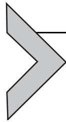

CHAPTER TWELVE

Oligo(dT)-primed RT-PCR Isolation of Polyadenylated RNA Degradation Intermediates

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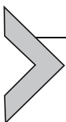
Abstract

The posttranscriptional modification of RNA by polyadenylation serves various purposes, among them to assist in RNA degradation (see an alternative protocol for measuring RNA degradation on Method for measuring mRNA decay rate in *Saccharomyces cerevisiae*). This function, once thought to occur in prokaryotic or organellar systems alone, is now known to operate in the nuclei and cytoplasm of eukaryotes as well (Slomovic et al., 2008; Slomovic et al., 2010; Houseley and Tollervey, 2009; Deutscher, 2006). Poly(A)-assisted RNA decay begins with the endonucleolytic cleavage of the transcript. Following this, a poly(A) or oligo(A) tail is added to 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 a tell-tale sign that poly(A)-assisted RNA decay occurs in the organism being studied. Determination of the tail nucleotide composition by DNA sequencing often aids the researcher in identifying the enzyme responsible for tail synthesis since tails can be either homopolymeric (exclusively A residues) or heteropolymeric (A-rich tails that may include other nucleotides). The following protocol, based on oligo(dT)-primed reverse transcription, describes the step-by-step detection and isolation of adenylated degradation intermediates in the study of poly(A)-assisted RNA decay.



1. THEORY

As described above, poly(A)-assisted RNA decay is a process in which RNA molecules are tagged with an oligo(A) or poly(A) tail which mechanistically assists exoribonucleases in identifying and degrading these

substrates. Research has shown that poly(A)-assisted RNA decay occurs in almost every life form. The general scheme of poly(A)-assisted RNA decay begins with endonucleolytic cleavage of the transcript, followed by tail addition and finally, 3′–5′ exonucleolytic degradation, albeit this process can vary between different biological systems. The adenosine tails that are produced during poly(A)-assisted RNA decay are termed 'transient,' since they are degraded along with the RNA fragment, as opposed to stable poly(A) tails which are found on the mature 3′ ends of most nuclear-encoded mRNAs and encourage transcript longevity. Transient tails can be called 'internal', as well, because when aligning the adenylated RNA fragment sequence with that of the mRNA's full-length sequence, the site at which the tail exists is internal, relative to the full sequence (upstream of the mature 3′ end).

Bound by their role in assisting in their 'host' RNA's decay, transient tails are degraded along with the RNA fragment at an extremely rapid rate. This leads to a very low abundance of adenylated degradation intermediates. Oligo(dT)-primed reverse transcription capitalizes on the poly(A) characteristic of these molecules and several steps of gene-specific PCR allow their isolation. However, a drawback of poly(A)-biased methods is that the oligo(dT) can anneal at any point along the adenosine tail. This can result in a reported tail length that does not reflect the *in vivo* length of the tail. Techniques such as circularized reverse transcription (see Circularized RT-PCR (cRT-PCR): analysis of RNA 5′ ends, 3′ ends, and poly(A) tails) can help overcome this drawback, although the low abundance of adenylated degradation intermediates can hamper their isolation when applying non-A-biased methods. Truncated molecules can be obtained using such techniques, but the majority of them lack poly(A) tails, either due to the fact that the tail was degraded prior to isolation or that the tail was yet to be added. However, in many cases, truncated molecules with a small number of adenosines (even as few as one adenosine) can be detected using circularized RT-PCR or other non-poly(A)-biased approaches. This serves as important data showing that the long poly(A) tails detected when using oligo(dT) priming do not necessarily accurately represent the tail population. Therefore, to get a more complete picture, it is important to apply both poly(A)-biased and nonbiased approaches.

***Note*: Oligo(dT)-primed RT-PCR can be used to analyze the mature 3′ end of an RNA molecule. In this case, the protocol varies in that the PCR product in Step 4 is a single gel band of an expected size rather than a smear. This product can be isolated from the gel and the protocol continued from Step 7.

2. EQUIPMENT

Biological hood
Electroporator
Spectrophotometer
Microcentrifuge
Heating block
PCR thermocycler
Shaker/incubator (37 °C)
UV camera
UV light box
Agarose gel electrophoresis equipment
Speed-vac (optional)
Micropipettors
Micropipettor tips
Scalpel
Electroporation cuvettes
Sterile plate spreader or glass balls
10-cm Petri plates
Toothpicks
10-ml glass vials
10–13-ml snap-cap polypropylene tubes (these tubes need to have caps that allow air to pass through while growing/shaking the bacteria within)
1.5-ml tubes
0.2-ml thin-walled PCR tubes
Whatman filter paper
0.45-μm PVDF filters

3. MATERIALS

Adapter oligo (without oligo(dT))
Gene-specific PCR primers
Purified water (deionized or milli-Q filtered)
Diethylpyrocarbonate (DEPC)
DEPC-treated water
Ethanol
Glacial acetic acid

Sodium acetate (NaOAc)
Sodium chloride (NaCl)
Dimethylformamide (DMF)
Bacto tryptone
Yeast extract
Bacto/Difco agar
Ampicillin
X-gal
Isopropyl β-D-1 thiogalactopyranoside (IPTG)
Tris base
Boric acid (H_3BO_3)
EDTA
Yeast tRNA
Agarose
Potassium chloride (KCl)
Ammonium sulfate [$(NH_4)_2SO_4$]
Magnesium chloride ($MgCl_2$)
Triton X-100
Bovine serum albumin (BSA)
dNTP mix (100 mM)
100-bp DNA ladder
Xylene cyanol
Bromophenol blue
Glycerol
Sodium dodecyl sulfate (SDS)
Ethidium bromide
Oligo(dT)-adapter oligo
Taq polymerase (with A overhang ability)
AffinityScript™ Reverse Transcriptase (Stratagene)*
XL1-blue competent cells (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)

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

3.1. Solutions & buffers

Step 3 10× PCR buffer

Component	Final concentration	Stock	Amount/100 ml
KCl	100 mM	2 M	5 ml
$(NH_4)_2SO_4$	60 mM	1 M	6 ml
$MgCl_2$	20 mM	1 M	2 ml
Tris–HCl, pH 8.2	200 mM	1 M	20 ml
Triton X-100	1%	10%	10 ml
BSA	0.1 mg ml^{-1}	100 mg ml^{-1}	0.1 ml

Add water to 100 ml

Step 4 5× TBE

Component	Final concentration	Stock	Amount/5 l
Tris base	445 mM		270 g
Boric acid	445 mM		137.5 g
EDTA, pH 8	10 mM	0.5 M	100 ml

Add water to 5 l (no sterilization needed)

5× DNA sample buffer

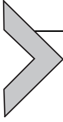
Component	Final concentration	Stock	Amount/40 ml
Xylene cyanol			0.25 g
Bromophenol Blue			0.25 g
SDS	0.3%	20%	0.6 ml
Glycerol			12.5 ml

Add water to 40 ml

Step 8 LB and LB-agar (plates)

Component	Final concentration	Stock	Amount/l
NaCl			10 g
Bacto tryptone			10 g
Yeast extract			5 g
For LB-agar plates, Bacto/Difco agar			15 g
Ampicillin	0.1 mg ml^{-1}	100 mg ml^{-1}	1 ml

Add water to 1 l and autoclave
*Add to warm (not hot) solution after autoclaving
**LB for electroporation is antibiotic-free and should be divided into glass vials with 5 ml in each and autoclaved



4. PROTOCOL

4.1. Duration

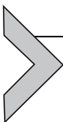
Preparation	About 1 day
Protocol	About 4–5 days

4.2. Preparation

1. This protocol can be used with RNA isolated from virtually any organism.
   The source for the RNA should be grown and ready for RNA purification.
2. Design and order all oligos used for reverse transcription and PCR (see Explanatory chapter: PCR -Primer design).
   The oligo(dT)-adapter should have the sequence:
   5′-GACTCGAGTCGACATCGAT$_{10}$-3′.
   The adapter oligo (without the poly(dT) tract) should have the sequence:
   5′-GACTCGAGTCGACATCGAT-3′.
   The melting temperature of the gene-specific primers should be ~60 °C.
3. LB-ampicillin agar plates can be prepared 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.



5. STEP 1 RNA PURIFICATION

5.1. Overview

Purify RNA from research organism (Fig. 12.1, Step 1).

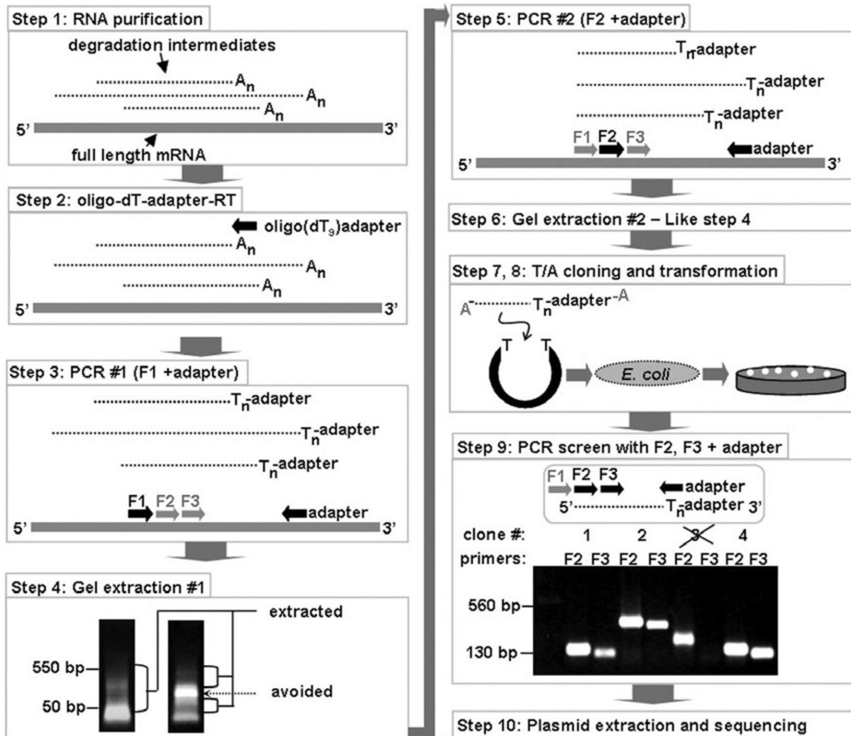


Figure 12.1 A summary of the oligo(dT)-primed isolation of adenylated degradation intermediates of poly(A)-assisted RNA decay. The summary is divided into steps parallel to those described in this protocol. *Step 1*. RNA purification: An mRNA is depicted with truncated, adenylated intermediates above. *Step 2*. Oligo(dT)-adapter RT: Total RNA is reverse transcribed using the oligo(dT)-adapter oligo. *Step 3*. PCR #1: PCR is performed on the cDNA using a gene-specific forward primer (F1) and the adapter oligo. F1, F2, and F3 are gene-specific forward primers each nested immediately downstream of the previous one. *Step 4*. Gel extraction: Run the PCR products on an agarose gel and extract the DNA running between 50 and 550 bp. In the figure, two example lanes are shown. The right lane shows a dominant band that should be avoided, as it is most likely due to nonspecific amplification or the mature 3′ end poly(A) tail of the full-length transcript. *Step 5*. PCR # 2: PCR is performed on the excised DNA using the gene-specific F2 primer together with the adapter oligo. *Step 6*. Gel extraction: Run PCR products on an agarose gel and isolate DNA as in Step 4. *Step 7*. Clone PCR products into a T/A cloning vector. *Step 8*. Transform XL1-blue competent cells and plate on LB-amp plates. *Step 9*. Screen white colonies by colony PCR. Prepare two PCR reactions for each colony: the first uses the F2 and adapter primers and the second uses the F3 and adapter primers. (Note: Be sure to replicate all screened colonies to a numbered master LB-amp plate before or during the PCR screening.) A positive colony (1, 2, and 4 in the figure) has a band in lane 2 that is slightly shorter than that found in lane 1. *Step 10*. Isolate plasmid mini-prep DNA and sequence the inserts.

5.2. Duration

1–2 h

1.1 Purify total RNA from desired source using an RNA purification kit. RNA can be resuspended in DEPC-treated water (RNase-free), elution buffer, or TE. The volume depends on the amount of starting material.

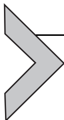
1.2 Measure RNA concentration using a spectrophotometer (see Explanatory Chapter: Nucleic Acid Concentration Determination). Keep RNA on ice and store at −20 °C or lower.

5.3. Tip

Ideal RNA concentration is 0.5–1 μg μl^{-1} and should be adjusted accordingly.

5.4. Tip

Total RNA can usually be used, even when examining RNA found in a particular organelle. However, sometimes a high background necessitates isolation of the organelle, followed by RNA purification. Polyadenylated RNA can be enriched but this is usually not required.



6. STEP 2 OLIGO(DT)-PRIMED REVERSE TRANSCRIPTION

6.1. Overview

Reverse-transcribe RNA using the oligo(dT)-adapter as a primer (Fig. 12.1, Step 2).

6.2. Duration

1–1.5 h

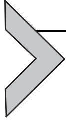
2.1 Typically, 0.5–5 μg total RNA is sufficient for reverse transcription.

Carry out the reaction using the oligo(dT)-adapter and a reverse transcriptase enzyme, such as AffinityScript™ (Stratagene), according to the manufacturer's instructions. Prepare a negative control reaction in which no reverse transcriptase is added in order to assess the presence of PCR products amplified from contaminating DNA.

2.2 Following reverse transcription, adjust the 3′ adapter-tailed cDNA volume to 50 μl with purified water and store at −20 °C.

6.3. Tip

Adapter sequences can be planned in order to introduce a restriction enzyme recognition site. However, no restriction enzyme sites are needed in this protocol. The poly(dT) tract at the 3′ end of the oligo(dT)-adapter should be at least nine nucleotides in length and need not exceed 18 nucleotides.



7. STEP 3 FIRST ROUND PCR (PCR #1)

7.1. Overview

Carry out PCR reactions on the cDNA template using a gene-specific forward primer (F1) together with the adapter oligo that consists of the adapter sequence alone without the poly(dT) tract (Fig. 12.1, Step 3).

7.2. Duration

1.5–2 h

3.1 Prepare PCR reaction with the following components:

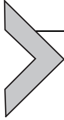
1-μl cDNA
2-μl 10× PCR buffer
1-μl dNTP mix (2 mM)
0.5-μl (50 ng) gene-specific forward primer (F1)
0.5-μl (50 ng) adapter oligo
0.3-μl Taq polymerase
Add water to 20 μl.

3.2 Run the following standard PCR program:

1. 5 min at 90 °C
2. 1 min at 90 °C
3. 1 min at 58 °C
4. 1 min at 72 °C
   30 cycles of Steps 2–4
5. 10 min at 72 °C
6. End at 15 °C

7.3. Tip

It is recommended that you run PCR reactions in duplicate or triplicate. Run a PCR reaction on the control RNA (the reaction lacking reverse transcriptase in Step 2).



8. STEP 4 GEL PURIFICATION OF PCR PRODUCTS

8.1. Overview

Run the PCR products from the first round PCR reaction on an agarose gel (see Agarose Gel Electrophoresis) and then isolate the DNA from the gel (Fig. 12.1, Step 4).

8.2. Duration

1–1.5 h

4.1 Combine the duplicate or triplicate reactions (40–60 μl total volume); run in a single gel lane to avoid excessive amounts of agarose in the gel extraction step.

4.2 Add 5× DNA sample buffer to samples, heat for 3 min at 80 °C, and place on ice for at least 5 min.

4.3 Prepare a 1% agarose gel by fully dissolving 1-g agarose in 100-ml 1× TBE buffer in a microwave oven. Add 5 μl of 10-mg/ml^{-1} ethidium bromide after cooling but before gel solidifies. Note that ethidium bromide is toxic. Wear gloves when handling the gel and take care to dispose of it properly.

4.4 Load sample and negative control side by side in the gel along with the DNA molecular weight ladder and run in 1× TBE buffer at 100 V for 20–30 min, until the xylene cyanol and bromophenol blue markers are separated by 1–2 cm.

4.5 Evaluate the separation of the DNA bands using a UV light box with proper UV protection (gloves, lab coat, glasses).

4.6 Use a scalpel to excise the portion of the gel extending from 50 to 550 bp according to the DNA ladder. This is the typical size range of polyadenylated degradation intermediates. These molecules should appear as a smear due to random 3′ end positions and varying tail lengths.

4.7 Purify the PCR products from the gel slice using a gel extraction kit. Elution volume should be <15 μl.

8.3. Tip

Sometimes, the DNA smear is not seen since it is not very abundant. Dominant bands should be avoided when possible as they usually represent artifacts resulting from nonspecific primer annealing or amplification of contaminating genomic DNA.

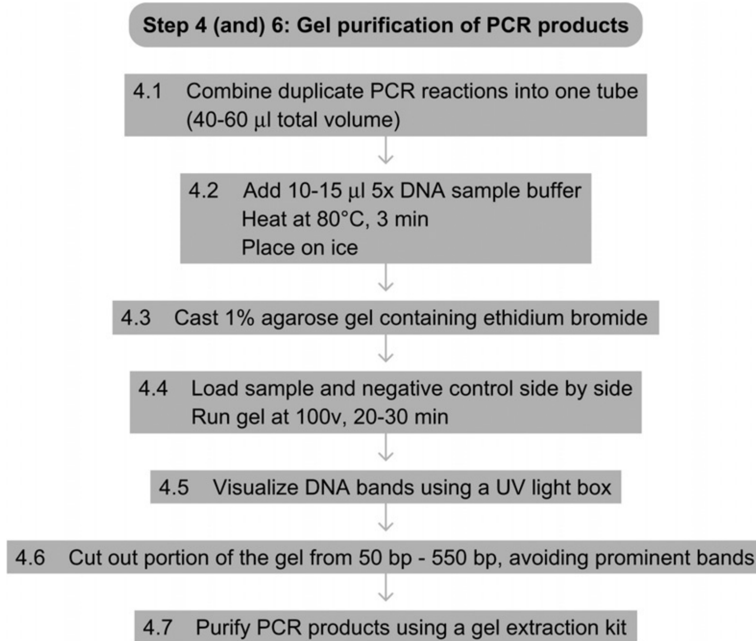


Figure 12.2 Flowchart for Step 4 (and 6).

Compare the sample lane with that of the negative control to aid in distinguishing which bands should be avoided when cutting the agarose gel.

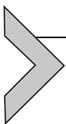
8.4. Tip

It is possible to simply clean the PCR products using a PCR clean-up kit instead of gel purification. The advantage of gel purification is that contamination by artifact bands due to nonspecific priming or amplification of genomic DNA during PCR, which would otherwise dominate in the T/A cloning step, can be minimized.

8.5. Tip

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

See Fig. 12.2 for the flowcharts of Steps 4 and 6.



9. STEP 5 SECOND ROUND PCR (PCR #2)

9.1. Overview

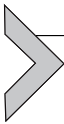
Carry out PCR reactions on the cDNA template using a gene-specific forward primer (F2) together with the adapter oligo. By using the nested

primer, amplification of any nonspecific products from the first PCR reaction will be minimized (Fig. 12.1, Step 5).

9.2. Duration

1.5–2 h

5.1 Carry out PCR reactions exactly as in Step 3 but using the F2 forward primer and the adapter oligo. Use 1 μl per reaction of the gel eluate (from Step 4.7) for the template DNA.



10. STEP 6 GEL PURIFICATION

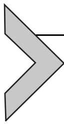
10.1. Overview

Run the PCR products on an agarose gel and purify them (Fig. 12.1, Step 6).

10.2. Duration

1–1.5 h

6.1 Carry out this step exactly as Step 4.



11. STEP 7 T/A CLONE THE PCR PRODUCTS

11.1. Overview

Clone the gel-purified second round PCR products into a T/A cloning vector (see Molecular Cloning) (Fig. 12.1 and Steps 7 and 8).

11.2. Duration

1 h (room temperature) – O/N (4 °C)
2 h (ethanol precipitation)

7.1 Clone the gel-purified second round PCR products into the pGEM®-T Easy T/A vector according to the manufacturer's instructions. The 10–15 μl of eluate from Step 6 can be used as is, or the eluate volume can be decreased in a Speed-vac dryer to a minimal volume of 4–5 μl.

7.2 After 1 h at room temperature or overnight at 4 °C, add water to the reaction to a final volume of 50 μl.

7.3 To precipitate the DNA, add one-tenth volume (5 μl) 3 M NaOAc, pH 5.5 and 2.5 volumes (125 μl) ice-cold absolute ethanol. Add 1-μl carrier yeast tRNA (10 mg ml^{-1}). Place at −20 °C for ≥1 h.

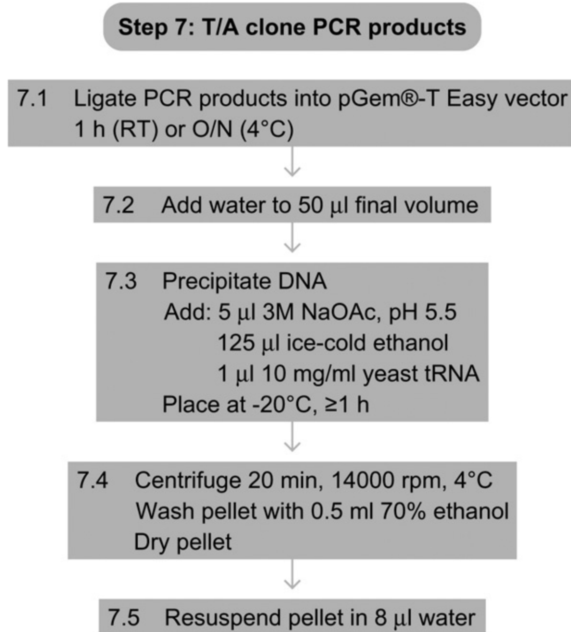
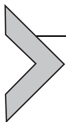


Figure 12.3 Flowchart for Step 7.

7.4 Centrifuge for 20 min at maximum speed, 4 °C. Carefully remove the supernatant and replace with 500 µl 70% ethanol. Centrifuge for 10 min, remove the supernatant, and dry the pellet in a Speed-vac or air-dry in a hood.

7.5 Resuspend the pellet in 8-µl water.

See Fig. 12.3 for the flowchart of Step 7.



12. STEP 8 ELECTROPORATION AND PLATING

12.1. Overview

Transform the T/A-cloned PCR products into competent cells (see Transformation of E. coli via electroporation). Plate cells and allow bacterial colonies to grow O/N at 37 °C (Fig. 12.1 and Steps 7 and 8).

12.2. Duration

1 h

O/N growth for bacterial colonies

8.1 Prepare LB agar plates ahead of time. After removing LB agar from the autoclave, let cool and add ampicillin (1 μl ml^{-1} of LB from 100 mg ml^{-1} stock). Plates are ready to use the following day.

8.2 Add 1 μl of the resuspended DNA (Step 7.5) in a cuvette containing 40–50-μl XL1-blue competent cells (or other blue/white competent cells) and electroporate.

8.3 Add 1 ml LB (no antibiotics) to cuvette and transfer electroporated cells to 1.5-ml tube. Incubate tubes in a 37 °C heat block or water bath for 1 h.

8.4 In a biological hood, add 60 μl X-gal (from 2% stock) and 20-μl IPTG (from 0.1 M stock) to LB+ampicillin (LB-amp) plates while transformed bacteria are incubating. Spread fluid on the agar surface with a sterile glass spreader or glass balls and let dry. One to three plates should be prepared for each sample.

8.5 Remove the tube from the 37 °C water bath, vortex briefly, and add 50, 100, or 200 μl to each plate using sterile techniques. Plate different volumes so there is a choice of bacterial colony densities for the next step. Incubate plates O/N in a 37 °C incubator.

12.3. Tip

If the bacteria are electroporated and plated at 2:00 P.M., the bacterial colonies will be ready at 9:00 A.M. the following morning. If 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.

See Fig. 12.4 for the flowchart of Step 8.

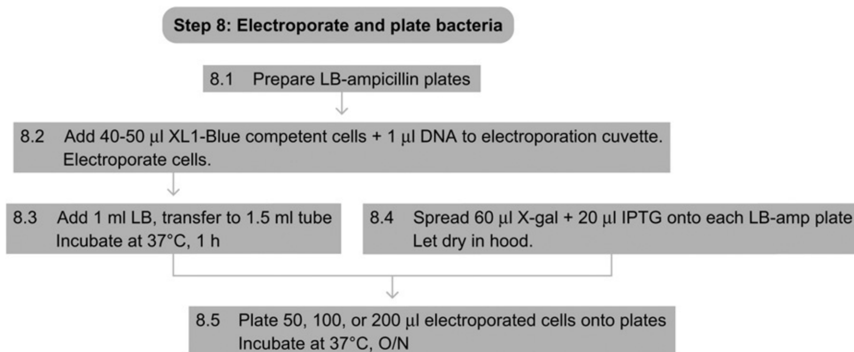
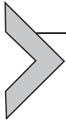


Figure 12.4 Flowchart for Step 8.



13. STEP 9 COLONY SCREENING BY PCR

13.1. Overview

Screen colonies by PCR to identify positive clones for plasmid DNA isolation and DNA sequencing (see Colony PCR) (Fig. 12.1, Step 9).

13.2. Duration

1–1.5 days

9.1 Remove plates from the 37 °C incubator and evaluate bacterial colony density. Choose plates with a colony density that allows picking single colonies without touching adjacent ones.

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

9.3 Place master plate in a 37 °C incubator for 6–24 h.

9.4 Prepare two PCR reactions for each colony to be screened as in Step 3.1. The first PCR reaction uses the F2 primer and the adapter oligo. The second PCR reaction uses the F3 primer (an oligo nested directly downstream of the F2 primer; see Fig. 12.1, Step 3) and the adapter oligo.

9.5 Pick each colony on the master plate with two toothpicks; swirl one in the first PCR tube and the other in the second PCR tube. Store the master plate at 4 °C.

9.6 Subject the samples to PCR as in Step 3.2.

9.7 Prepare an agarose gel as before. Run the paired PCR reactions side by side on the gel.

9.8 Examine the gels using a UV light box. Positive clones are identified by comparing the gel band of the first member of the pair to that of the second member of the pair. Since the F3 primer is nested directly downstream of the F2 primer, in a positive clone, the gel band from the second reaction is ~20 bp shorter than the first. All other cases should be disregarded.

See Fig. 12.5 for the flowchart of Step 9.

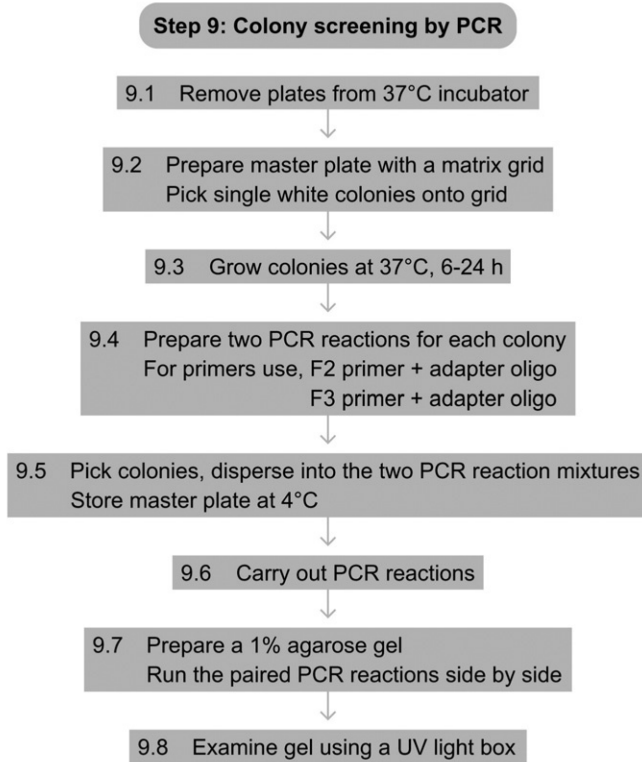
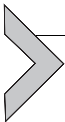


Figure 12.5 Flowchart for Step 9.



14. STEP 10 PLASMID DNA ISOLATION AND DNA SEQUENCING

14.1. Overview

Isolate plasmid DNA from positive colonies using a plasmid DNA mini-prep kit (see also Isolation of plasmid DNA from bacteria) and sequence the DNA.

14.2. Duration

1–4 days

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

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

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

10.4 Sequence plasmid inserts: evaluate tail length, nucleotide composition, and poly(A) site after validating that the analyzed insert indeed originates from the subject transcript.

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Explanatory chapter: PCR -Primer design.
Explanatory Chapter: Nucleic Acid Concentration Determination.
Agarose Gel Electrophoresis.
Molecular Cloning.
Transformation of E. coli via electroporation.
Colony PCR.
Isolation of plasmid DNA from bacteria.