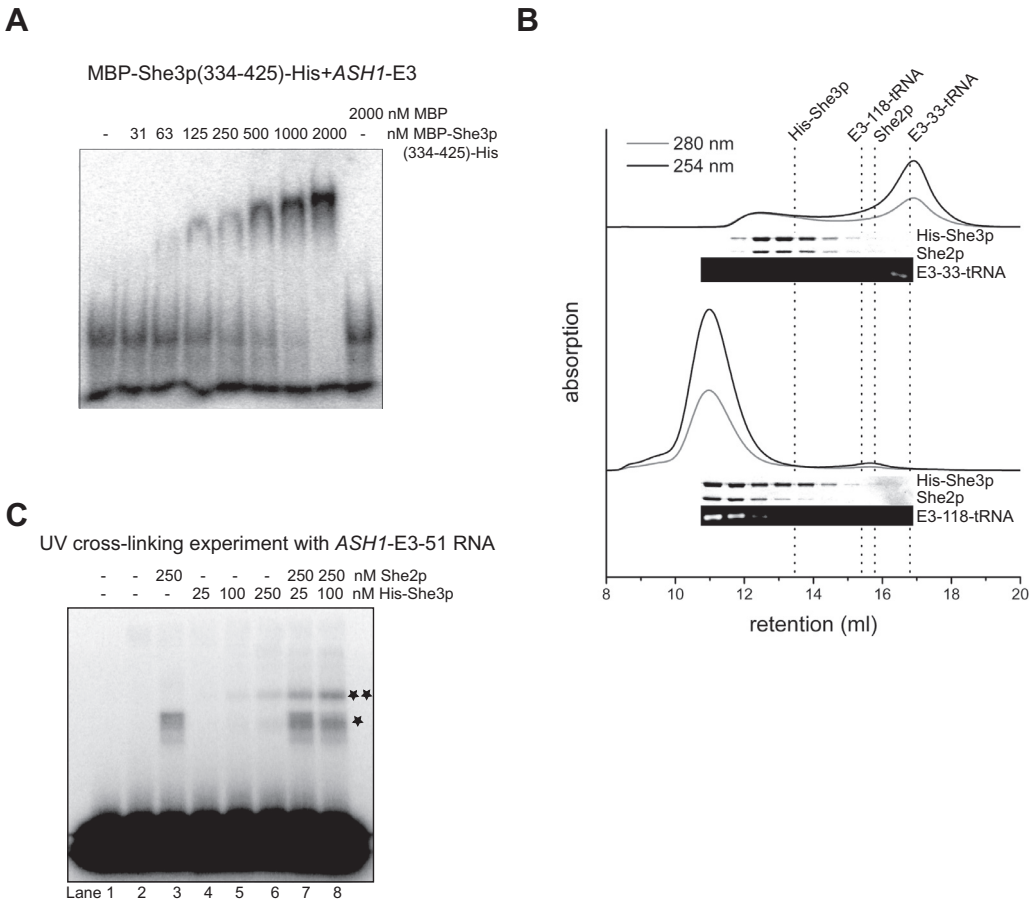

Fig. 4. Different techniques for the detection of RNA–protein interactions. (A) EMSA with a radioactively labeled *ASH1*-mRNA fragment and the RNA-binding domain of She3p. At the bottom, free radioactive nucleotides are visible, which are usually not shown in publications figures. Above these nucleotides, free RNA is observed. Whereas in the very left lane all RNA is unbound, the following lanes towards the right show greater mobility shifts in response to increasing amounts of protein. At 2000 nM concentration, She3p binds all RNA. (B) Size-exclusion chromatography followed by SDS PAGE and agarose-gel electrophoresis for the respective detection of proteins and RNA in different elution fractions. In the upper part an unspecific RNA was added to the co-complex of She2p and She3p. Whereas this RNA did not co-elute with both proteins, the addition of a specifically binding RNA fragment (lower part) resulted in co-elution of all three components, indicating the formation of a stable ternary complex. (C) UV-crosslinking experiments can be used to identify direct protein–RNA interactions. UV light only crosslinks RNA with proteins when they are in direct contact with each other. In the present example crosslinking data confirmed that both proteins of the ternary She2p-She3p-RNA complex (see B) directly contact the RNA. A single asterisk marks the mobility shift of the She2p-RNA crosslink and a double asterisk indicates the She3p-RNA crosslink. Because of the low efficiency of UV crosslinking, no trimeric crosslinks between She2p, She3p, and RNA are observed. Figures A-C were taken from Ref. [24].

Table 3
Summary of methods and techniques mentioned in the text along with respective references for further reading.

	Technique (chapter)	Purpose	Comments	Citations
Production of RNA	*In vitro* transcription (chapter 2.2)	*In vitro* production of RNAs with up to thousands of bases length	Usually requires subsequent purification	[10]
	Total chemical synthesis (chapter 2.3)	Production of RNAs with up to 50–60 bases length	Might require additional purification	[19]
	tRNA scaffold and related techniques (chapter 2.4)	Recombinant expression of mg amounts of RNA fused to an RNA scaffold	Requires subsequent purification	[20,21,25–27]
	End-cleavage by ribozymes, DNAzymes, or protein enzymes (chapter 2.2)	Cleavage at 5′ or 3′ ends to produce homogenous transcripts	Higher experimental effort, might require additional purification	[11–14,16–18]
Purification and quality control of RNA	Precipitation and extraction (chapter 3.1)	Crude purification by removing small molecule contaminations such as free nucleotides, protection groups, or salt	Large and small scale purification, might leave traces of precipitant	[7,9,30–33]
	Anion-exchange chromatography (chapter 3.2)	Isolation of RNA with defined size (native and denaturing purification) and homogenous folding state (native purification). Quality control	Often the most efficient large-scale purification strategy	[34–38]
	Size-exclusion chromatography (SEC) (chapter 3.3)	Isolation of RNA with defined size and homogenous folding state. Quality control of folded RNA	Alternative to anion-exchange chromatography for large-scale purification	[39–42]
	Polyacrylamide gel electrophoresis (PAGE) (chapter 3.4)	Isolation of RNA with defined size. Quality control of unfolded or folded RNA	Unparalleled resolution for purification and quality control. Amount of RNA is limited	[43]
	Capillary electrophoresis (CE) (chapter 3.5)	Quality control of RNAs, interaction studies with proteins	High resolution, only small amounts of RNA required. CE equipment required	[46,47]
	Refolding of RNA (chapter 4)	Production of homogenous, functional folding state of a given RNA	Several buffers and temperature conditions should be tested	[52]
Protein–RNA interaction studies	Electrophoretic mobility shift assay (EMSA) (chapter 6)	Visualization of interaction, estimation of equilibrium dissociation constants and stoichiometries	Biochemical standard technique for RNA–protein interactions	[9,59,60]
	Filter binding assay (chapter 6)	More precise determination of equilibrium dissociation constants and stoichiometries than by EMSA	Biochemical alternative to EMSA. Also detects more transient interactions	[9,61,62]
	Isothermal titration calorimetry (ITC) (chapter 6)	Determination of equilibrium dissociation constants, stoichiometries, enthalpy and entropy	Precise technique, requires large amounts of sample	[63]
	Surface plasmon resonance (SPR) (chapter 6)	Determination of equilibrium dissociation constants, on- and off-rates. In some cases stoichiometries can be determined	Precise technique, requires less sample than ITC	[64,65]
	Analytical ultracentrifugation (AUC) (chapter 6)	Determination of equilibrium dissociation constants, molecular weight, and sedimentation rates	Precise technique, sample requirement depends on type of AUC equipment	[66]
	Microscale thermophoresis (MST) (chapter 6)	Determination of equilibrium dissociation constants. Stoichiometries and dissociation constants might be determined	Precise, relatively new technique, requiring comparably low amounts of sample	[67,68]
	Fluorescence spectroscopy/ anisotropy (chapter 6)	Determination of equilibrium dissociation constants	Precise technique, requires comparably low amounts of sample	[69]
	Static light scattering (SLS) (chapter 6)	Determination of molecular weight	Precise technique, requires larger amounts of sample	[70]

of interest to columns for extensive washing with 500 mM sodium chloride [56,57]. In extreme cases, an increase up to 2 M sodium chloride or the use of high concentrations of phosphate buffer may be tried. For DNA-binding proteins it has been reported that washing steps with 2–3 M urea effectively remove nucleic-acid contaminations without denaturing the protein of interest [58]. This approach can be readily adapted to RNA-binding proteins.

Regardless of the method used, a final quality control step should be included to ensure successful removal of nucleic-acid contaminations. The ratio of the absorbance at 280 nm and 260 nm wavelength should be measured at the end of the purification of every protein. This has proven to be a fast and efficient method for quality control. Values for "pure" proteins range between OD_{260}/OD_{280}: 0.5–0.6, whereas nucleic-acid contaminated proteins have higher values.

In case of RNA degradation during the assembly of RNA–protein complexes, RNases might have been co-purified as contaminations with proteins. The presence of RNases can be tested by commercial kits, such as RNaseAlert (Ambion). In case RNase activity is observed (either via RNA degradation during complex assembly or by a kit), the protein purification strategy should be revised and optimized. In addition or as alternative, it can be attempted to inhibit RNases with a mixture of RNAse inhibitors (e.g. SUPERase In from Life technologies; RiboLock from Fermentas; RiboGuard from Biozym).

6. Analysis of RNA–protein interactions

There are several methods to detect RNA–protein interactions and determine binding affinities, stoichiometries and even the molecular weight as well as on- and off-rates of complexes. A comprehensive description of most available biochemical techniques can be found in reference [9]. One of the first experiments many labs perform to detect RNA–protein interactions is an EMSA [9,59,60]. In this experiment a constant, low amount of radioactively or fluorescence-labeled RNA is incubated with increasing amounts of an interacting protein. To exclude unspecific RNA binding to the protein an unrelated competitor RNA (e.g. tRNA, or 5S rRNA) is included in the buffer. In the subsequent native PAGE the RNA–protein complex migrates slower than the free RNA and thus leads to a shift in migrating band height (Fig. 4A). Besides the detection of such interactions, this technique allows the determination of binding constants and stoichiometries of RNP components [59]. If a complex with more than one protein is probed by EMSA, the presence of each protein in the shifted complex can be

confirmed by the addition of specific antibodies [9]. Since the binding of an antibody further increases the molecular weight of the shifted complex, a so-called supershift will be observed in case the protein is present in the RNP.

Since in EMSAs the separation of free RNA from RNPs takes several minutes to hours, transient protein–RNA interactions may not be detected by this method. In such cases, alternative techniques such as filter-binding assays [9,61,62] or biophysical approaches like isothermal titration calorimetry [63], surface-plasmon resonance [64,65], analytical ultracentrifugation [66], microscale thermophoresis [67,68], or fluorescence spectroscopy/anisotropy [69] can be applied. Whereas filter-binding assays can be performed to obtain equilibrium dissociation constants of RNPs in standard-laboratory settings, the biophysical methods require special equipment and experienced scientists. However, these methods offer a multitude of information such as molecular weight of complexes, their stoichiometries, on- and off-rates, entropy, and enthalpy (Table 3). The choice of biophysical technique depends on the sample properties as well as on the desired information to be obtained.

Another interesting possibility to assess protein–RNA interactions is the separation of RNPs from unbound molecules by size-exclusion chromatography. Since the RNP elutes at higher molecular weight than its individual components, it can be easily identified. A great advantage here is that the elution fractions of the RNP can be analyzed by PAGE and all participating components be identified (Fig. 4B). Inserting a static light scattering detector behind the column further increases the amount of information derived from size-exclusion chromatography experiments. The combined information of both analysis methods allows the exact determination of molecular weights of the eluted RNPs [70].

For the study of multi-protein RNPs it might also be of interest to know which protein directly contacts the RNA and which ones are associated only indirectly with the RNA. To address such questions an RNA–protein crosslinking approach might be used [71]. Since UV light induces the formation of covalent bonds between the RNA and protein only if they directly contact each other, this approach can be used to provide evidence for such direct interactions. Their presence can be detected as mobility shift of radioactively labeled RNA in a denaturing SDS–PAGE (Fig. 4C). UV crosslinking has recently been combined with mass spectrometry after trypsin and RNase digestion [72]. This powerful technique allows the identification of peptides within the protein that make direct contacts to RNA.

7. Conclusions

Although RNA biochemistry will remain more difficult than work with DNA or proteins, it is obvious that a number of technological advances helped to make this field more approachable. It is therefore conceivable that remaining difficulties, such as the lacking accuracy of RNA structure predictions or a comprehensive understanding of the functional roles of RNA modifications, will improve in future and further help to open up the field.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (DFG) and the Bayerisch-Französisches Hochschulzentrum (BFHZ).

References

[1] A. Kornberg, Science 131 (1960) 1503–1508.
[2] N.P. Higgins, C.L. Peebles, A. Sugino, N.R. Cozzarelli, Proc. Natl. Acad. Sci. USA 75 (1978) 1773–1777.
[3] A.V. Pisarev, A. Unbehaun, C.U. Hellen, T.V. Pestova, Methods Enzymol. 430 (2007) 147–177.
[4] J. Sambrook, D.W. Russell, Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 2001.
[5] A. Graebsch, S. Roche, D. Niessing, Proc. Natl. Acad. Sci. USA 106 (2009) 18521–18526.
[6] D.P. Bartel, J.W. Szostak, Science 261 (1993) 1411–1418.
[7] D.M. Wallace, Methods Enzymol. 152 (1987) 41–48.
[8] O.C. Uhlenbeck, RNA 1 (1995) 4–6.
[9] C.W.J. Smith, RNA: Protein Interactions – A Practical Approach, Oxford University Press, Oxford, 1998.
[10] J.F. Milligan, D.R. Groebe, G.W. Witherell, O.C. Uhlenbeck, Nucleic Acids Res. 15 (1987) 8783–8798.
[11] S.R. Price, N. Ito, C. Oubridge, J.M. Avis, K. Nagai, J. Mol. Biol. 249 (1995) 398–408.
[12] A.R. Ferre-D'Amare, J.A. Doudna, Nucleic Acids Res. 24 (1996) 977–978.
[13] H.K. Cheong, E. Hwang, C. Cheong, Methods Mol. Biol. 941 (2012) 113–121.
[14] A. Salvail-Lacoste, G. Di Tomasso, B.L. Piette, P. Legault, RNA 19 (2013) 1003–1014.
[15] J.S. Kieft, R.T. Batey, RNA 10 (2004) 988–995.
[16] R.T. Batey, J.S. Kieft, RNA 13 (2007) 1384–1389.
[17] A.Y. Keel, L.E. Easton, P.J. Lukavsky, J.S. Kieft, Methods Enzymol. 469 (2009) 3–25.
[18] G. Di Tomasso, P. Lampron, P. Dagenais, J.G. Omichinski, P. Legault, Nucleic Acids Res. 39 (2011) e18.
[19] W.S. Marshall, R.J. Kaiser, Curr. Opin. Chem. Biol. 8 (2004) 222–229.
[20] L. Ponchon, F. Dardel, Nat. Methods 4 (2007) 571–576.
[21] L. Ponchon, G. Beauvais, S. Nonin-Lecomte, F. Dardel, Nat. Protoc. 4 (2009) 947–959.
[22] M.P. Deutscher, Nucleic Acids Res. 34 (2006) 659–666.
[23] Z. Li, S. Reimers, S. Pandit, M.P. Deutscher, EMBO J. 21 (2002) 1132–1138.
[24] M. Müller, R.G. Heym, A. Mayer, K. Kramer, M. Schmid, P. Cramer, H. Urlaub, R.P. Jansen, D. Niessing, PLoS Biol. 9 (2011) e1000611.
[25] L. Ponchon, M. Catala, B. Seijo, M. El Khouri, F. Dardel, S. Nonin-Lecomte, C. Tisne, Nucleic Acids Res. 41 (2013) e150.
[26] L. Ponchon, G. Beauvais, S. Nonin-Lecomte, F. Dardel, Methods Mol. Biol. 941 (2012) 9–18.
[27] F.H. Nelissen, E.H. Leunissen, L. van de Laar, M. Tessari, H.A. Heus, S.S. Wijmenga, Nucleic Acids Res. 40 (2012) e102.
[28] Y. Liu, V.G. Stepanov, U. Strych, R.C. Willson, G.W. Jackson, G.E. Fox, BMC Biotechnol. 10 (2010) 85.
[29] X. Zhang, A.S. Potty, G.W. Jackson, V. Stepanov, A. Tang, Y. Liu, K. Kourentzi, U. Strych, G.E. Fox, R.C. Willson, JMR 22 (2009) 154–161.
[30] P.N. Hengen, Trends Biochem. Sci. 21 (1996) 224–225.
[31] J.J. Barlow, A.P. Mathias, R. Williamson, D.B. Gammack, Biochem. Biophys. Res. Commun. 13 (1963) 61–66.
[32] G. Cathala, J.F. Savouret, B. Mendez, B.L. West, M. Karin, J.A. Martial, J.D. Baxter, DNA 2 (1983) 329–335.
[33] G. Cathala, C. Brunel, Nucleic Acids Res. 18 (1990) 201.
[34] M.J. Jahn, D. Jahn, A.M. Kumar, D. Soll, Nucleic Acids Res. 19 (1991) 2786.
[35] J. Koubek, K.F. Lin, Y.R. Chen, R.P. Cheng, J.J. Huang, RNA (2013), http://dx.doi.org/10.1261/rna.038117.113 [epub ahead of print].
[36] T.P. Shields, E. Mollova, L. Ste Marie, M.R. Hansen, A. Pardi, RNA 5 (1999) 1259–1267.
[37] O. Duss, C. Maris, C. von Schroetter, F.H. Allain, Nucleic Acids Res. 38 (2010) e188.
[38] L.E. Easton, Y. Shibata, P.J. Lukavsky, RNA 16 (2010) 647–653.
[39] P.J. Lukavsky, J.D. Puglisi, RNA 10 (2004) 889–893.
[40] S.A. McKenna, I. Kim, E.V. Puglisi, D.A. Lindhout, C.E. Aitken, R.A. Marshall, J.D. Puglisi, Nat. Protoc. 2 (2007) 3270–3277.
[41] I. Kim, S.A. McKenna, E. Viani Puglisi, J.D. Puglisi, RNA 13 (2007) 289–294.
[42] J.C. Paillart, E. Skripkin, B. Ehresmann, C. Ehresmann, R. Marquet, J. Biol. Chem. 277 (2002) 5995–6004.
[43] S.A. Woodson, E. Koculi, in: J.N. Abelson, M.I. Simon (Eds.), Biophysical, Chemical, and Functional Probes of RNA Structure, Interactions and Folding: Part B, Elsevier, 2009, pp. 190–208.
[44] S.M. Hassur, H.W. Whitlock Jr., Anal. Biochem. 59 (1974) 162–164.
[45] A.L. Zarzosa-Alvarez, A. Sandoval-Cabrera, A.L. Torres-Huerta, R.M. Bermudez-Cruz, JoVE 43 (2010) e2136.
[46] K.R. Mitchelson, J. Cheng, Capillary Electrophoresis of Nucleic Acids, Humana Press, Totowa, New Jersey, 2001.
[47] K.R. Mitchelson, J. Cheng, Capillary Electrophoresis of Nucleic Acids, Humana Press, Totowa, New Jersey, 2010.
[48] N.G. Walter, Methods 49 (2009) 85–86.
[49] N.B. Leontis, E. Westhof, Curr. Opin. Struct. Biol. 13 (2003) 300–308.
[50] E.A. Doherty, R.T. Batey, B. Masquida, J.A. Doudna, Nat. Struct. Biol. 8 (2001) 339–343.
[51] N.B. Leontis, A. Lescoute, E. Westhof, Curr. Opin. Struct. Biol. 16 (2006) 279–287.
[52] H. Weidner, D.M. Crothers, Nucleic Acids Res. 4 (1977) 3401–3414.
[53] E. Hartswood, J. Brodie, G. Vendra, I. Davis, D.J. Finnegan, RNA 18 (2012) 729–737.
[54] H. Jambor, C. Brunel, A. Ephrussi, RNA 17 (2011) 2049–2057.
[55] M.D. Moore, W.S. Hu, AIDS Rev. 11 (2009) 91–102.
[56] A. Graebsch, S. Roche, D. Kostrewa, J. Soding, D. Niessing, PLoS One 5 (2010) e13402.
[57] M. Müller, K. Richter, A. Heuck, E. Kremmer, J. Buchner, R.P. Jansen, D. Niessing, RNA 15 (2009) 2002–2012.

[58] S. Tan, Y. Hunziker, L. Pellegrini, T.J. Richmond, J. Mol. Biol. 297 (2000) 947–959.
[59] S.P. Ryder, M.I. Recht, J.R. Williamson, Methods Mol. Biol. 488 (2008) 99–115.
[60] K.T. Gagnon, E.S. Maxwell, Methods Mol. Biol. 703 (2011) 275–291.
[61] D. Niessing, S. Hüttelmaier, D. Zenklusen, R.H. Singer, S.K. Burley, Cell 119 (2004) 491–502.
[62] I. Wong, T.M. Lohman, Proc. Natl. Acad. Sci. USA 90 (1993) 5428–5432.
[63] A.L. Feig, Methods Enzymol. 468 (2009) 409–422.
[64] Y. Yang, Q. Wang, D. Guo, Mol. Biotechnol. 40 (2008) 87–93.
[65] M. Ritzefeld, N. Sewald, J. Amino Acids 2012 (2012) 816032.
[66] C.J. Wong, K. Launer-Felty, J.L. Cole, Methods Enzymol. 488 (2011) 59–79.
[67] K. Zillner, M. Jerabek-Willemsen, S. Duhr, D. Braun, G. Langst, P. Baaske, Methods Mol. Biol. 815 (2012) 241–252.
[68] P. Baaske, C.J. Wienken, P. Reineck, S. Duhr, D. Braun, Angew. Chem. Int. Ed. Engl. 49 (2010) 2238–2241.
[69] R. Favicchio, A.I. Dragan, G.G. Kneale, C.M. Read, Methods Mol. Biol. 543 (2009) 589–611.
[70] R.P. Rambo, J.A. Tainer, RNA 16 (2010) 638–646.
[71] R. Reed, M.D. Chiara, Methods 18 (1999) 3–12.
[72] C. Schmidt, K. Kramer, H. Urlaub, J. Proteomics 75 (2012) 3478–3494.