



Circularized RT-PCR (cRT-PCR): Analysis of the 5' Ends, 3' Ends, and poly(A) Tails of RNA

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Abstract

Many techniques that are used to characterize individual RNA molecules can potentially alter the original transcript sequence or its posttranscriptional modifications, such as polyadenylation. Methods that are designed to define the ends of an RNA molecule, for example, oligonucleotide ligation, avoid altering the transcript sequence but can usually fulfill only one objective per experiment (e.g., define the 5' or the 3' end). In contrast, not only does circularized reverse transcription coupled with PCR (cRT-PCR) preserve the original 5' and 3' ends of the transcript and posttranscriptionally added extensions, but also the material from one experimental procedure can be utilized in order to characterize both the 5' and 3' ends. Furthermore, if suitable oligonucleotide

primers are designed, cRT-PCR can be used to isolate truncated, adenylated (and non-adenylated) molecules that are intermediates in RNA decay (Slomovic and Schuster, 2008).



1. THEORY

One of the most basic and integral stages in the study of a particular protein is that of the messenger RNA that encodes it. By characterizing the RNA molecule, much can be learned about the expression levels under different physiological conditions, cellular localization and targeting, turnover rate, and alternative splicing which, of course, leads to alternative polypeptide products. RNA analysis is not limited to protein encoding mRNAs, but is also of central importance in defining ribozymes, regulatory RNA, miRNA, and noncoding RNA, among others. Additionally, posttranscriptional modifications such as adenylation or uridylation can influence transcript longevity, stability, and translatability.

Before carrying out cRT-PCR, it is important to know certain things about the target RNA molecule. For example, since RNA ligase-based circularization is the foundation of cRT-PCR, it is important to know whether the target RNA is 5' capped or triphosphorylated since these modifications can hinder circularization (ligation of the 5' and 3' ends). In such cases, the 5' cap must be removed or cleaved prior to ligation. In contrast, for polycistronic mRNAs that are processed after transcription, the 5' end can readily be ligated to the 3' end without additional steps. In another example, if posttranscriptional extensions, such as adenylation, are the focus of the study, cRT-PCR-labeling can be accurately carried out if the mature 5' and 3' end positions (mature transcript length) are known. In such cases, the poly(A) tail length can be deduced by subtracting the predicted RNA product size from the observed product size. If the extremities are not known, they can be revealed via cRT-PCR sequencing. cRT-PCR can be coupled with a variety of downstream applications. For example, one can isolate, clone, and sequence the products of the reaction. Alternatively, one can radiolabel the products and separate them by SDS-PAGE in order to gain a more global statistical view of the sizes of the RNAs.

cRT-PCR can be used not only to elucidate the processes that govern the life of an RNA molecule but also to reveal those that govern its death. Among the various RNA decay pathways, a nearly ubiquitous one

is poly(A)-assisted RNA decay, a process that operates in prokaryotes, organelles, and the nuclei of eukaryotic cells (Deutscher, 2006; Slomovic and Schuster, 2008). Poly(A)-assisted RNA decay is initiated by the endonucleolytic cleavage of the transcript followed by the addition of a poly(A) or oligo(A) tail at the 3' end of the cleavage product. This tag serves as a 'landing pad' for 3'-5' exoribonucleases that then begin to digest the RNA fragment. Truncated RNA molecules that have undergone tail addition but have yet to be degraded are called degradation intermediates. The detection of such intermediates is considered to serve as a tell-tale sign that a poly(A)-assisted RNA decay pathway operates in the studied organism. Sequencing of the tail nucleotide composition can aid the researcher in identifying the enzyme responsible for tail synthesis since tails can be either homopolymeric (exclusively A's) or heteropolymeric (A-rich tails that may include other nucleotides). One of the techniques used to isolate such intermediates is oligo(dT)-primed RT-PCR (see [Oligo\(dT\)-primed RT-PCR isolation of polyadenylated RNA degradation intermediates](#)). However, this method involves annealing an oligo(dT) primer to the poly(A) tail and this can occur at any point along the tail; potentially altering the original tail length and composition. cRT-PCR can be used in addition to oligo(dT)-based methods in order to provide a nonbiased documentation of these degradation intermediates.

The following protocol describes the step-by-step analysis of the 5' end, 3' end, and poly(A) tail of an RNA molecule using cRT-PCR ([Fig. 13.1](#)). As described above, in addition to these applications, cRT-PCR can be used to isolate degradation intermediates of poly(A)-assisted RNA decay. In this case the primers are situated toward the middle of the RNA molecule rather than near the ends ([Fig. 13.2](#)).



2. EQUIPMENT

- Centrifuge
- Microcentrifuge
- Dry heating block
- PCR thermocycler
- Shaking incubator
- Incubator
- Electroporator
- UV/vis spectrophotometer
- Agarose gel electrophoresis equipment

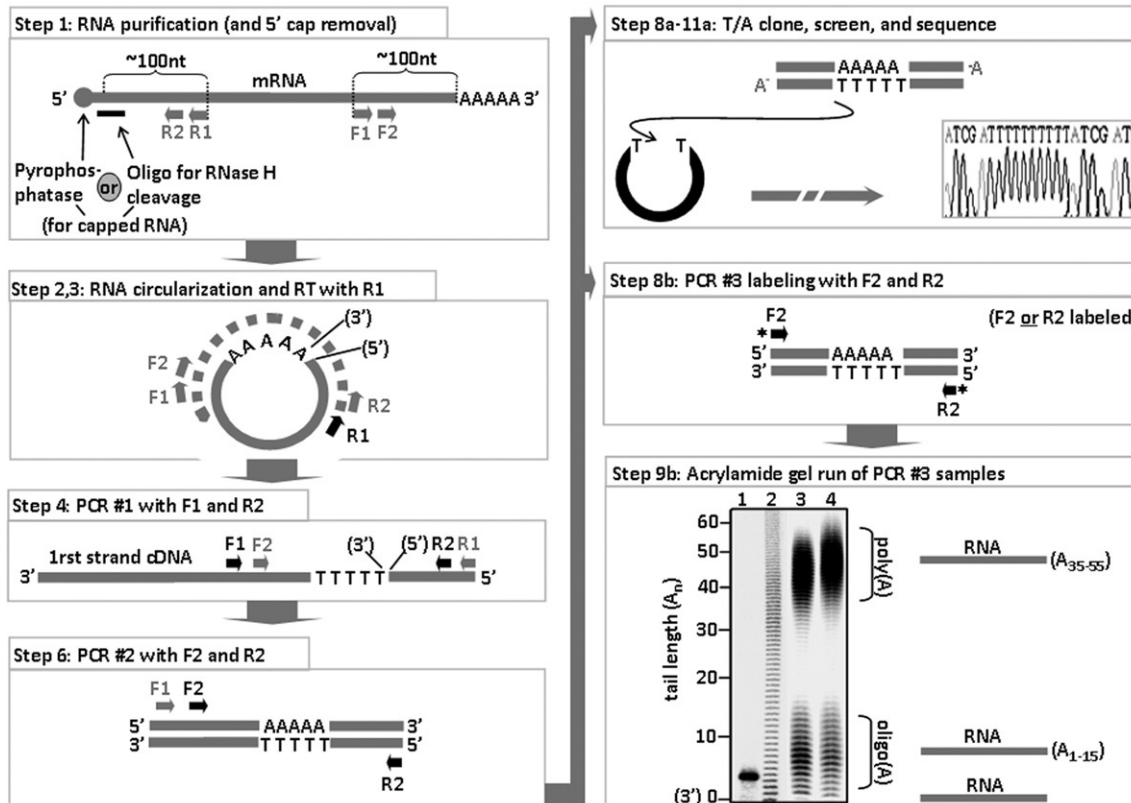


Figure 13.1 A schematic drawing of the steps of CRT-PCR RNA analysis are shown. Step1: The target mRNA is shown with the locations of the forward and reverse primers. Steps 2, 3: The 5' and 3' ends are joined with T4 RNA ligase and the RNA is subjected to gene-specific reverse transcription. Step 4: PCR using the F1 and R2 primers. The amplified region includes the adjoined 5' and 3' ends. Step 6: PCR using the F2 and R2 primers. To clone and sequence individual PCR products, follow Steps 8a–11a. To globally assess the lengths of the poly(A) tails, follow Steps 8b–9b. In the figure, an experiment studying the effect of RNAi silencing of the human mitochondrial PNPase on the poly(A) tail of the mitochondrial ND3 mRNA is shown. Lane 1: DNA oligonucleotide marker, Lane 2: An alkaline hydrolysis-prepared RNA nucleotide ladder, Lane 3: mock RNAi-treated cells, Lane 4: PNPase RNAi-treated cells. In this example, an oligoadenylated and polyadenylated population of the ND3 mRNA can be seen.

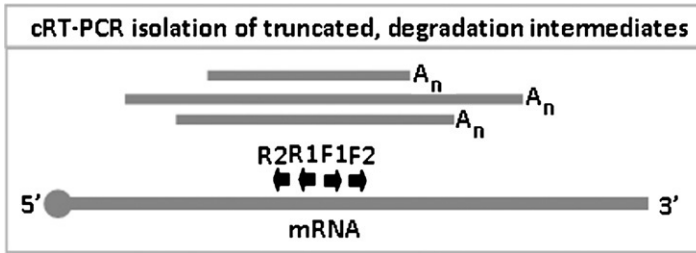


Figure 13.2 cRT-PCR is used to isolate degradation intermediates of poly(A)-assisted RNA decay. The four primers are grouped close to one another within the mRNA sequence (Slomovic and Schuster, 2006).

Polyacrylamide gel electrophoresis equipment
 Phosphorimager or film processor
 Phosphorimager cassette or autoradiography film
 Gel dryer (vacuum)
 Plexiglass shield
 UV camera
 UV light box
 Micropipettors
 Speed-vac
 Micropipettor tips
 Plate spreader or glass balls
 Scalpel
 ~10-ml vials
 1.5-ml microcentrifuge tubes
 0.5-ml microcentrifuge tubes
 0.2-ml thin-walled PCR tubes
 Electroporator cuvettes
 10-cm Petri dishes
 12-ml sterile polypropylene snap-cap tubes
 Toothpicks
 Whatman 3MM chromatography paper
 0.45 μm PVDF filters



3. MATERIALS

Potassium chloride (KCl)
 Ammonium sulfate $[(\text{NH}_4)_2\text{SO}_4]$
 Magnesium chloride (MgCl_2)
 Triton X-100

Bovine serum albumin (BSA)
Tris base
Boric acid (H_3BO_3)
EDTA
Diethylpyrocarbonate (DEPC)
Ethanol
Phenol/Chloroform (1:1)
Glacial acetic acid
Sodium acetate (NaOAc)
N,N-Dimethylformamide (DMF)
5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal)
Isopropyl β -D-1 thiogalactopyranoside (IPTG)
Yeast tRNA
Agarose
dNTP mix (100 mM)
Gene-specific PCR primers
100-bp DNA ladder
Formamide
Xylene cyanol
Bromophenol blue
Glycerol
Sodium dodecyl sulfate (SDS)
Ethidium bromide
Sodium chloride (NaCl)
Yeast extract
Bacto tryptone
Bacto/Difco agar
Ampicillin
[γ - ^{32}P]-ATP
40% Acrylamide (19:1 acrylamide/bis-acrylamide)
Urea
TEMED
Ammonium persulfate (APS)
T4 RNA ligase (Ambion)
RNase-inhibitor (RNAguard, GE Lifesciences)
DNase 1 (Ambion)
Taq polymerase (with A overhang ability)
Polynucleotide kinase
Tobacco acid pyrophosphatase
RNase H

XL1-Blue competent cells (Stratagene)
 AffinityScript™ Reverse Transcriptase (Stratagene)*
 pGEM®-T Easy T/A cloning vector (Promega)
 HiYield™ Gel/PCR DNA extraction kit (RBC Bioscience)
 GeneJET™ Plasmid Miniprep kit (Fermentas Life Sciences)
 MasterPure™ RNA Purification kit (Epicentre Biotechnologies)

3.1. Solutions & buffers

Step 4 10× PCR buffer

Component	Final concentration	Stock	Amount
Tris-HCl, pH 8.2	200 mM	1 M	200 ml
KCl	100 mM	2 M	50 ml
(NH ₄) ₂ SO ₄	60 mM	1 M	60 ml
MgCl ₂	20 mM	1 M	20 ml
Triton X-100	1 %		10 ml
BSA	0.1 mg/ml	100 mg ml ⁻¹	1 ml

Add water to 1 l

Step 5 5× TBE

Component	Final concentration	Stock	Amount
Tris base	446 mM		270 g
Boric acid	445 mM		137.5 g
EDTA, pH 8	10 mM	500 mM	100 ml

Add water to 5 l (no sterilization needed)

5× DNA sample buffer

Component	Final concentration	Stock	Amount
Xylene cyanol			0.25 g
Bromophenol Blue			0.25 g
SDS	0.3%	20%	0.625 ml
Glycerol			12.5 ml

Add water to 40 ml

*Kits/enzymes listed here are suggested due to validated efficiency but similar kits produced by other manufacturers can be used instead.

Step 9a LB and LB-agar (plates)

Component	Final concentration	Stock	Amount
NaCl			10 g
Bacto tryptone			10 g
Yeast extract			5 g
Bacto/Difco agar (for LB-agar plates)			15 g
* Ampicillin	0.1 mg ml ⁻¹	100 mg ml ⁻¹	1 ml

Add water to 1 l and autoclave

LB for electroporation is antibiotic-free and should be divided into glass vials with 5 ml in each and autoclaved

*Add to warm (not hot) media after autoclaving and then pour plates

Step 9b Acrylamide gel Solution A

Component	Final concentration	Stock	Amount
40% acrylamide/bis-acrylamide (19:1)	15%		100 ml
Urea	7 M		112 g
5 × TBE	1 ×		53.4 ml

Add water to 267 ml

Acrylamide gel Solution B

Component	Final concentration	Stock	Amount
Urea	7 M		420 g
5 × TBE	1 ×		200 ml

Add water to 1 l

Formamide loading dye

Component	Final concentration	Stock	Amount
Formamide			49 ml
EDTA		0.5 M	1 ml
Xylene cyanol			13 mg
Bromophenol blue			13 mg



4. PROTOCOL

4.1. Duration

Preparation	About 1 day
Protocol	About 4–5 days

4.2. Preparation

1. This protocol can be used with RNA from virtually any organism. The particular research model organism should be grown and ready for RNA purification.
2. All oligonucleotides used for reverse transcription and PCR must be designed (see Explanatory chapter: PCR -Primer design) and ordered. The gene-specific primers should have melting temperatures of ~ 60 °C. If RNase H is to be used to cleave the 5' cap, an appropriate oligonucleotide must be designed.
3. LB-agar plates can be prepared during the protocol or ahead of time.

4.3. Caution

Gloves and RNase-free material should always be used when working with RNA. Take preventive measures when working with UV light to minimize UV-induced damage.

4.4. Caution

Consult your institute Radiation Safety Officer for proper ordering, handling, and disposal of radioactive materials.



5. STEP 1 RNA PURIFICATION

5.1. Overview

Purify RNA from research organism (Fig. 13.1, Step 1).

5.2. Duration

1–2 h

- 1.1 Purify total RNA from research organism using an RNA purification kit or Trizol reagent. RNA can be resuspended in elution buffer,

TE buffer, or DEPC-treated water. The volume depends on the amount of starting material.

- 1.2 Measure RNA concentration using a spectrophotometer (see [Explanatory Chapter: Nucleic Acid Concentration Determination](#)). RNA should be kept on ice and stored at -20 or below.

5.3. Tip

The NanoDrop 2000 spectrophotometer (Thermo Scientific) can measure the concentration using just 1 μl of RNA eluate.

5.4. Tip

The ideal RNA concentration is 0.5–1 $\mu\text{g } \mu\text{l}^{-1}$ and should be adjusted accordingly.

5.5. Tip

Usually total RNA is used, even when examining RNA that is found within an organelle. However, sometimes isolation of the organelle followed by RNA purification is necessary.



6. STEP 2 CIRCULARIZATION OF RNA MOLECULES

6.1. Overview

Ligate the 5' and 3' ends of the RNA to circularize it. When studying unprocessed RNAs it may be necessary to remove the 5' cap by oligonucleotide-directed RNase H cleavage of the 5' end (Fig. 13.1, Steps 2–3).

6.2. Duration

1–1.5 h

2 h (optional, for RNase H cleavage of the 5' end)

- 2.1 (optional) Remove the 5' cap using either RNase H (together with an oligonucleotide that will anneal to a designated position along the RNA sequence) or tobacco acid pyrophosphatase. We recommend a protocol available online (<http://www.mcb.arizona.edu/parker/PROTOCOLS/RnaseHDigestions.htm>).

- 2.2 Ligate the ends of the RNA to circularize them.

Mix:

3–5 μg total RNA

DEPC-treated water to 15 μ l

Heat at 70 °C, 2 min, and then chill on ice for 5 min.

Add:

2 μ l 10 \times T4 RNA ligase buffer

1 μ l DNase 1

1 μ l T4 RNA ligase

1 μ l RNase inhibitor

Incubate at 37 °C, 1 h.

Add 80 μ l DEPC-treated water and 100 μ l phenol/chloroform (1:1). Vortex samples and centrifuge at 13000 rpm for 2 min at room temperature. Transfer the upper phase to a new tube.

2.3 Add one-tenth volume (10 μ l) 3 M NaOAc, pH 5.5, and 2.5 volumes (250 μ l) ice-cold absolute ethanol to precipitate the circularized RNA. Put at -20 °C for at least 1 h.

2.4 Centrifuge at 14 000 rpm at 4 °C for 20 min. Carefully remove the supernatant and replace with 500 μ l 70% ethanol. Centrifuge at 14 000 rpm at 4 °C for 10 min, remove the supernatant, and air-dry the pellet. Resuspend the pellet in 14.2 μ l DEPC-treated water.

See [Fig. 13.3](#) for the flowchart of Step 2.



7. STEP 3 REVERSE TRANSCRIPTION USING A GENE-SPECIFIC REVERSE PRIMER (R1)

7.1. Overview

Reverse transcribe the RNA using a gene-specific primer (R1), positioned ~100 nucleotides from the 5' end of the transcript or the RNase H cleavage site. A cDNA comprising the adjoined 5' and 3' ends is produced ([Fig. 13.1](#), Steps 2–3).

7.2. Duration

1–1.5 h

3.1 Mix:

14.2 μ l circularized RNA

1.5 μ l DEPC-treated water

1 μ l R1 primer (~500 ng)

3.2 Heat at 65 °C for 5 min. Set at room temperature for ~10 min to anneal primers to the RNA.

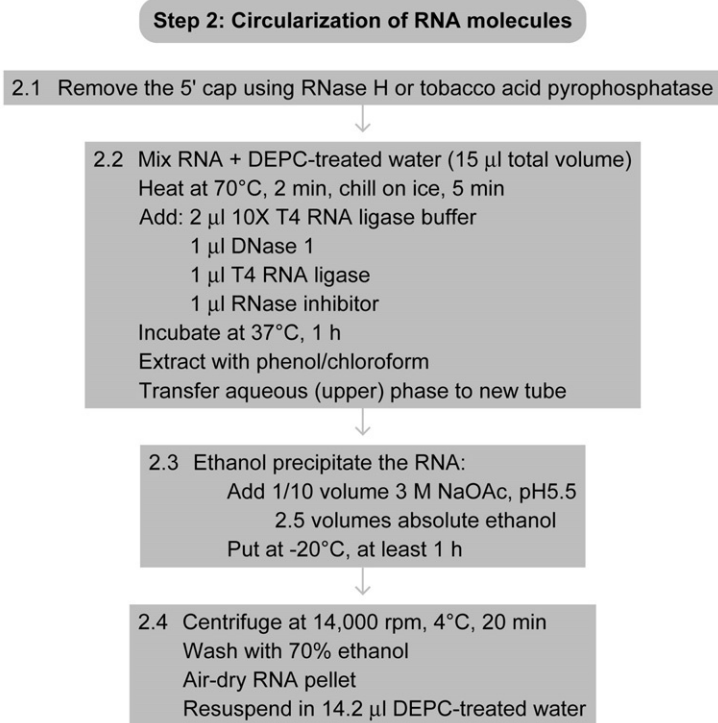


Figure 13.3 Flowchart of Step 2.

3.3 Add:

- 2 µl 10× reverse transcriptase buffer
- 0.8 µl dNTP mix (25 mM)
- 0.5 µl RNase inhibitor
- 1 µl reverse transcriptase (AffinityScript)

3.4 Incubate at 42–55 °C for 1 h. Heat inactivate enzyme at 70 °C for 15 min.

3.5 Add DEPC-treated water to a total volume of 50 µl.

See [Fig. 13.4](#) for the flowchart of Step 3.



8. STEP 4 PCR USING FORWARD PRIMER (F1) AND REVERSE PRIMER (R2)

8.1. Overview

Perform PCR on the cDNA template using a gene-specific reverse primer (R2) and forward primer (F1). This amplifies the relevant region and

Step 3: Reverse transcription using a gene-specific primer (R1)

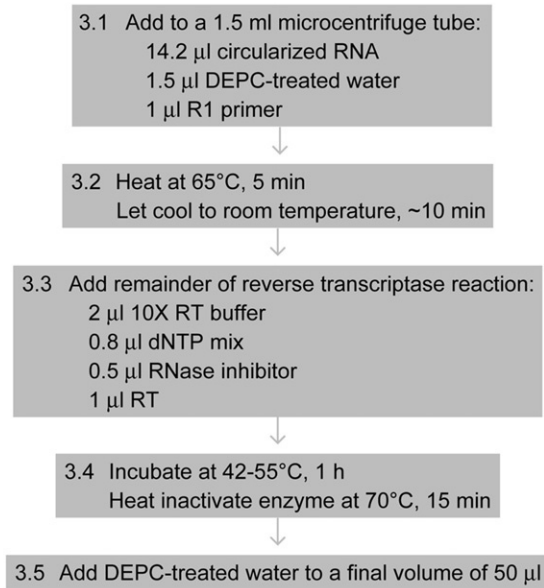


Figure 13.4 Flowchart of Step 3.

contributes to specificity since the R2 primer is ‘nested’ under the R1 primer (Fig. 13.1, Step 4).

8.2. Duration

1.5–2 h

4.1 Prepare the following PCR reaction:

1 µl cDNA

2 µl 10× PCR buffer

1 µl dNTP mix (2 mM)

0.5 µl (50 ng) gene-specific forward primer (F1)

0.5 µl (50 ng) gene-specific reverse primer (R2)

0.3 µl Taq polymerase (with A overhang capability)

Water to 20 µl

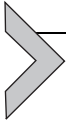
4.2 Run the following standard PCR program:

5 min at 90 °C

- 30 cycles:
- 1 min at 90 °C
 - 1 min at 58 °C
 - 1 min at 72 °C
 - 10 min at 72 °C
 - Pause/end at 15 °C

8.3. Tip

It is recommended that the PCR be carried out in duplicate or triplicate.



9. STEP 5 GEL PURIFICATION OF PCR PRODUCTS

9.1. Overview

Run the PCR products on an agarose gel (see Agarose Gel Electrophoresis) and then purify them.

9.2. Duration

~1 h

- 5.1 Prepare 1% agarose gel by fully dissolving 1 g agarose in 100 ml 1× TBE buffer in microwave. Cool slightly and then add 5 µl ethidium bromide and pour gel.
- 5.2 Combine duplicate or triplicate reactions in order to run in a single gel lane.
- 5.3 Add 4 µl of 5× DNA sample buffer, heat at 80 °C for 3 min and place on ice for 5 min.
- 5.4 Load sample on gel along with the 100 bp DNA molecular weight marker and run in 1× TBE buffer at 100 V for 20–30 min (until xylene cyanol and bromophenol blue markers are separated by 1–2 cm).
- 5.5 Evaluate gel separation and DNA profile with UV light box using proper UV protection (gloves, lab coat, glasses).
- 5.6 Excise the region of the gel containing the PCR product and isolate the DNA from the gel slice using a gel extraction kit. Elution volume should be <15 µl.

9.3. Tip

In some cases it is possible to simply perform a PCR cleanup instead of carrying out gel purification. The advantage of gel purification is that artifactual bands (due to non-specific priming during PCR) that would otherwise predominate in later stages can be avoided.

9.4. Tip

If the transcript has a known or estimated sequence length, the expected PCR product size (or size range) can be calculated from the transcript sequence and the positions of the primers. Therefore, one can usually identify the correct gel band to be isolated from the gel (although an above and below margin of reasonable size should be included in the excised piece in order to avoid losing valuable data). If there is more than one band, they can be removed and transferred to separate tubes.

9.5. Tip

Some types of agarose can impair the cloning process. This should be considered when choosing the agarose.

See Fig. 13.5 for the flowchart of Steps 4 and 5.

Step 4: PCR using forward (F1) and reverse (R2) primers

4.1 Add to 0.2 ml PCR tube:
 1 μ l cDNA
 2 μ l 10X PCR buffer
 1 μ l dNTP mix
 0.5 μ l each primer, F1 and R2
 0.3 μ l Taq polymerase
 14.7 μ l water

4.2 Run standard PCR program:
 5 min at 90°C
 30 cycles 1 min at 90°C
 1 min at 58°C
 1 min at 72°C
 10 min at 72°C
 end at 15°C

5.2-5.3 Combine duplicate PCR reactions
 Add 4 μ l 5X DNA sample buffer
 Heat at 80°C, 3 min, chill on ice, 5 min

Step 5: Gel purification of PCR products

5.1 Prepare 1% agarose gel

5.4-5.5 Load samples on agarose gel
 Run gel at 100 V, 20-30 min
 Check separation of bands using a UV light box

5.6 Excise portion of gel containing PCR products
 Purify DNA using gel extraction kit
 Elute in 15 μ l

Figure 13.5 Flowchart of Steps 4 and 5.



10. STEP 6 SECOND ROUND PCR

10.1. Overview

Carry out a second round of PCR using a gene-specific reverse primer (R2) and forward primer (F2). This step contributes to the specificity since the F2 primer is 'nested' under the F1 primer used in the first PCR reaction (Fig. 13.1, Step 6).

10.2. Duration

1.5–2 h

6.1 This step is carried out exactly as in Step 4 but with the R2 and F2 primers. Use 1 μ l of the gel eluate from Step 5 as the template DNA.



11. STEP 7 GEL PURIFICATION OF PCR PRODUCTS

11.1. Overview

The PCR products are run on an agarose gel (see Agarose Gel Electrophoresis) and then purified.

11.2. Duration

~1 h

7.1 Carry out this step exactly as in Step 5.

7.2 Reduce the sample volume to ~6 μ l in a Speedvac.

Note: At this point, the protocol branches along two alternative pathways. To clone and sequence the PCR products, follow Steps 8a–11a. To radio-label the PCR products to globally assess the size range of the poly(A) tails, follow Steps 8b–9b. Each pathway can use half of the material from Step 7.



12. STEP 8A T/A CLONE THE SECOND ROUND PCR PRODUCTS

12.1. Overview

Clone the purified second round PCR products into a cloning T/A vector (Fig. 13.1, Steps 8a–11a).

12.2. Duration

3 h – overnight

- 8a.1** Ligate the PCR products into the pGEM[®]-T Easy vector.
To a 0.5-ml microcentrifuge tube add:
- 5 μ l 2 \times T4 DNA Ligase Rapid Ligation Buffer
 - 1 μ l pGEM[®]-T Easy vector
 - 3 μ l PCR product DNA
 - 1 μ l T4 DNA ligase
- 8a.2** Incubate at room temperature for 1 h or, for a maximal number of transformants, overnight at 4 °C. Add water to a final volume of 50 μ l.
- 8a.3** Add one-tenth volume (5 μ l) 3 M NaOAc, pH 5.5, 2.5 volumes (125 μ l) ice-cold absolute ethanol, and 1 μ l yeast tRNA to precipitate the DNA. Put at -20 °C for at least 1 h.
- 8a.4** Centrifuge at 14 000 rpm at 4 °C, for 20 min. Carefully remove the supernatant and replace with 500 μ l 70% ethanol. Centrifuge at 14 000 rpm at 4 °C for 10 min and remove the supernatant, and air-dry the pellet.
- 8a.5** Resuspend the pellet in 8 μ l water.



13. STEP 9A ELECTROPORATE AND PLATE BACTERIA

13.1. Overview

Electroporate the cloned PCR products into competent cells (see Transformation of *E. coli* via electroporation), and then plate the bacteria onto selective plates (Fig 13.1, Steps 8a–11a).

13.2. Duration

1 h (plus overnight incubation)

- 9a.1** Add 1 μ l of the ligated DNA to an electroporation cuvette along with 40–50 μ l XL1-Blue competent cells and electroporate.
- 9a.2** Add 1 ml LB (without antibiotics) to cuvette and transfer electroporation reaction to 1.5-ml microcentrifuge tube. Incubate at 37 °C for 1 h.
- 9a.3** Using sterile techniques, add 60 μ l X-gal (from 2% stock) and 20 μ l IPTG (from 0.1 M stock) to LB + ampicillin (LB-amp) plates while transformed bacteria are incubating. Spread fluid on agar surface with sterile glass spreader or glass balls and let it dry. One to three plates should be prepared for each sample.
- 9a.4** Remove the tube from 37 °C water bath; vortex briefly; and add 50, 100, or 200 μ l to each plate using sterile techniques. Plate different

volumes so there is a choice of bacterial colony densities for the next step. Incubate the plates O/N in a 37-C incubator.

13.3. Tip

If the bacteria are electroporated and plated at 2:00, the bacterial colonies will be ready at 9:00 the following morning. If the plates are left at 37 °C for much longer than this, 'satellite' colonies will appear and hamper the screening of individual colonies in the next step.



14. STEP 10A SCREEN CLONES BY COLONY PCR

14.1. Overview

Screen colonies by PCR (see Colony PCR) to identify positive clones for plasmid DNA isolation and DNA sequencing (Fig. 13.1, Steps 8a–11a).

14.2. Duration

1–1.5 days

- 10a.1** Remove plates from 37-C incubator and evaluate bacterial colony density. Choose plates with a colony density that allows picking single colonies without touching adjacent ones.
- 10a.2** Prepare master LB-amp plates (X-gal and IPTG are not needed). The master plate should be divided into a matrix with each transferred colony assigned a number. Use a toothpick or pipette tip to touch white colonies and transfer them to the LB-amp master plate. It is suggested that you start by picking 48 colonies.
- 10a.3** Place the master plate in a 37-C incubator for 6–24 h.
- 10a.4** Prepare a PCR reaction for each colony to be screened as in Step 4.1. Use the R2 and F2 oligonucleotides as primers.
- 10a.5** Pick each colony on the master plate with a toothpick and swirl in a PCR tube. Store the master plate at 4 °C.
- 10a.6** Subject the samples to PCR as in Step 4.2.
- 10a.7** Pour an agarose gel as before. Run the samples in the gel, examine with UV light table, and identify colonies with relevant inserts by their molecular weight.

14.3. Tip

Colonies that survived the ampicillin selection contain the pGEM vector: blue colonies are empty vectors lacking inserts and white colonies contain an insert.



15. STEP 11A PLASMID DNA ISOLATION AND DNA SEQUENCING

15.1. Overview

Isolate plasmid DNA from positive colonies (see Isolation of plasmid DNA from bacteria) using a plasmid DNA mini-prep kit and sequence the DNA (Fig. 13.1, Steps 8a–11a).

15.2. Duration

1–4 days

11a.1 Prepare LB-amp by adding ampicillin to LB media (1 μl per ml of LB from 100 mg ml^{-1} stock). For each clone add 4–5 ml of LB-amp to a \sim 10–13-ml tube.

11a.2 Pick positive colonies from the master plate using toothpicks. Swirl toothpick in the LB-amp. Incubate the tubes overnight at 37 °C, with shaking.

11a.3 Isolate plasmid DNA from each clone using a plasmid DNA mini-prep kit.

11a.4 Sequence plasmid inserts: evaluate the 5' and 3' ends, the length of the poly(A) or (U) tail, and nucleotide composition.

See Fig. 13.6 for the flowchart of Steps 8a–11a.



16. STEP 8B RADIOLABEL A PRIMER AND USE IT IN PCR

16.1. Overview

End-label either the F2 or R2 oligo with $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ and use it in PCR (Fig. 13.1, Step 8b).

16.2. Duration

3–4 h

8b.1 Choose which oligonucleotide, either F2 or R2, to 5' end-label.

8b.2 Set up the following reaction:

1 μl oligonucleotide (100 ng)

4 μl $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$

1 μl 10 \times PNK buffer

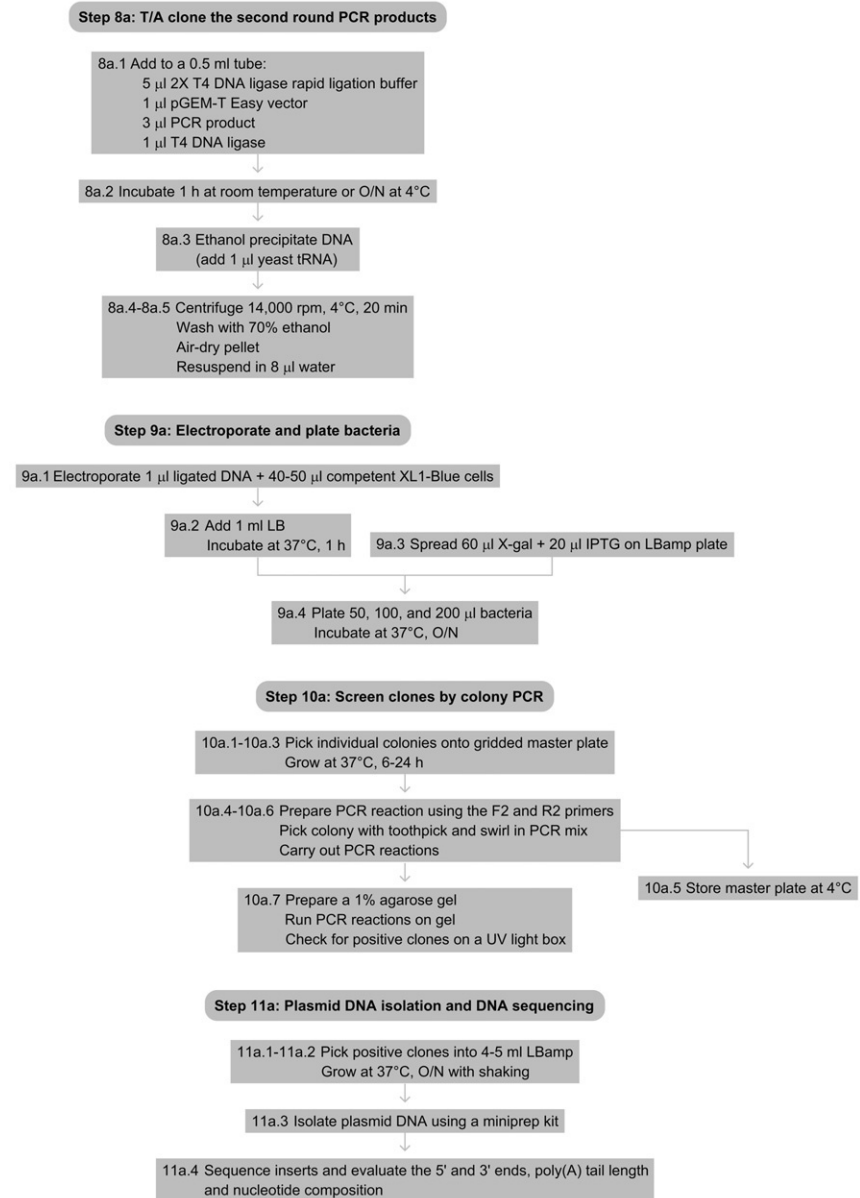


Figure 13.6 Flowchart of Steps 8a–11a.

1 μl polynucleotide kinase (PNK)

3 μl water

Incubate at 37 °C for 1 h. Inactivate enzyme at 85 °C for 2 min.

Add 40 μl water, one-tenth volume (5 μl) 3 M NaOAc, pH 5.5, and 2.5 volumes (125 μl) ice-cold absolute ethanol. Put at -20 °C for at least 1 h.

Centrifuge at 14 000 rpm at 4 °C for 20 min, wash with 70% ethanol, and air-dry pellet. Resuspend in 10 μl water. Labeled primer is now at $\sim 10 \text{ ng } \mu\text{l}^{-1}$.

8b.3 Prepare the following PCR reaction:

1 μl DNA (second round PCR products from Step 7.2)

2 μl 10 \times PCR buffer

1 μl dNTP mix (2 mM)

1 μl [³²P]-labeled primer (F2 or R2)

1 μl second gene-specific primer (either R2 or F2, 10 $\text{ng } \mu\text{l}^{-1}$)

0.3 μl Taq polymerase

Water to 20 μl

8b.4 Run the following PCR program:

5 min at 90 °C

6 cycles:

1 min at 90 °C

1 min at 58 °C

1 min at 72 °C

10 min at 72 °C

Pause/end at 15 °C

16.3. Tip

To decide which oligonucleotide to end-label, F2 or R2, perform two PCR reactions using cDNA from the studied organism as template each containing only one of the two primers. Analyze the products by agarose gel electrophoresis and for step 8b, use the oligo that does not amplify products by itself.



17. STEP 9B ANALYZE PCR PRODUCTS BY POLYACRYLAMIDE GEL ELECTROPHORESIS

17.1. Overview

Run PCR products on a polyacrylamide gel and visualize using a phosphorimager (Fig. 13.1, Step 9b).

17.2. Duration

7–8 h

- 9b.1** Prepare an 8% polyacrylamide sequencing gel (50 ml volume). Combine 26.7 ml acrylamide gel Solution A with 23.3 ml acrylamide gel Solution B. Add 400 μ l 20% APS, 40 μ l TEMED, and pour the gel.
- 9b.2** Prerun the gel at 35 mA for 30 min and clean out the wells. Add 5 μ l formamide loading dye to 10 μ l of each sample. Heat samples at 85 °C for 3 min, chill on ice for 5 min, and load on gel.
- 9b.3** Run the gel at 35 mA with an aluminum heat-diffusing plate. Once the gel run is completed, carefully transfer the gel to two pieces of Whatman 3MM chromatography paper and dry in a vacuum gel dryer at 80 °C for 1 h. Continue under vacuum for another 30 min to cool slowly.
- 9b.4** Place dry gel in a phosphorimager cassette overnight and scan on a phosphorimager.

17.3. Tip

A midi gel (25 ml) can be used instead of a sequencing gel if one is not available, although it may provide lower gel resolution.

17.4. Tip

The remainder of the PCR sample can be stored at –20 °C with proper shielding for ³²P.

17.5. Tip

The xylene cyanol and bromophenol blue can assist in judging when to stop the gel run. On an 8% polyacrylamide gel, xylene cyanol runs at 19 nucleotides and bromophenol blue runs at 75 nucleotides.

17.6. Tip

During PCR Taq polymerase disengages from the template many times before reaching the end, thus a ladder of gel bands at the resolution of a single nucleotide may be visible, despite extension stage of PCR. This ladder can be utilized along with a radiolabeled DNA or RNA molecule of a known size run in the gel. In analyzing the gel results, focus on the intensity of the gel bands. Bands that represent the true sizes of the molecules can be distinguished from those due to Taq falling off the template by their greater intensity. A range of these bands is expected, as they represent

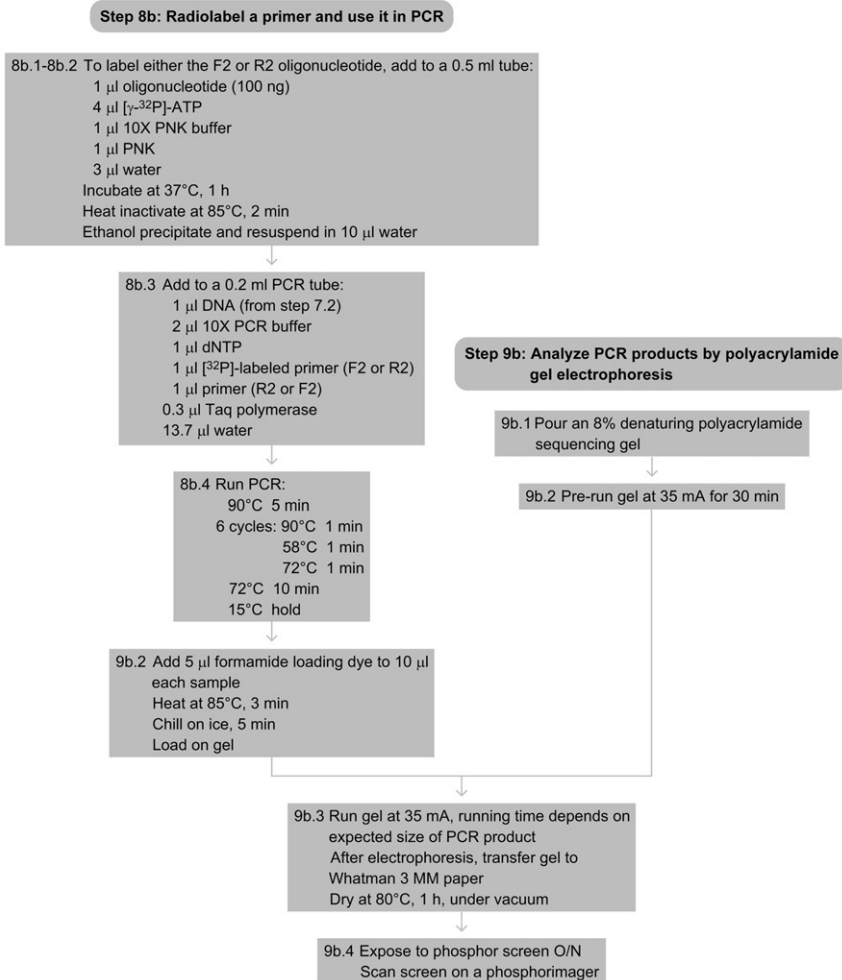


Figure 13.7 Flowchart of Steps 8b–9b.

a population of the target mRNA, each with a tail of differing length, with the average tail-length appearing as a region of more intense gel bands (Fig. 13.1, Step 9b).

See Fig. 13.7 for the flowchart of Steps 8b–9b.

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[Oligo\(dT\)-primed RT-PCR isolation of polyadenylated RNA degradation intermediates.](#)

Explanatory chapter: PCR -Primer design.

[Explanatory Chapter: Nucleic Acid Concentration Determination.](#)

Agarose Gel Electrophoresis.

Transformation of E. coli via electroporation.

Colony PCR.

Isolation of plasmid DNA from bacteria.