



Fluorescently Labeling Synthetic RNAs

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Abstract

This protocol covers the steps required to incorporate *N*-hydroxysuccinamide (NHS) functionalized fluorophores into synthetic RNAs containing a residue derivatized with a primary amine. This method has been widely used to label RNA oligonucleotides that are used directly, targeted to a complementary RNA using base pairing rules, or covalently ligated to a RNA of interest (Ha et al., 1999; Hodak et al., 2005; Baum and Silverman, 2007; Sattint et al., 2008; Akiyama and Stone, 2009; Solomatin and Herschlag, 2009). While this technique is quite general, the details of a particular experiment can vary, therefore, it is always important to keep in mind that other labeling strategies are available and should potentially be considered.



1. THEORY

In the biological sciences, fluorescence detection is among the most prevalent techniques in modern experimental design. In recent years, the use of site-specifically incorporated fluorophores for detection of RNA has gained in popularity relative to the traditional techniques of radioisotope labeling (typically with ^{32}P , see [RNA Radiolabeling](#)) or the use of nonspecific intercalating dyes. Fluorescence detection can have sensitivity that exceeds radiolabeling and, when stored properly, labeled samples retain sensitivity for long periods of time. Fluorescence also avoids the special handling and institutional requirements associated with the use of radioactive isotopes. Moreover, the great diversity of fluorescent dyes available allows the simultaneous detection of multiple species. This property allows experimental possibilities not realizable with radiolabeling. For instance, it is possible to specifically incorporate two fluorophores into RNAs of considerable size in order to monitor conformational changes using fluorescence resonance energy transfer (FRET) (Ha et al., 1999).

Site-specifically labeling RNA requires the incorporation of an unnatural nucleic acid into a synthetic oligonucleotide. The incorporated nucleoside is either fluorescent or has a synthetic handle which can easily be derivatized. Direct incorporation of a fluorophore during oligonucleotide synthesis is likely the most common method of fluorescent labeling and is available from most suppliers of synthetic RNA oligonucleotides. However, postsynthesis modification of an unnatural nucleic acid affords some advantages:

1. More control of fluorophore location and position. This can be important for maintaining the optimal photophysical properties of the fluorophores.

2. Greater choices of fluorophores.
3. Potentially less expensive (particularly if multiple RNA sequences are to be labeled with the same fluorophore).

There are multiple functional handles available for postsynthesis labeling of RNA. This protocol is for derivatizing a primary amine incorporated into a synthetic nucleic acid with a NHS-activated fluorophore. There are many RNA base, sugar, or phosphate modifications that incorporate a primary amine capable of being modified. Some examples of these modified RNAs are shown in [Fig. 15.1](#).

All of these modifications can be conjugated to a fluorophore. However, the local chemical environment of the modification or the surrounding oligonucleotide can cause the reactivity of the primary amine to vary. For instance, it has been observed that 2'-amino groups have decreased reactivity when compared to the other examples shown above, likely because of steric hindrance. While it is important to be aware of these limitations when designing an oligonucleotide to be labeled, initial considerations should focus on picking the location of fluorophore attachment best suited for the desired application, since sufficient labeling can likely be economically feasible with scaled-up reactions.

NHS-activated fluorophores are highly reactive toward primary amines, which allows for the coupling reaction to proceed in mild reaction conditions. While this is beneficial from the standpoint of working with a biological macromolecule, it necessitates the taking of considerable care when working with NHS-reactive fluorophores. They are highly reactive

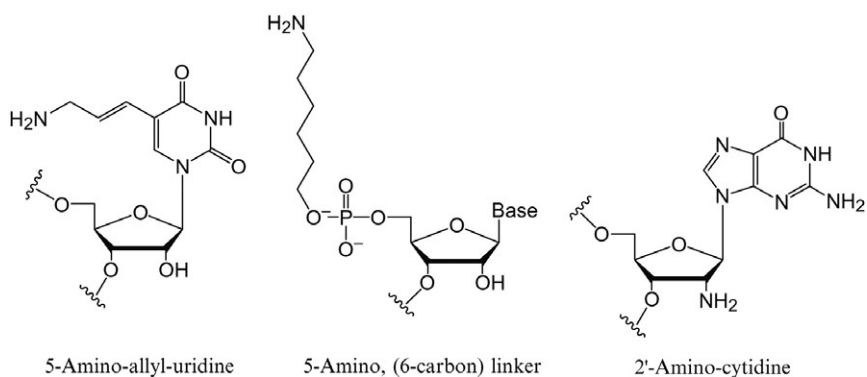


Figure 15.1 Examples of common primary amine-modified nucleotides suitable for use in this protocol.

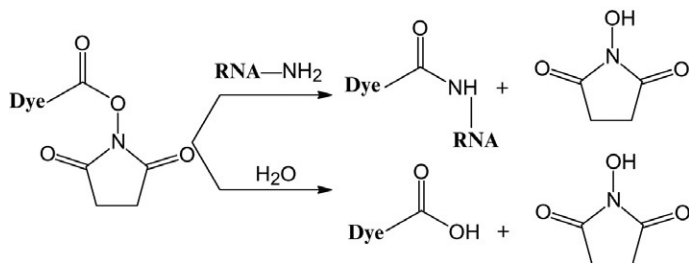


Figure 15.2 NHS-activated fluorophores are capable of reacting with unprotonated primary amines to yield the desired fluorescently labeled oligonucleotide as well as with water to yield inactive dye. Care must be taken to avoid unnecessary exposure of NHS fluorophores to water. *Figure modified from Solomatin et al.(2009).*

toward water, as shown in Fig. 15.2, and toward other nucleophilic species, for example, amines present in buffer components such as Tris.

The protocol design needs to consider the high reactivity of NHS esters toward water and that the unprotonated primary amine is the reactive species. Specifically, reactions are carried out in 50% organic solvent and high oligonucleotide concentrations with the aim of decreasing side reactions. Additionally, the solution is buffered at an elevated pH, typically between pH 8 and 9, to increase the concentration of the reactive unprotonated primary amine. When these factors are accounted for, this protocol has generated a >80% yield of labeled oligonucleotide with only a 2:1 excess of fluorophore over the oligonucleotide.



2. EQUIPMENT

- Refrigerated microcentrifuge (4 °C)
- Polyacrylamide gel electrophoresis (PAGE) equipment
- UV/Vis spectrophotometer
- Flat bed fluorescence scanner (e.g., GE Healthcare Typhoon™) (optional)
- Water bath or heating block
- Freezer (−20 °C)
- Refrigerator (4 °C)
- Micropipettors
- Micropipettor tips (DMSO resistant)
- 0.5-ml microcentrifuge tubes (DMSO resistant)
- 1.5-ml microcentrifuge tubes
- Ultrafiltration spin columns (optional)

Plastic wrap
Sterile single use scalpels
0.2- μm syringe filter
Syringe



3. MATERIALS

Primary amine-modified RNA oligonucleotide
Fluorescent dye, *N*-hydroxysuccinimide (NHS) ester form
Dimethyl sulfoxide (DMSO, anhydrous)
Sodium phosphate monobasic monohydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$)
Sodium phosphate dibasic heptahydrate ($\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$)
Formamide
Sodium acetate (NaOAc)
Ethanol
RNase/DNase-free water
Sodium chloride (NaCl)
2-Amino-2-hydroxymethyl-propane-1,3-dio (Tris base)
Hydrochloric acid (HCl)
ethylenediaminetetraacetic acid pH 8.0 (EDTA)
Denaturing PAGE loading buffer
Materials for Denaturing PAGE (gel composition and thickness will need to be adjusted depending on the oligonucleotide being labeled)
Nucleic acid gel stain such as SYBR[®] (Invitrogen)
Dry ice

3.1. Solutions & buffers

Step 2 500-mM phosphate buffer, pH 8.7

Component	Amount
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	0.1 g
$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$	13.2 g

Dissolve in a total of 100-ml ultrapure water.

Step 3 TEN buffer

Component	Final concentration	Stock	Amount
Sodium chloride	300 mM	2 M	7.5 ml
Tris-HCl, pH 7.4	10 mM	1 M	500 μl
EDTA	1 mM	500 mM	100 μl

Add water to 50 ml.



4. PROTOCOL

4.1. Preparation

Consult your supplier of the amine-functionalized RNA oligonucleotides for the requirements for any final deprotection steps. If additional steps are required, oligonucleotides should be deprotected in accordance with the supplier's protocols and completely dried prior to labeling.

There is a wide range of NHS-derivatized fluorescence dyes compatible with this protocol. The experimentalist's choice of dye will depend on many factors such as their previous experience with a dye or downstream experimental considerations such as spectral overlap with other fluorophores to be used in conjunction with the dye.

4.2. Duration

Preparation	1 day
Protocol	14 hours over 3 days

See [Fig. 15.3](#) for the flowchart of the complete protocol.

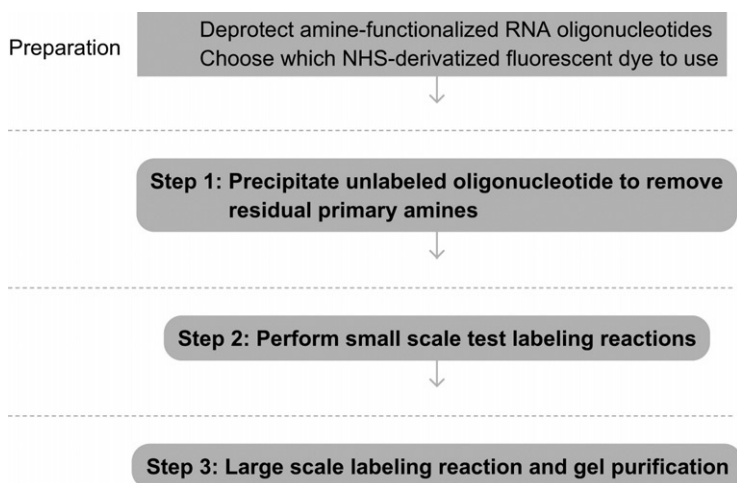


Figure 15.3 Flowchart of the complete protocol, including preparation.



5. STEP 1 PRECIPITATE UNLABELED OLIGONUCLEOTIDE

5.1. Overview

To ensure high labeling efficiencies, it is critical to remove residual primary amines associated with the unlabeled oligonucleotide. Buffers containing primary amines can be carried over from synthesis steps and will quench the labeling reaction. Precipitating the unlabeled oligonucleotide prior to labeling steps is a good way of removing contaminants (see [RNA purification – precipitation methods](#)).

5.2. Duration

Overnight + 3 h

- 1.1 Resuspend the dried oligonucleotide in 50 μl of water. Add 5 μl of 3 M Na acetate, pH 5.3, and 165 μl of ice-cold ethanol. Transfer the solution to a 1.5-ml microcentrifuge tube. Place the solution at $-20\text{ }^{\circ}\text{C}$ overnight.
- 1.2 Centrifuge at $>12\ 000 \times g$, $4\text{ }^{\circ}\text{C}$, for 30 min in a microcentrifuge. Typically, a visible pellet will form towards the bottom of the tube.
- 1.3 Immediately pipet off the supernatant, being careful not to disturb the pellet. Keep the supernatant until you have verified that most of the oligonucleotide was recovered in the pellet.
- 1.4 Add 50 μl of 70% ice-cold ethanol to the tube, being careful not to disturb the pellet. Centrifuge again at $>12\ 000 \times g$, $4\text{ }^{\circ}\text{C}$, for 15 min.
- 1.5 Immediately pipet off the supernatant, being careful not to disturb the pellet. Keep the supernatant until you have verified the concentration of the oligonucleotide.
- 1.6 Cover the tube and air-dry the pellet. Let the pellet dry completely; this typically takes 3 h.
- 1.7 Resuspend the pellet in a volume of water sufficient that the final concentration of oligonucleotide is approximately 5 mM. Use 10 μl of water for an RNA oligonucleotide synthesized at a 100-nmol scale.
- 1.8 Remove 0.5 μl of oligonucleotide and dilute it in 9.5 μl of water. Use dilutions of this stock to determine the concentration of the oligonucleotide. At this time, verify whether the supernatants from the earlier precipitation steps are devoid of large quantities of the oligonucleotide. If you have recovered less than 90% of your oligonucleotide, consider

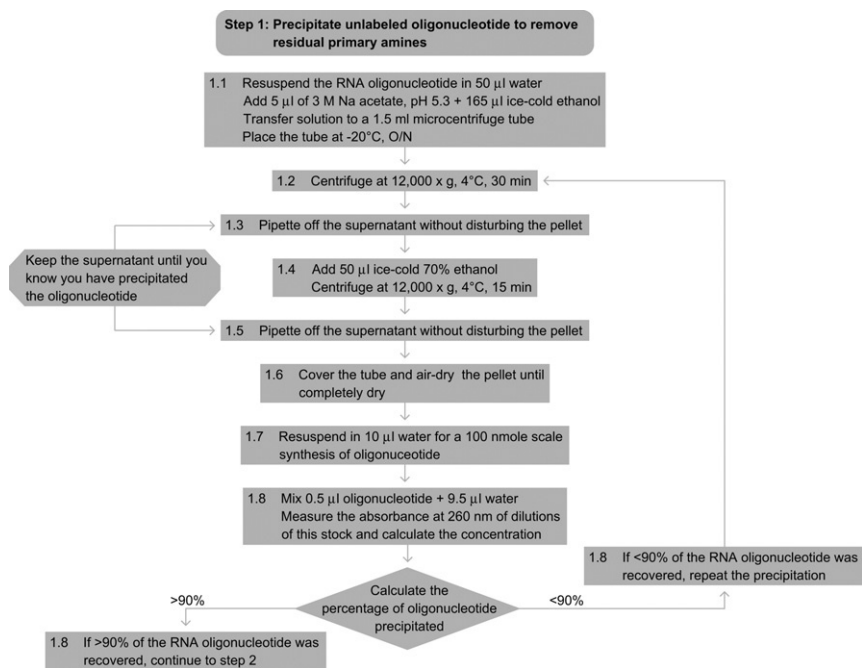


Figure 15.4 Flowchart of Step 1.

repeating Steps 1.2–1.8. If you continue to get poor yields, consult a more comprehensive protocol on oligonucleotide precipitation. See Fig. 15.4 for the flowchart of Step 1.



6. STEP 2 TEST-LABELING REACTIONS (OPTIONAL)

6.1. Overview

It can be informative to carry out test-labeling reactions prior to a large-scale labeling reaction. This can help ensure the efficient use of oligonucleotide and dye. If poor yields are obtained at a small scale, there is the potential to optimize the reaction before proceeding with large-scale labeling reactions. However, this is only advisable if the labeling dye is available in small packets. Some manufacturers only supply large quantities of dye (e.g., packets of 1 or 5 mg), which are not suitable for small-scale test reactions. Unfortunately, due to the high lability of the NHS ester, these dyes cannot reliably be divided into smaller aliquots. For this step, the authors use Amersham™ CyScribe PostLabeling Kits. These are small packets that contain 40 nmol of Cy3 or Cy5 dye and contain >75% active NHS esters.

6.2. Duration

3 h

- 2.1 Prepare and prerun a denaturing polyacrylamide (PAGE) gel (see [Analysis of RNA by analytical polyacrylamide gel electrophoresis](#)) such that single nucleotide resolution can be obtained for an oligonucleotide of the length being labeled. The conjugated fluorophore results in a gel shift equivalent to about five nucleotides.
- 2.2 Have ready a 37 °C water bath or heating block.
- 2.3 Set up the test reactions listed below (concentrations of dye and oligonucleotide may need to be adjusted depending on what you have available). The order in which the components are added is important to avoid unnecessary hydrolysis of the NHS ester. First combine the aqueous components (oligonucleotide, water, and phosphate buffer) in 0.5-ml microcentrifuge tubes:

Component	Concentration	1×	0.25×	0.0625×	0.0625×/no dye
Oligo	~5 mM	0.8 µl	0.2 µl		
Oligo (0.2 dilution)	~1 mM			0.25 µl	0.25 µl
Water			0.6 µl	0.55 µl	0.55 µl
Phosphate buffer, pH 8.7	500 mM	0.2 µl	0.2 µl	0.2 µl	0.2 µl

Second resuspend the dye in 3.5 µl DMSO and immediately add 1 µl of the dye to the aqueous components. Add 1 µl DMSO to the 0.0625×/no dye sample.

Incubate at 37 °C for 1 h.

- 2.4 Add 30 or 6 µl of water to the 1× and 0.25× reactions, respectively. This will make the oligonucleotide concentrations the same in all of the reactions. Transfer ~500 ng of oligonucleotide (or a quantity sufficient to be visualized with the gel stain you will be using) to tubes containing denaturing gel loading buffer.
- 2.5 Load samples onto the denaturing polyacrylamide gel prepared in Step 2.1. Run the gel for a time sufficient that a difference of five nucleotides can be resolved. When the gel is finished running, stain it according to the manufacturer's instructions for the stain you are using.
- 2.6 Two bands should be seen in the samples subjected to the oligonucleotide labeling reaction. The faster running band should run at the same

position as the 0.0625×/no dye control, and represents unlabeled RNA. The slower running band corresponds to labeled RNA.

2.7 Ideally you should get >80% modification of the 1× reaction. However, this is not always obtained. At this point it will be necessary to consider for a large scale reaction if one of the current reaction conditions can economically provide enough material for your future experiments. If a satisfactory condition is found, proceed to step 3. Alternatively, the following should be considered:

1. The precipitation step did not sufficiently remove primary amines. Consider additional purification steps, for example, a second precipitation, purification using a desalting spin column, or dialysis.
2. The dye had an unexpectedly low activity. It is possible that the NHS ester could have been largely hydrolyzed prior to starting the labeling reaction. This could be a result of either a defective batch from the manufacturer or poor handling by the user. Identify and eliminate steps that could have led to premature hydrolysis of the NHS ester and/or try a dye from another vendor.
3. The oligonucleotide you are trying to label has inherently poor reactivity. Consider using as a positive control an oligonucleotide you have previously successfully labeled or one of the oligonucleotides used by Sattin et al. (2008).

After considering these points, you may want to change your experimental design accordingly and repeat Step 2.

6.3. Tip

The gel prepared should be able to resolve a difference of five nucleotides between the unlabeled and labeled oligonucleotides. The protocol you choose to use will depend on the equipment available and the length of oligonucleotide you are labeling. Denaturing PAGE is an extensively used technique and the available equipment can vary considerably between laboratories. Consult the manufacturer's instructions for the PAGE apparatus available and any experienced users of PAGE in your lab. Manufacturers of PAGE equipment and supplies typically provide extensive explanations of the theory and practice of PAGE. Additionally, the protocol by Albright and Slatko (2001) is a useful starting point.

6.4. Tip

For efficient labeling, it is important to work at high concentrations of oligonucleotide and dye. As a consequence, the reaction volumes are unusually small.

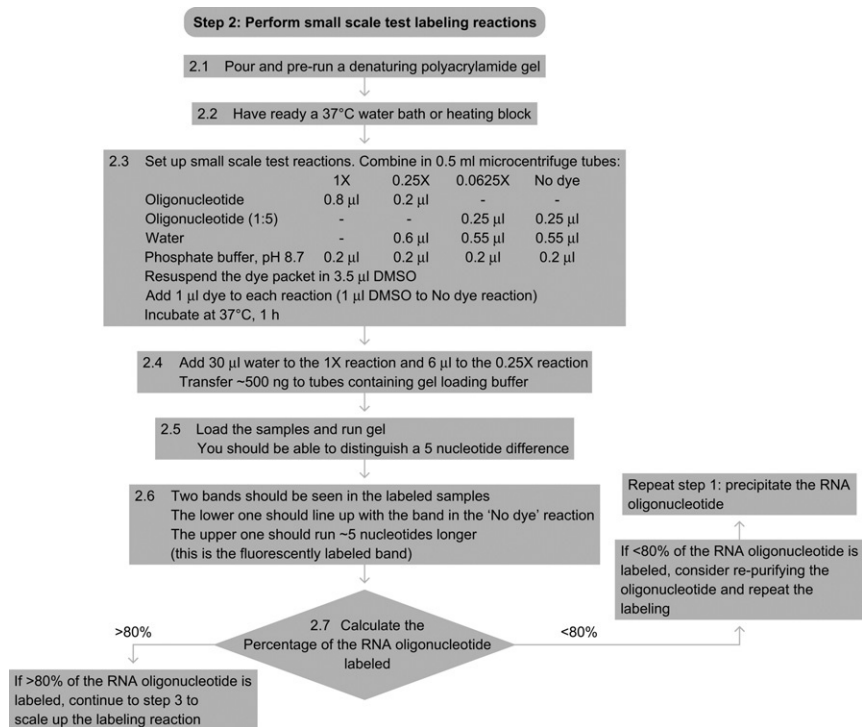


Figure 15.5 Flowchart of Step 2.

6.5. Tip

Save this gel or an image of this gel. It can be a useful guide in determining the position of your RNA in the gel purification of the large-scale reaction.

6.6. Tip

If the fluorophore you are using absorbs in the visible range, there will likely be a large number of bands visible on the gel. This is typical even when high labeling efficiencies are obtained and likely arises from unreactive dye aggregates.

See [Fig. 15.5](#) for the flowchart of Step 2.

7. STEP 3 LARGE-SCALE LABELING REACTION AND GEL PURIFICATION

7.1. Overview

This is a linear scale-up of the reactions outlined in Step 2. If you did not do Step 2, use the suggested concentrations of dye and oligonucleotide from the

supplier as a guide for the reaction conditions. The labeled oligonucleotide is then PAGE-purified (see [RNA purification by preparative polyacrylamide gel electrophoresis](#) and Purification of DNA Oligos by Denaturing Polyacrylamide Gel Electrophoresis (PAGE)) to eliminate excess dye and to separate labeled from unlabeled oligonucleotide.

7.2. Duration

About 3 days

- 3.1 Determine the amount of labeled oligonucleotide you will need for your future experiments. Take into account the labeling efficiency (from Step 2) and then double that amount to account for a 50% loss during gel purification. It is also prudent to factor in an additional safety factor of twofold, to account for unanticipated losses. Therefore, you should probably start with five times the amount of oligonucleotide that you want labeled. With this in mind, pick a dye-labeling packet of sufficient quantity to meet the needs of this labeling reaction. Alternatively, if small labeling packets are available, it can be convenient to do a simple linear scaling of the reaction conditions determined in Step 2.
- 3.2 Prepare and prerun a denaturing polyacrylamide gel, scaled up to account for the significantly larger quantity of oligonucleotide being loaded. This can be done by increasing the thickness of the gel, the width of the loading wells, or a combination of both.
- 3.3 Have a 37 °C water bath or heating block ready.
- 3.4 Set up the large-scale labeling reaction according to the requirements determined in Step 3.1. The reaction conditions listed below are for a ninefold linearly scaled up version of the 1 × reaction listed in Step 2.3. The order in which the components are added is important.
Add to a 1.5-ml microcentrifuge tube:

Oligonucleotide (~5 mM)	7.2 μl
Phosphate buffer, pH 8.7	1.8 μl

Then resuspend the dye in DMSO (use 3-μl DMSO for each small labeling packet) and immediately add the 9 μl of dye to the reaction. Incubate at 37 °C for 1 h.

- 3.5 Add loading buffer to the sample and load onto the denaturing polyacrylamide gel. Run the gel so that the indicator dyes are in the same positions as in Step 2.

- 3.6** When the gel has finished running, disassemble the gel apparatus. Open the gel plates carefully so that the gel remains stuck to one of the plates. Immediately cover it with plastic wrap, this will help prevent contamination of the gel by RNases.
- 3.7** You must now excise the region of the gel that contains your RNA. If the dye is not visible or is hard to see, a flat bed fluorescence scanner should be used to image the gel. If this is necessary, a full-size printout should be taped to the back of the gel using reference marks on the gel plate for alignment.

There can be dozens of bands on the gel, but only one of them is your RNA. If you completed Step 2, use the gel completed in that step as a guide for where your RNA is running on the preparative gel. Additionally, the location of the indicator dyes should provide an approximate location of your RNA. Even with these guides it can be hard to confidently identify the labeled RNA, so it is best to excise all the reasonable choices in the region that should contain your RNA and to save the gel until you have verified that one of the excised bands contains the labeled oligonucleotide of interest.

Bands of interest should be excised using a sterile scalpel. It is best to cut a rectangle around the region of interest, and then with the edge of the scalpel, peel-back the plastic wrap. At this point, the uncovered gel piece is vulnerable to contamination so it is prudent to work quickly, being careful not to introduce unnecessary contamination (e.g., coughing on an exposed gel piece). Using the scalpel, transfer the gel piece to a microcentrifuge tube. Repeat this step with a fresh scalpel for each band of interest.

- 3.8** Use a pipette tip to crush the gel pieces. This increases the surface area of the gel pieces and increases the recovery of the labeled oligonucleotide.
- 3.9** Place the tubes on dry ice to freeze the crushed gel pieces.
- 3.10** Add 300 μ l of TEN buffer to the frozen gel pieces and allow them to completely thaw. Complete two more freezing and thawing cycles. After the final cycle, place the samples at 4 °C overnight. The freeze/thaw cycles help increase recovery of labeled oligonucleotide.
- 3.11** Transfer the supernatant to a new 1.5-ml microcentrifuge tube, trying not to remove the gel pieces. Add an additional 300 μ l of TEN buffer.
- 3.12** Measure the absorbance at 260 nm of 1 μ l of each of the samples. The sample containing the oligonucleotide will give a reading at 260 nm.

- 3.13** Repeat Step 3.11 0 to 5 more times, depending on your desired yields. You can inspect the color of the gel piece or check the absorbance at 260 nm to see whether further elution is warranted.
- 3.14** Pool the eluted fractions and filter the sample using an appropriately sized syringe and a 0.22- μm syringe filter. To maximize recovery of the sample, pass an additional 500 μl of TEN buffer through the syringe filter and add this to the sample. This step eliminates the residual gel pieces from the sample.
- 3.15** Add three volumes of ethanol and place the sample at $-20\text{ }^{\circ}\text{C}$ overnight (the 300-mM sodium in the TEN buffer used to elute the oligonucleotide is sufficient to precipitate it).
- 3.16** Centrifuge the sample at $12\,000 \times g$, $4\text{ }^{\circ}\text{C}$, for 30 min. Wash with 70% ethanol, centrifuge as above, and air-dry the pellet.
- 3.17** Validate the labeled product by running an analytical denaturing polyacrylamide gel (see [Analysis of RNA by analytical polyacrylamide gel electrophoresis](#)). You can use the sample left over from the test labeling reactions to validate where your purified product should run on the gel. RNA that has been PAGE-purified can be of $>99\%$ purity. The RNA should be ready for future experiments.

7.3. Caution

Polyacrylamide gel electrophoresis is a shock hazard that could lead to significant personal injury or death. Turn off the power supply and disconnect the leads before loading the gel or disassembling it.

7.4. Caution

Care should always be taken when working with acrylamide as the monomer is a neurotoxin. DMSO is an organic solvent and therefore, waste containing DMSO is hazardous. Consult your institution's office of environmental health and safety regarding proper handling and disposal of these materials.

7.5. Tip

A reaction scaled up ninefold over the $1\times$ reaction in Step 2.3 would use three small labeling packets (compared to the example of one packet used for the three reactions in Step 2.3) instead of purchasing a larger 1-mg dye packet.

7.6. Tip

If possible, carry out the labeling reaction and all subsequent steps in conditions where light exposure can be kept to a minimum. This will reduce unnecessary photobleaching.

7.7. Tip

Remove 0.2–0.5 μ l of this reaction for an analytical gel to evaluate the quality of the labeling and purification. This is particularly important if the small-scale reaction was skipped.

7.8. Tip

Run the excess from the test labeling reaction on the denaturing polyacrylamide gels so the RNA does not go to waste.

7.9. Tip

Precipitation of the sample prior to loading onto the purification gel can help remove dye aggregates that obscure the identification of labeled RNA.

7.10. Tip

There should be enough oligonucleotide in 1 μ l of sample to identify whether it is present. If none of the tubes give a reading at 260 nm, increase the amount of sample being tested. If still no 260 nm absorbance is detected, go back to the gel and try again at identifying the correct band. If you come to this situation, it would also make sense to run an analytical gel of the small sample you kept from the large-scale labeling reaction to verify whether the labeling reaction was successful and that there is a band to cut out.

7.11. Tip

The final precipitation is done to ensure a consistent starting point for future experiments. Alternatively, ultrafiltration spin columns can be used for concentration and buffer exchanges. If spin columns are used, consult the manufacturer's instructions on proper use.

7.12. Tip

If there is significant degradation, it likely results from contamination introduced when the bands were excised from the gel and eluted. If necessary, repeat the PAGE purification, minimizing the handling steps that lead to degradation.

See [Fig. 15.6](#) for the flowchart of Step 3.

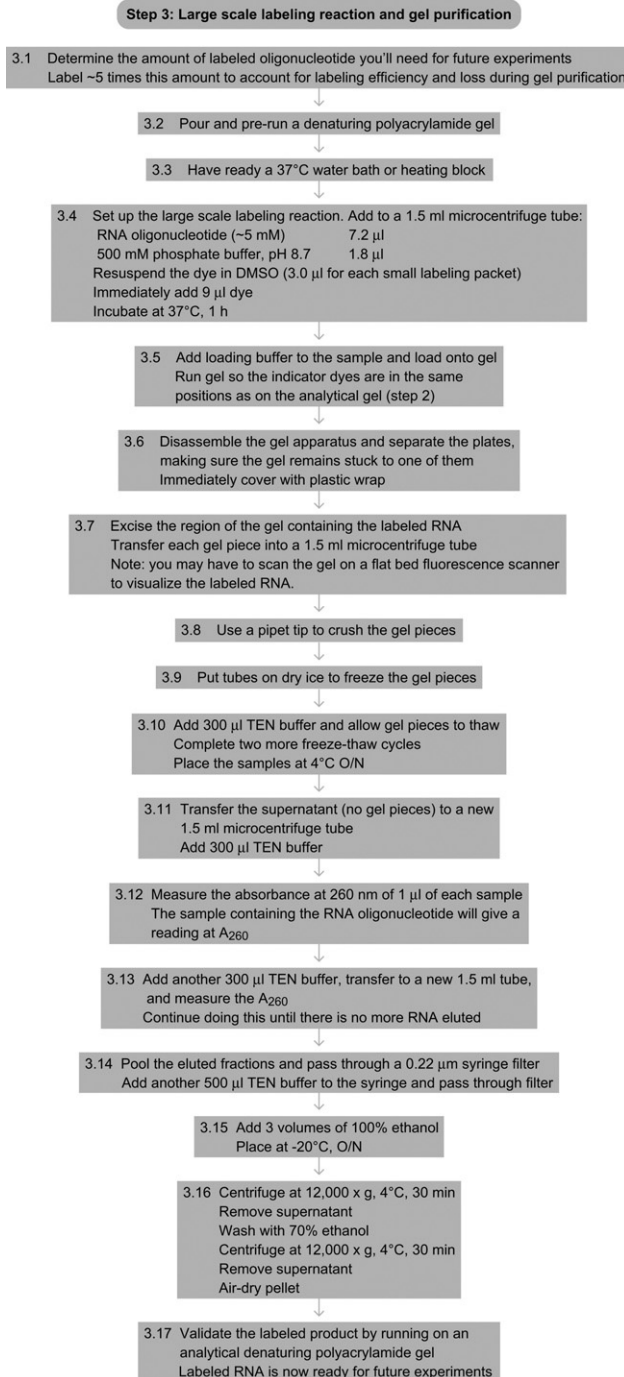


Figure 15.6 Flowchart of Step 3.

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RNA Radiolabeling.

RNA purification – precipitation methods.

Analysis of RNA by analytical polyacrylamide gel electrophoresis.

RNA purification by preparative polyacrylamide gel electrophoresis.

Purification of DNA Oligos by Denaturing Polyacrylamide Gel Electrophoresis (PAGE).