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In vitro analysis of RNA interference in *Drosophila melanogaster*

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Abstract

Double-stranded RNA (dsRNA) triggers the destruction of mRNA sharing sequence with the dsRNA, a phenomenon termed RNA interference (RNAi). The dsRNA is converted by endonucleolytic cleavage into 21- to 23-nt small interfering RNAs (siRNAs), which direct a multiprotein complex, the RNA-induced silencing complex to cleave RNA complementary to the siRNA. RNAi can be recapitulated in vitro in lysates of syncytial blastoderm Drosophila embryos. These lysates reproduce all of the known steps in the RNAi pathway in flies and mammals. Here we explain how to prepare and use *Drosophila* embryo lysates to dissect the mechanism of RNAi.
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1. Introduction

In animals, double-stranded RNA (dsRNA) induces potent and specific gene silencing by a phenomenon known as RNA interference (RNAi). When the complete sequence of an animal's genome is known, RNAi can be used to induce a loss-of-function phenotype for any gene, without recourse to labor-intensive, traditional genetic methods. RNAi can also be used to verify or complement existing genetic mutations. The rapid adoption of RNAi as a surrogate genetics tool stems from both its potency and its specificity [1–5]. Despite apparent differences in mechanism, the RNAi pathway is widely conserved, and RNAi has been demonstrated in such widely diverged eukaryotes as *Neurospora*, trypanosomes, and mammals [1,3,6–15]. However, while many researchers routinely use RNAi as a tool, the actual mechanism by which it works remains incompletely understood.

RNAi is triggered by the introduction of dsRNA, typically longer than 200 bp [4]. The dsRNA is converted into 21- to 23-nt RNA duplexes, termed small interfering RNAs (siRNAs), by ATP-dependent endonucleolytic cleavage [16,17]. siRNAs are the specificity determinants of the RNAi pathway [17–19]. The RNase III enzyme, Dicer, mediates this initial cleavage event [20], yielding double-stranded siRNAs with 2-nucleotide 3′ overhangs, 5′-terminal phosphates, and 3′ hydroxyls [17,21]. The initial processing of dsRNA can be bypassed by synthetic oligonucleotides bearing the same structure as Dicer-produced siRNAs [3,17,18]. In *Drosophila* these siRNAs are ultimately incorporated into in an ~550-kDa ribonucleoprotein complex (RNP) that mediates target cleavage [22,23], although a smaller complex with target-cleaving activity has also been identified [21]. This smaller complex likely corresponds to a minimal active RNA-induced silencing complex and contains single-stranded siRNA [21]. In addition, in vitro experiments have identified an ~360-kDa RNP that contains double-stranded siRNA but is not yet competent for mediating target cleavage [21]. Although the 360-kDa complex is inactive, its formation seems to be a prerequisite for downstream events in the RNAi pathway.

2. Preparation of translationally active *Drosophila* embryo lysates that recapitulate RNAi in vitro

A cell-free system for studying RNAi in vitro was first described by Tuschl and co-workers in 1999 [24]. This

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lysate, which is prepared from early *Drosophila melanogaster* syncytial blastoderm embryos, efficiently translates ^{7m}G-capped exogenous mRNAs and recapitulates in vitro RNAi triggered by both long dsRNA and synthetic or native siRNAs. The use of syncytial blastoderm embryos allows the identification of 5′ cleavage products during the RNAi reaction, because ^{7m}G-capped mRNA lacking a poly(A) tail is stable in *Drosophila* embryos prior to the midblastula transition, which occurs at 25 °C about 2 h after egg-laying.

Rearing large populations of fruit flies and the preparation of yeast paste and apple juice agar have been described elsewhere [25]. Zero- to 2-h-old fly embryos are collected on yeasted apple juice agar from population cages maintained at 25 °C and 70–80% relative humidity. Embryos are harvested using a wet, $1\frac{1}{2}$-in. (~3.75-cm), synthetic-bristle paint brush and washed into a set of stacked nylon-mesh sieves. The sieve is designed so that flies, larvae, and debris are retained on the upper sieve (500-μm nylon mesh; McMaster–Carr) and embryos are captured by the lower (70-μm nylon mesh; McMaster–Carr). The upper sieve is removed, and the embryos in the lower sieve are dechorionated by immersion in 50% (v/v) bleach, prepared immediately before use, for 4–5 min, washed extensively with cold water to remove all traces of bleach, blotted dry with paper tissues (Kimwipes; Kimberly–Clark) from beneath the sieve, and transferred to a chilled, tared Potter–Elvehjem tissue grinder (Kontes). Embryos are lysed in 1 ml lysis buffer (see Appendix A) containing 5 mM DTT and 1 mg/ml complete "mini" protease inhibitor tablets (Roche) per gram of damp embryos. The lysate is then clarified by centrifugation at 14,000×g for 25 min at 4 °C. The supernatant is aliquoted into chilled microcentrifuge tubes, flash frozen in liquid nitrogen and stored at −80 °C. Lysate typically retains activity for at least 6 months.

3. Preparation of double-stranded RNA

3.1. PCR strategy for producing transcription templates

We typically use PCR to generate DNA templates for the transcription of RNA. The 5′ PCR primer is designed to include at its 5′ end a T7 promoter sequence, although T3 or SP6 could, in principle, be used instead. The T7 promoter is followed by 15–20 bases of sequence from the desired transcript RNA. In addition to the final transcribed G of the T7 promoter, one or two additional guanosines should be included immediately after the promoter sequence to ensure efficient transcription. A 20-nt, 3′ PCR primer is designed to be complementary to the target sequence 200–1000 bases downstream of the 5′ sequences. Two separate primer pairs are used to generate templates for the transcription of sense and antisense RNA strands. We typically pool 5 to 10 100-μl PCR reaction for each DNA template. The PCR products are precipitated directly from the reaction by the addition of 2.5 volumes of absolute ethanol and 1/10 volume of 3 M sodium acetate. The precipitate is recovered by centrifugation, washed in 70% ethanol, dried under vacuum, and redissolved in 1× T7 transcription buffer to give a stock of DNA template 10-fold more concentrated than the original PCR reaction.

3.2. In vitro transcription with T7 RNA polymerase

To generate uniformly ^{32}P-radiolabeled RNA, we typically assemble a 50-μl reaction containing 5-μl 10× T7 buffer (see Appendix A), 0.5 μl 1 M DTT, 2.5 μl 10 mM ATP, 2.5 μl 100 mM UTP, 2.5 μl 100 mM CTP, 4 μl 100 mM GTP, 25 μl [α-^{32}P]ATP (40 mCi/ml, 3000 Ci/mmol, available from ICN as a custom order), 2.5 μl PCR template (as described above), 1 μl RNasin (Promega), 2.5 μl H_2O, and 2 μl recombinant, histidine-tagged T7 polymerase. For the production of nonradioactive RNA, we typically prepare a 100-μl reaction containing 10 μl 10× T7 transcription buffer, 3 μl PCR template (described above), 0.5 μl 1 M DTT, 5 μl 100 mM ATP, 5 μl 100 mM CTP, 5 μl 100 mM UTP, 8 μl 100 mM GTP, 61.5 μl H_2O, and 2 μl T7 polymerase.

3.3. Gel purification of in vitro-transcribed RNA

Gel purification is an essential step to eliminate the DNA template and ensure full-length RNA. After incubation at 37 °C for 2 h, reactions are stopped by adding an equal volume of 8 M urea stop mix (see Appendix A), heated at 95 °C for 2 min, and then purified by electrophoresis on a preparative denaturing acrylamide gel. RNA is detected by UV shadowing or phosphorimagery. The RNA-containing portion of the gel is excised and transcripts are recovered by soaking the gel slice in 1–2 ml of 2× PK buffer (see Appendix A) tumbling overnight at room temperature. The eluate is then phenol extracted and the RNA precipitated with 2.5 volumes of absolute ethanol. The RNA precipitate is resuspended in pure, sterile H_2O. Diethyl pyrocarbonate (DEPC)-treated water should not be used as it is impossible to get rid of all the DEPC, which can inhibit RNAi and translation.

After purification, the concentration of each transcript is determined. We typically measure the absorbance at 260 nm of 2 μl RNA stock diluted into 198 μl H_2O. Concentration is calculated by the equation

$$C = (A_{260} \times \text{dilution factor}) / (10,313 \times \text{number of nucleotides}),$$

where C is in molar and the dilution factor in this instance is 100.

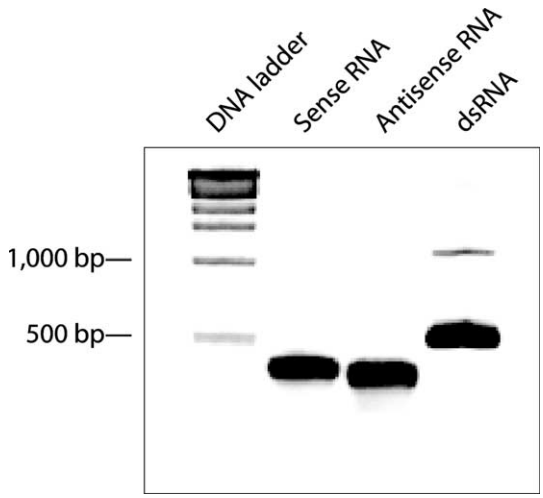

Fig. 1. Native gel analysis of dsRNA. 1 μM stocks of in vitro-transcribed sense and antisense RNAs (501 nt each) were annealed in 1× lysis buffer. dsRNA formation was confirmed by electrophoresis through a 2% native agarose gel. The markers are a 1 kb DNA ladder (Gibco BRL). The gel was stained with ethidium bromide.

3.4. Annealing the in vitro-transcribed dsRNA

Equal volumes of 1 μM stocks of each sense and antisense RNA strands are annealed by mixing them with an equal volume of 2× lysis buffer. The mixture is heated for 5 min at 95 °C and then annealed overnight at 37 °C. To confirm double-strand formation, we typically analyze a small portion of the dsRNA on a native agarose gel run in 1× TBE buffer. ssRNA for both sense and antisense strands of the dsRNA should be run adjacent to the dsRNA product. dsRNA, unlike single-stranded RNA, typically runs with the same mobility as dsDNA, so we use dsDNA markers as size standards on the gel. The RNA is visualized by staining the gel with ethidium bromide or, for [^{32}P]RNAs, by drying the gel under vacuum onto a positively charged nylon membrane and detecting the RNA by phosphorimagery (Fig. 1).

4. Analysis of Dicer activity

A typical Dicer activity assay is performed as follows: for a 10-μl reaction, add 5 μl *Drosophila* lysate, 3 μl 40× reaction mixture (see Appendix A), 1 μl water, and 1 μl 50 nM uniformly ^{32}P-labeled dsRNA (5 nM final concentration). The reaction is incubated for 1–3 h at 25 °C. After an appropriate time, an aliquot of the reaction mixture should be quenched in 100 μl 2× PK buffer. Reactions are deproteinized by adding 10 μl 20 μg/μl proteinase K and 1 μl 20 μg/μl glycogen carrier (Roche) to each aliquot. After 10–60 min at 65 °C, the sample is phenol extracted and the RNA precipitated with 3 vol absolute ethanol. The precipitate is washed with 80% ethanol and the pellet redissolved in 20 μl formamide loading dye (see Appendix A). Dicer products are resolved by electrophoresis on a 15% denaturing polyacrylamide sequencing gel. 5′-^{32}P-radiolabeled synthetic RNA oligonucleotides are used as size markers. We typically include 14-, 19-, 21-, 25-, and 34-nt RNA markers (Fig. 2).

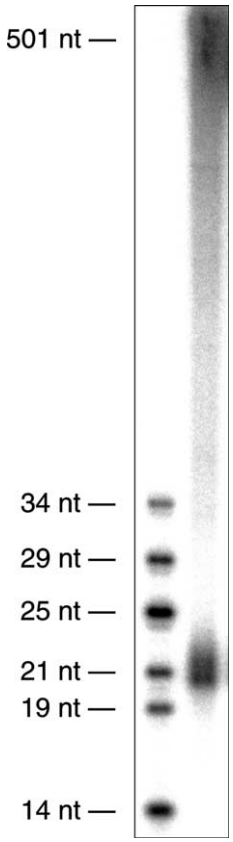

Fig. 2. A Dicer activity assay. Radiolabeled 501-nt dsRNA was incubated with *Drosophila* embryo lysate in a standard reaction. The reaction products were resolved by electrophoresis through a 15% sequencing gel. The markers are 5′-radiolabeled synthetic RNAs.

5. Design and preparation of synthetic siRNAs

Native siRNAs contain 5′-terminal phosphates and 2-nt 3′ overhangs [17]. Because native siRNAs are generated from much longer dsRNA, they form a pool of siRNA sequences spanning the length of the dsRNA. Therefore, they induce target RNA cleavage at a diverse array of sites. In contrast, synthetic siRNAs correspond to a single 21-nt sequence and trigger target cleavage at a corresponding single site [17,18,21]. How to design synthetic siRNAs, as well as where to purchase them, has been described elsewhere [26,27]. Synthetic siRNAs typically bear a 5′ hydroxyl, but are rapidly phosphorylated in the *Drosophila* embryo lysate by an endogenous kinase [21].

6. ^{32}P radiolabeling and purification of synthetic siRNAs

6.1. 5′ Radiolabeling of siRNA

A typical 10-μl siRNA 5′-end-labeling reaction contains 100 pmol (in 1 μl of water) of a single siRNA strand, 5 μl of [γ-^{32}P]ATP (6000 Ci/mmol, ⩾10 mCi/ml; NEN or ICN), 1 μl of T4 polynucleotide kinase (PNK; New England Biolabs (NEB)), 2 μl of PNK reaction buffer (NEB), and 1 μl of H_2O. The reaction is incubated at 37 °C for 1 h and then adjusted to 100 μl with water and run through a G-25 spin column (Roche) to remove unincorporated [^{32}P]ATP. The column flowthrough is precipitated with 3 vol of absolute ethanol, 1/10 vol of 3 M sodium acetate, and 1 μl of 20 mg/ml glycogen carrier. The precipitate is washed with 80% ethanol and then redissolved in 10 μl of formamide loading dye. The sample is then heated to 95 °C for 2 min and purified by electrophoresis on a 15% denaturing polyacrylamide gel. The radiolabeled siRNA is then visualized by phosphorimagery, excised from the gel, and eluted by tumbling overnight in 1 ml of 2× PK buffer. The eluate is phenol extracted, precipitated with 3 vol of absolute ethanol and then washed with 80% ethanol. Finally, the resulting precipitate is dissolved in 10 μl of H_2O. Typically, our yield is about 40% of the input siRNA.

6.2. 3′ Radiolabeling of siRNA

To label the 3′ end of an siRNA strand, we use radioactive cordycepin (3′ deoxyadenosine triphosphate) and poly(A) polymerase. Our typical reaction mixture contains 100 pmol (in 1 μl of water) of single-stranded siRNA, 2 μl of 5× reaction buffer (United States Biochemical), 5 μl of [^{32}P]cordycepin (5000 Ci/mmol, 10 mCi/ml; NEN), 1 μl of poly(A) polymerase, and 1 μl of H_2O. The reaction is incubated at 30 °C for 20 min. The ^{32}P-radiolabeled siRNA is purified as described above for 5′ siRNA radiolabeling.

6.3. Annealing the siRNA strands

To anneal the two complementary, 21-nt RNA strands, add equimolar concentrations of the antisense and sense siRNAs to an equal volume of 2× lysis buffer. Heat the mixture to 90 °C for 1 min, and cool at 37 °C for 1 h. At this point, the siRNA can be stored at −20 °C until needed.

A small portion of the duplexed siRNA should be resuspended in native loading dye (see Appendix A), resolved by electrophoresis on a 15% native polyacrylamide gel, and visualized by phosphorimagery. To ensure the siRNA does not denature during electrophoresis, the temperature of the gel should not exceed 25 °C. We clamp to the front glass plate a heat-dissipating aluminum plate bearing an inexpensive, self-stick, LCD thermometer (McMaster–Carr) on its face to ensure the gel does not exceed 25 °C. The ^{32}P-radiolabeled single-stranded siRNA used in the annealing reaction should be run in adjacent lanes of the gel as markers (Fig. 3).

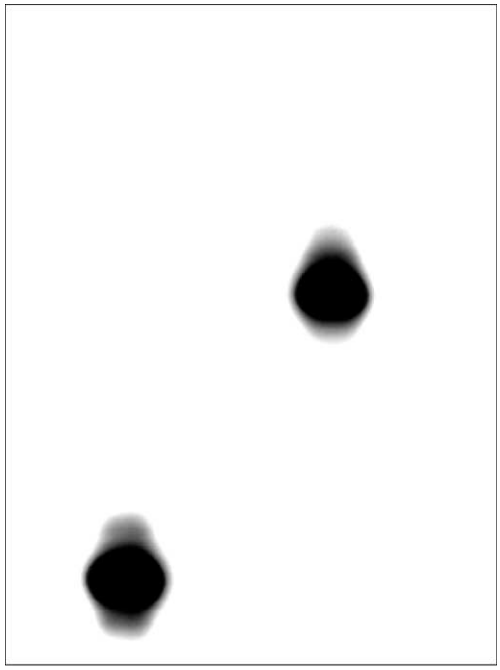

Fig. 3. Analysis of siRNA duplex formation. 5′-^{32}P-radiolabeled single-stranded or annealed siRNAs were electrophoresed in adjacent lanes of a 15% native polyacrylamide gel.

7. Analysis of RNAi in vitro using a translation-based assay

The usefulness of RNAi lies in its ability rapidly and specifically to destroy a target mRNA. RNAi can be monitored either by directly following the fate of the target mRNA or by using a dual luciferase-based translation assay [24]. The dual luciferase strategy provides less information about the mechanism of the RNAi pathway, but has the virtues of including an internal control and of high throughput. This assay relies on two evolutionarily distinct luciferase reporter genes from the firefly *Pyralis photinus* (*Pp*-luc) and the sea pansy *Renilla reniformis* (*Rr*-luc). In this system, dsRNA or siRNA can be used to target either of the two mRNAs, effectively knocking down expression of that target. Expression of the other, nonhomologous target is left unaltered and functions as an internal specificity control. Results are usually expressed by normalizing the expression of the targeted luciferase to the control

luciferase (e.g., *Pp*-luc/*Rr*-luc). A commercial kit for analyzing the dual luciferase expression is available from Promega (DLR Assay System) and single-tube and plate-reading luminometers are widely available. The assay is usually performed in triplicate, and errors are propagated by standard methods.

7.1. Design and preparation of capped mRNA

Because translation in the *Drosophila* embryo lysate is cap-dependent [24], mRNAs for *Pp*-luc or *Rr*-luc are transcribed in the presence of ^{7m}G(5′)ppp(5′)G cap analog. Although PCR templates can be used, we typically use plasmid transcription templates linearized with an appropriate restriction enzyme immediately after a 25- to 30-nt poly(A) stretch. We use 3 μl of a 2 μg/μl stock of linearized plasmid transcription template for a 100-μl reaction. From 100 mM stock solutions, add 5 μl each of ATP, CTP, and UTP, plus 1 μl of GTP. To this, add 10 μl of 10× T7 reaction buffer, 0.5 μl of 1 M DTT, 26.5 μl of water, 1 μl of RNasin (40 U/ml; Promega), 40 μl of 10 mM cap analog (^{7m}G(5′)ppp(5′)G; New England Biolabs), and 2 μl of the appropriate recombinant bacteriophage RNA polymerase (e.g., SP6 or T7). Incubate for 2 h at 37 °C. Stop the reaction by adding 7 μl of 500 mM EDTA to redissolve the insoluble Mg^{2+} · inorganic pyrophosphate precipitate. Precipitate the RNA transcripts with 2.5 M LiCl for 1 h on ice, centrifuge at 13,000×g for 10 min, wash with 80% ethanol, and resuspend pellet in 75 μl of H_2O. Examine 5 μl on a denaturing 2% agarose gel and visualize with ethidium bromide. To the remaining 70 μl add 10 μl of RQ1 DNase buffer (Promega), 5 μl of RQ1 DNase (Promega) and incubate for 30 min at 37 °C. Proteinase K digest (see above), phenol extract, and precipitate with 2.5 vol of absolute ethanol. Resuspend purified transcripts in H_2O, and determine concentrations by absorbance at 260 nm.

7.2. Translation-based dual-luciferase RNAi assay

For each reaction, place 5 μl *Drosophila* embryo lysate, 3 μl 40× reaction mix, 0.01–5 nM (final concentration) each capped luciferase transcript (as a mix in 1 μl water), and 100 nM siRNA or 5 nM long dsRNA corresponding to one of the two luciferase mRNA transcripts. As a translation efficiency control, a separate reaction should be made, in which lysis buffer is substituted for siRNA or dsRNA trigger. Incubate reactions at 25 °C for up to 3 h. At each appropriate time point, place a small aliquot into Passive Lysis Buffer (Promega) to quench the reaction. The DLR Assay System kit from Promega provides all the reagents necessary to generate a separate luminescent signal from either reporter gene. This assay effectively provides a quick and quantitative method for examining the effectiveness and specificity of RNAi. It works best with a dual-dispensing, plate-reading luminometer.

8. Analysis of RNAi in vitro using an mRNA cleavage assay

An alternative method for analyzing RNAi efficiency and specificity in vitro has been described [17,18,21]. This assay relies on the accumulation of the stable, 5′-capped cleavage products formed during RNAi. It is important to note that the accumulation of these 5′ cleavage products is proportional to the decrease in target mRNA only during the early, linear phase of the RNAi reaction. This phenomenon may likewise be true in vivo [28]. Each synthetic siRNA directs mRNA cleavage at a single site 10–11 nt from the siRNA 5′ end, across from the center of the siRNA. By using cap-radiolabeled mRNA, the 5′ cleavage product can be readily detected. The presence of an appropriately sized 5′ cleavage product is diagnostic of RNAi, providing a positive result that bolsters confidence that the disappearance of the target mRNA

Fig. 4. In vitro RNAi reaction. A standard RNAi reaction was performed using a 501-nt, ^{32}P-cap-radiolabeled target RNA and 50 nM siRNA duplex corresponding to nucleotides 62–81 of the target. Reaction products were resolved by electrophoresis through an 8% denaturing polyacrylamide sequencing gel. The 72-nt 5′ cleavage product is diagnostic of siRNA-directed RNAi.

reflects RNAi rather than other nonsequence-specific RNA-degrading processes.

8.1. Design and preparation of cap-radiolabeled mRNA

Design and preparation of the target mRNA sequence should be performed as described under 'In vitro transcription with T7 RNA polymerase,' above. Following gel purification, 10 pmol of the uncapped mRNA target (in 1.75 μl of H_2O) is added to 5 μl of [α-^{32}P]GTP (3000 Ci/mmol, 40 mCi/ml, custom order; ICN), 1 μl of 10× guanylyl transferase buffer (see Appendix A), 0.5 μl of 0.1 mM DTT, 0.25 μl of 20 U/μl RNasin (Promega), 1 μl of 10 mM *S*-adenosyl methionine (SAM; Sigma) in SAM dilution buffer, and 0.5 μl of recombinant, heterodimeric vaccinia virus guanylyl transferase (e.g., Ambion). Incubate for 2 h at 37 °C, adjust the reaction to 100 μl with H_2O, and pass through a G-50 spin column (Roche). Gel purification of the radiolabeled target is performed by electrophoresis as described.

8.2. In vitro RNAi reaction using cap-radiolabeled mRNA

A standard RNAi reaction containing the 5′-^{32}P-cap-radiolabeled target mRNA is performed essentially as described for the translation-based assay. A 10-μl reaction is set up as follows: 5 μl *Drosophila* embryo lysate is added to 3 μl 40× reaction mix, 1 μl target mRNA (stock concentration 10–50 nM), and 1 μl synthetic siRNA (stock concentration 250–1000 nM) or dsRNA (stock concentration 50–100 nM). A control reaction substituting 1 μl 1× lysis buffer for the siRNA or dsRNA trigger serves as a control for nonspecific mRNA degradation. Incubate for up to 3 h at 25 °C and quench at each time point with 100 μl 2× PK buffer. Deproteinize as described above, add 1 μl 20 mg/ml glycogen carrier, and phenol extract. Precipitate with 3 vol absolute ethanol, wash with 80% ethanol, and redissolve in 20 μl formamide loading dye. Analyze by electrophoresis on a denaturing polyacrylamide sequencing gel, and visualize the 5′ cleavage products by phosphorimagery (Fig. 4). Radiolabeled markers are readily prepared by cap-radiolabeling commercially available 100 nt RNA ladder marker mixes with guanylyl transferase and [α-^{32}P]GTP as described above.

Appendix A

1. *1× lysis buffer*
   100 mM potassium acetate
   30 mM Hepes–KOH, pH 7.4
   2 mM magnesium acetate

2. *10× T7 transcription buffer*
   400 mM Tris–Cl, pH 7.9
   25 mM spermidine
   260 mM $MgCl_2$
   0.1% w/v Triton X-100

3. *10× PCR buffer*
   100 mM Tris–Cl, pH 8.3
   500 mM KCl
   15 mM $MgCl_2$
   0.1% w/v gelatin

4. *2× proteinase K buffer*
   200 mM Tris–Cl, pH 7.5
   25 mM EDTA, pH 8.0
   300 mM NaCl
   2% w/v sodium dodecyl sulfate

5. *2× urea stop mix/loading dye*
   8 M urea
   25 mM EDTA, pH 8.0
   0.025% w/v xylene cyanol FF
   0.025% w/v bromophenol blue

6. *Formamide loading dye*
   98% w/v deionized formamide
   10 mM EDTA, pH 8.0
   0.025% w/v xylene cyanol
   0.025% w/v bromophenol blue

7. *Native loading dye*
   3% w/v Ficoll-400
   0.04% w/v bromophenol blue
   2 mM Tris–Cl, pH 7.4

8. *10× Guanylyl transferase buffer*
   500 mM Tris–Cl, pH 8.0
   60 mM KCl
   12 mM $MgCl_2$
   25 mM DTT

9. *SAM dilution buffer*
   (To make 50 ml, add 6.96 μl 36 M H_2SO_4 and 5 ml absolute ethanol to 45 ml deionized water.)

10. *40× reaction mix*
    50 μl water
    20 μl 500 mM creatine monophosphate (Fluka; prepared fresh from powder)
    20 μl amino acid stock (Sigma; 1 mM each amino acid)
    2 μl 1 M DTT
    2 μl 20 U/μl RNasin (Promega)
    4 μl 100 mM ATP
    1 μl 100 mM GTP
    6 μl 2 U/μl creatine phosphokinase (Cal-Biochem), prepared by diluting 2 μl of a 20 U/μl stock in 18 μl 1 × lysis buffer containing 2 mM DTT
    16 μl 1 M potassium acetate

References

[1] J.R. Kennerdell, R.W. Carthew, Cell 95 (1998) 1017–1026.

[2] A. Grishok, H. Tabara, C. Mello, Science 287 (2000) 2494–2497.
[3] S.M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, Nature 411 (2001) 494–498.
[4] A. Fire, S. Xu, M.K. Montgomery, S.A. Kostas, S.E. Driver, C.C. Mello, Nature 391 (1998) 806–811.
[5] L. Timmons, A. Fire, Nature 395 (1998) 854.
[6] K. Ui-Tei, S. Zenno, Y. Miyata, K. Saigo, FEBS Lett. 479 (2000) 79–82.
[7] L. Misquitta, B.M. Paterson, Proc. Natl. Acad. Sci. USA 96 (1999) 1451–1456.
[8] H. Ngo, C. Tschudi, K. Gull, E. Ullu, Proc. Natl. Acad. Sci. USA 95 (1998) 14687–14692.
[9] J.U. Lohmann, I. Endl, T.C. Bosch, Dev. Biol. 214 (1999) 211–214.
[10] A. Sánchez-Alvarado, P.A. Newmark, Proc. Natl. Acad. Sci. USA 96 (1999) 5049–5054.
[11] N.J. Caplen, S. Parrish, F. Imani, A. Fire, R.A. Morgan, Proc. Natl. Acad. Sci. USA 98 (2001) 9742–9747.
[12] J.C. Clemens, C.A. Worby, N. Simonson-Leff, M. Muda, T. Maehama, B.A. Hemmings, J.E. Dixon, Proc. Natl. Acad. Sci. USA 97 (2000) 6499–6503.
[13] N.J. Caplen, J. Fleenor, A. Fire, R.A. Morgan, Gene 252 (2000) 95–105.
[14] E. Billy, V. Brondani, H. Zhang, U. Muller, W. Filipowicz, Proc. Natl. Acad. Sci. USA 98 (2001) 14428–14433.
[15] S. Yang, S. Tutton, E. Pierce, K. Yoon, Mol. Cell. Biol. 21 (2001) 7807–7816.
[16] P. Zamore, T. Tuschl, P. Sharp, D. Bartel, Cell 101 (2000) 25–33.
[17] S.M. Elbashir, W. Lendeckel, T. Tuschl, Genes Dev. 15 (2001) 188–200.
[18] S.M. Elbashir, J. Martinez, A. Patkaniowska, W. Lendeckel, T. Tuschl, EMBO J. 20 (2001) 6877–6888.
[19] A.J. Hamilton, D.C. Baulcombe, Science 286 (1999) 950–952.
[20] E. Bernstein, A.A. Caudy, S.M. Hammond, G.J. Hannon, Nature 409 (2001) 363–366.
[21] A. Nykänen, B. Haley, P.D. Zamore, Cell 107 (2001) 309–321.
[22] S.M. Hammond, E. Bernstein, D. Beach, G.J. Hannon, Nature 404 (2000) 293–296.
[23] S.M. Hammond, S. Boettcher, A.A. Caudy, R. Kobayashi, G.J. Hannon, Science 293 (2001) 1146–1150.
[24] T. Tuschl, P.D. Zamore, R. Lehmann, D.P. Bartel, P.A. Sharp, Genes Dev. 13 (1999) 3191–3197.
[25] J.C. Sisson, in: W. Sullivan, M. Ashburner, R.S. Hawley (Eds.), Drosophila melanogaster, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2000, pp. 541–555.
[26] http://www.mpibpc.gwdg.de/abteilungen/100/105/sirna.html.
[27] S. Elbashir, J. Harborth, K. Weber, T. Tuschl, Methods Findings Exp. Clin. Pharmacol. 26 (2002) 199–213.
[28] T. Holen, M. Amarzguioui, M.T. Wiiger, E. Babaie, H. Prydz, Nucleic Acids Res. 30 (2002) 1757–1766.