

Chapter 2

PPAR-Alpha Cloning, Expression, and Characterization

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Abstract

Peroxisome proliferator-activated receptor α (PPAR α) is a member of the nuclear/steroid receptor gene superfamily that also comprises β , δ , and γ isoforms. PPAR α is a ligand-activated transcription factor that plays an important role in the regulation of many genes involved in key metabolic processes. Today, PPAR α has been cloned from mammalian, marsupial, and a number of marine species and its expression has been found to be relatively tissue- and species-specific. Here, we describe the methods for cloning of PPAR α genes by RT-PCR and RACE approaches and related protocols for studying the expression of cloned PPAR α cDNAs in mammalian cell systems.

Key words: PPAR α cloning, RT-PCR, RACE, Immunoblots, Cos-7 cell expression system

1. Introduction

The peroxisome proliferator-activated receptors (PPARs) belong to a subfamily of the nuclear receptor gene that involves in energy and metabolic processes, inflammation, proliferation, and differentiation (1). Of the PPAR subfamily, which also comprises β , δ , and γ isoforms, PPAR α is an important ligand-activated transcription factor that plays an essential role in lipid metabolism and energy homeostasis (2). PPAR α has been found to be abundantly expressed in liver, kidney, heart, and skeletal muscle (3) and its expression is relatively species-specific. For examples, in rat and mouse livers, PPAR α mRNA is highly expressed, while in human and guinea-pig livers, PPAR α mRNA is much less abundant (4, 5).

PPAR α was first isolated from mouse liver (6) and subsequently cloned from *Xenopus* (7, 8), rat (9), guinea-pig (4), human (5),

koala (10), chicken (11), and some marine species (12–15). In species studied thus far, PPAR α can be activated by a class of structurally diverse compounds collectively classified as peroxisome proliferators (PPs). Fatty acids, fatty acid derivatives, and non-metabolizable fatty acids have also been identified as potent activators of PPAR α (6, 9). *Xenopus* PPAR α has also been suggested to be activated by naturally occurring fatty acids (9).

PPAR α regulates the effects of PPs via transcriptional activation of PPAR α target genes. PPAR α proteins from different species have been found to have similar function when studied in vitro (4). PPAR α binds as a heterodimer with the retinoid X receptor (RXR) to a peroxisome proliferator responsive element (PPRE) located in the promotor region of PPAR α target genes (8, 16). PPRE has been identified in genes encoding peroxisomal β -oxidation enzymes and cytochrome P450 CYP4A subfamily, in particular human, rat, and *Xenopus* acyl CoA oxidase (AOX), rabbit CYP4A6, rat CYP4A1, as well as several other peroxisome proliferator-inducible genes (8, 16–33). To date PPRE has been found in 88 target genes as described by Lemay and colleagues (34). Evidence for the direct binding of PPs to PPAR α has also been reported (35, 36).

In certain species such as mouse and rat, PPs cause peroxisome proliferation, which is characterized by a profound increase in number and size of peroxisomes in liver parenchymal cells and an increase in liver weight. In addition, exposure to PPs induces expression of peroxisomal β -oxidation enzymes, CYP4As, and other susceptible genes (4, 34, 37, 38). A transgenic mouse line with a PPAR α gene disruption exhibits no peroxisome proliferation, unchanged constitutive expression of fatty acid-metabolizing enzymes, and no increase in liver size after PP treatment (39, 40).

As PPAR α controls the regulation of many genes which encode enzymes involved in key metabolic processes, it is essential to accomplish adequate primary information on PPAR α . The molecular data on PPAR α genes across species will enable further studies to investigate the mechanism underlining the regulatory roles of PPAR α and fully establish the effects of PPs on cellular function and processes via modulation of PPAR α . Thus, technique for successful generation of such data will be invaluable. In this book chapter, we describe the methodologies that have been applied successfully for the isolation and cloning of PPAR α by our laboratory, including step-by-step procedures for primer designs, RT-PCR approaches and optimization, gene cloning, and gene sequencing and analyses. Related methods for studying the expression of cloned PPAR α cDNAs in mammalian cells and for identification of cDNA-expressed PPAR α proteins and PPAR α from cellular fractions will be briefly discussed.

2. Materials

2.1. Cloning of PPAR α cDNAs by RT-PCR Approaches

2.1.1. Isolation of RNA by Guanidinium Thiocyanate-Phenol-Chloroform Extraction (41, 42)

1. Denaturing solution: 4 M guanidium thiocyanate, 25 mM sodium citrate, pH 4.0, 0.5% (w/v) *N*-laurylsarcosine (sarkosyl), 0.1 M 2-mercaptoethanol (2-ME) (see Note 1). Weigh 94.56 g guanidium thiocyanate and transfer to a 200 mL graduated cylinder or a glass beaker. Add diethyl pyrocarbonate (DEPC)-treated water to a volume of about 170 mL and mix to dissolve. Then add 10 mL of 0.5 M sodium citrate and 5 mL of 20% (w/v) sarkosyl. Make up to 200 mL with water. Store at 25°C (room temperature), in a bottle wrapped with aluminum foil. Just before use, add 1.4 μ L of 14.26 M 2-ME to 200 mL of the solution. Store at room temperature (see Note 2).
2. 2 M Sodium acetate, pH 4.0. Dissolve 27.2 g sodium acetate. $3\text{H}_2\text{O}$ in 80 mL of DEPC-treated water. Adjust to the pH of 4.0 with glacial acetic acid. Make up to 100 mL with DEPC-treated water. Store at room temperature (see Note 3).
3. Phenol (DEPC-treated water saturated). Dissolve 50 g phenol crystal in about 1 L distilled water at 65°C. Remove as much as possible the upper phase water by aspiration. Store at 4°C (see Note 4).
4. Chloroform:isoamyl alcohol (49:1) (v/v). Prepare just before use. Mix 98 mL of chloroform with 2 mL of isoamyl alcohol (see Note 5).
5. Isopropanol. Use straight from the manufacturer container.
6. 80% v/v Ethanol. Add 80 mL absolute ethanol to 20 mL DEPC-treated water.

2.1.2. Purification of mRNA and cDNA

Purification of mRNA from total RNAs and synthesis of first-strand cDNA (Amersham Pharmacia Biotech, Buckinghamshire, UK) (see Note 6):

1. Purification of mRNA. The kit (Amersham) provides sufficient columns and reagents for 2 or 4 mRNA purifications including:
 - (i) Oligo(dT)-cellulose columns containing oligo(dT)-cellulose suspended in 0.15% Kathon™ CG/1CP Biocide storage buffer.
 - (ii) High salt buffer containing 10 mM Tris-HCl, pH 7.4, 1 mM ethylenediaminetetraacetate (EDTA), and 0.5 M NaCl.
 - (iii) Sample buffer containing 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 3 M NaCl.
 - (iv) Low salt buffer containing 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.1 M NaCl.

- (v) Elution buffer containing 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA.
 - (vi) 5–10 mg/mL glycogen solution.
Additional reagents to be prepared:
 - (vii) 0.1% v/v DEPC-treated water: Add 1 mL of DEPC to 1 L Milli-Q grade water. Shake vigorously to get the DEPC into solution, allowed to stand overnight at room temperature, then autoclave for 15 min at 121°C (see Note 7).
 - (viii) TE (Tris-EDTA) buffer: 10 mM Tris-HCl, pH 7.4, 1 mM EDTA in DEPC-treated water. This buffer can be prepared by adding stock solutions of 1 mL of 1 M Tris-HCl, pH 7.4, and 200 μ L of 0.5 M EDTA, pH 8.0 to 90 mL DEPC-treated water. Make up to 100 mL with DEPC-treated water, then autoclave for 15 min at 121°C. Store at room temperature. To prepare 1 M Tris-HCl, pH 7.4 stock solution, dissolve 121.1 g Tris base in 800 mL DEPC-treated water. Adjust pH to 7.4 by adding 70 mL HCl. Make up to 1 L with DEPC-treated water, then autoclave for 15 min at 121°C. For preparation of 0.5 M EDTA, pH 8.0, dissolve 186.1 g EDTA.2H₂O in 800 mL DEPC-treated water. Adjust pH to 8.0 with approximately 20 g NaOH pellets. Add DEPC-treated water to 1 L, then autoclave. Store the stock solutions at room temperature.
 - (ix) 5.5 M Guanidinium thiocyanate: Weigh 118.2 g guanidinium thiocyanate and transfer to a 200 mL graduated cylinder or a glass beaker. Add DEPC-treated water to a volume of about 190 mL and mix to dissolve, then make up to 200 mL with water. Store at room temperature.
 - (x) Glycogen (1% w/v).
2. Synthesis of first-strand cDNA. The kit (Amersham) provides all the reagents to generate full-length first-strand cDNA from an RNA template, utilizing a number of primers. First-strand cDNA synthesized from this kit could be used as a template for DNA amplification by most polymerase chain reaction (PCR) applications. The kit contains sufficient reagents for up to 55 first-strand cDNA syntheses including:
- (i) Bulk First-Strand cDNA Reaction Mixes containing cloned FPL Cpure™ Murine reverse transcriptase, RNAGuard™ (porcine), RNase/DNase-free Bovine serum albumin (BSA), deoxyadenosine triphosphate (dATP), dCTP (cytidine), dGTP (guanosine), and dTTP (thymidine) in aqueous solution.
 - (ii) 200 mM dithiothreitol (DTT) aqueous solution.

- (iii) pd(N6) primer containing 0.2 µg/mL random hexadeoxynucleotides in aqueous solution.
- (iv) Not I d(T)₁₈ bifunctional primer containing 5 µg/mL 5' d[AACTGGAAGAATTCGCGGCCGCAGGAA T₁₈]-3' in aqueous solution.
- (v) DEPC-treated RNase-free water.

Additional DEPC-treated water is required and can be prepared as in previous step (Subheading 2.1.2 step I and vii).

2.1.3. RT-PCR and RACE

1. Oligonucleotide primers: Custom-made (Sigma Life Science, St Louis, MO, USA) (see Note 8).
2. Rapid amplification of cDNA ends (5'-; 3'-RACE) amplification kits (Clontech Laboratories Inc, Palo Alto, CA; Ambion International, Austin, TX; GibcoBRL Life Technologies, Rockville, MD) (USA).
3. *Tag* DNA polymerases (Qiagen Pty Ltd., VIC, Australia), Clontech Advantage 2 polymerase mix, Ultra-pure deoxyribonucleoside triphosphate (dNTPs) (100 mM, Amersham Pharmacia Biotech, Buckinghamshire, UK), Clontech 50× dNTP mix, 5× Loading buffer (Promega Corporation, Madison, WI, USA; Amersham; Sigma).
4. Mineral oil, 0.5 mL sterile PCR reaction tubes, PCR pipettors and pipette tips, DNA markers (Promega), thermal cycler (Perkin-Elmer GeneAmp 480, Waltham, MA, USA), Air-cooled thermal cycler PC-960 (Corbett Research, Australia).

2.1.4. PPAR α Cloning, Sequencing, and Analyses

1. DNA electrophoresis low-melt agarose gel: 1% w/v low-melt agarose, 1× Tris–acetate (TAE) buffer. Prepare the gel by melting 1 g agarose in 80 mL 1× TAE in a 100 mL glass bottle, allow the solution to cool down, make up to 100 mL with TAE buffer. Concentrated stock solution 50× TAE can be prepared by dissolving 242 g Tris base in about 0.5 L sterile or Milli-Q water, add 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA (pH 8.0), make up to 1 L with Milli-Q water. Prepare 0.5 M EDTA, pH 8.0 as in previous step (Subheading 2.1.2 step I and viii).
2. DNA SubCell™ electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA).
3. DNA gel extraction kits: QIAEX II gel extraction kit (Qiagen); 5× stop/loading buffer, 0.1 µg/mL ethidium bromide (EtBr) (Promega).
4. Shimadzu UV-1601UV-visible spectrophotometer (Shimadzu Corporation, Tokyo, Japan).
5. Sub-cloning vectors and competent cells for DNA transformation: pGEM®-T vector system, pCMV-Script®, pCI-neo,

Epicurian Coli® XL10-Gold™ ultracompetent cells (Promega; Stratagene, La Jolla, CA, USA), isopropyl-β-D-thio-galactoside (IPTG; 0.4 M), X-gal (50 mg/mL; Progen Industries Ltd, Brisbane, QLD, Australia). Alternately, prepare competent cells in-house using DH5α or XL1-blue *Escherichia coli* (Stratagene).

6. Restriction enzymes, DNA markers, T₄ DNA ligase (Invitrogen Life Science, VIC, Australia), Plasmid DNA purification kits (QIAGEN, QIAfilter plasmid Midi/Maxi; Qiagen). Alternately, plasmid DNAs can be purified in-house.
7. Plasmid DNA purification components. Resuspending buffer (50 mM glucose, 25 mM, Tris-HCl, pH 8.0, 10 mM EDTA), freshly prepared lysis solution 2.0 M NaOH, 1% w/v sodium dodecyl sulphate (SDS), neutralizing solution (3 M potassium acetate, pH 4.8), TE equilibrated phenol (pH 8)/chloroform (1:1). Prepare the solutions as in previous step.
8. M13 universal, M13 reverse, T3, T7, T7_{PCI} sequencing primers (Geneworks, Adelaide, Australia), ABI Prism™ Big-Dye reaction mix (Perkin Elmer).
9. Perkin Elmer ABI Prism 377 sequencer.
10. Cell culture and cDNA expression components. Luria Bertani broth (LB; 1% w/v trypton, 0.5% w/v yeast extract, 1% w/v NaCl, pH 7), antibiotics, Dulbecco's Modified Eagle Medium (DMEM; supplemented with 10% fetal bovine serum (FBS), 50 U/ML penicillin/streptomycin, 4 mM l-glutamine), 50 mM CaCl₂, sterile glycerol (Sigma), Cos-7 cells (African green monkey kidney cells), Lipofectamine reagent (Gibco-BRL), 0.1 M phosphate buffer (pH 7.4).
11. Orbital mixer incubator 0M10 (Ratek Instrument Pty, Boronia, VIC, Australia).

2.2. Characterization of PPARα from Cytosolic, Nuclear Fractions, and cDNA-Expressed PPARα Proteins

Cytosolic and nuclear extracts are used to detect PPAR-alpha protein from tissues. The cell fractions should be prepared from fresh tissues.

2.2.1. Cell Fraction Extraction Buffers

1. Nuclear extract homogenization buffer: 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 25 mM KCl, 1 mM phenylmethanesulfonyl fluoride (PMFS), 1 mM DTT, 10% (v/v) glycerol. This buffer can be prepared by adding stock solutions of 2 mL of 1 M Tris-HCl, pH 7.4, 200 μL of 0.5 M EDTA, pH 8, 2.5 mL of 1 M KCl, 1 mL of 0.1 M PMFS, 200 μL of 0.5 M DTT, and 10 mL glycerol to 90 mL milli-Q water. Make up to 100 mL with water. Store at room temperature. Prepare 1 M Tris-HCl, pH 7.4 and 0.5 M EDTA stock solutions as in previous steps.

For preparation of 1 M KCl stock solution, dissolve 7.46 g KCl in 100 mL Milli-Q water. To prepare 0.1 M PMFS, dissolve 1.74 g in isopropanol. To make 0.5 M DTT stock solution, dissolve 7.71 g DTT in 100 mL Milli-Q water. Store DTT stock solution at -20°C , other stock solutions at room temperature (25°C).

2. Homogenization buffer with 0.4 M NaCl: Freshly prepared by dissolving 2.33 g NaCl in 100 mL nuclear extract homogenization buffer.
3. Potassium phosphate 0.1 M buffer (pH 7.4) with 1.15% w/v KCl. Prepare two stock solutions of 1 M K_2HPO_4 and 1 M KH_2PO_4 . To obtain phosphate 0.1 M buffer (pH 7.4), mix 80.2 mL 1 M K_2HPO_4 to 19.8 mL 1 M KH_2PO_4 , then dissolving 1.15 g KCl in 100 mL of this phosphate buffer. To prepare the 1 M K_2HPO_4 and 1 M KH_2PO_4 stock solutions, dissolve 45.6 g K_2HPO_4 in 200 mL Milli-Q water and 34.0 g KH_2PO_4 in 250 mL water, respectively.
4. Teflon or glass Potter Elvehjem homogenizer (Omni International, Kennesaw, GA, USA).
5. Reagents for measurement of protein concentration: Freshly prepared protein measurement solution (0.28 M Na_2CO_3 , 0.1 M NaOH, 0.8 mM CuSO_4 , 1.4 mM potassium sodium tartrate), Folin and Ciocalteus reagent (1 in 3 dilution in water), BSA (1 mg/mL).

2.2.2.2. SDS Polyacrylamide Gel Components

Prepare as stock solutions:

1. Resolving gel buffers: 1.5 M Tris-HCl, 0.4% (w/v) SDS, pH 8.8. Weigh 181.7 g Tris and 4 g SDS and transfer to a 1 L graduated cylinder or a glass beaker containing about 100 mL distilled or Milli-Q water (see Note 9). Add water to 900 mL, mix until Tris and SDS dissolved (see Note 10), and adjust pH to 8.8 with concentrated HCl (see Note 11). Make up to 1 L with water. Store at 4°C (see Note 12).
2. Stacking gel buffer: 0.5 M Tris-HCl, 0.4% (w/v) SDS, pH 6.8. Weigh 60.6 g Tris and 4 g SDS. Prepare a 1 L solution as in previous step (Subheading 2.2.2 step 1) for the resolving gel buffer. Store at 4°C .
3. Acrylamide:bis-acrylamide solution (29.2:0.8 acrylamide:bis-acrylamide): Dissolve 29.2 g acrylamide and 0.8 g bis-acrylamide in about 40 mL water in a 100 mL graduated cylinder. Make up to 100 mL with water and filter through a $0.45\ \mu\text{m}$ filter (see Note 13). Store at 4°C , in a bottle wrapped with aluminum foil (see Note 14).
4. SDS polyacrylamide gel electrophoresis (PAGE) running buffer: 0.25 M Tris-HCl, pH 8.3, 0.192 M glycine, 0.1% (w/v)

SDS. Weigh 3 g Tris, 14.4 g glycine, and 1 g SDS, mix, and make it to 1 L with water.

5. SDS lysis buffer: 0.2 M Tris-HCl, 10.2% (w/v) SDS, 17% (v/v) 2-ME, 0.03% (w/v) bromophenol blue (BPB), 33% (v/v) glycerol. Dissolve 3 g SDS and 10 mg BPB in 12.5 mL stacking gel buffer, add 5 mL 2-ME and 10 mL glycerol, and mix well. Leave one aliquot at 4°C for current use and store remaining aliquots at -20°C (see Note 12).
6. *N,N,N,N*-tetramethyl-ethylenediamine (TEMED; 10% v/v). Dilute 200 µL TEMED in 2 mL water.
7. 2%, w/v SDS: Dissolve 2 g SDS in 100 mL water.

2.2.3. Immunoblotting Components

Prepare as stock solutions:

1. Western blot transfer buffer: 0.025 M Tris, 0.192 M glycine, 20% (v/v) methanol. Weigh 7.7 g Tris-HCl, 9.2 g Tris, and 72 g glycine, mix, and dissolve in about 3.5 L water. Add 1 L methanol and make up to 5 L with water.
2. Tris buffered saline (5× TBS): 2.5 NaCl, 0.1 M Tris-HCl, pH 7.5. Weigh 10.2 g Tris-HCl, 2.4 g Tris, and 146 g NaCl, mix and make to 1 L with water. Adjust pH to 7.5 with HCl.
3. Tween-20 (10% v/v). Measure 10 mL Tween-20 and make to 100 mL with 1× TBS (see Note 15). Freshly prepared before use:
4. Nitrocellulose membranes (NitroBind, Micron Separations Inc, MA, USA).
5. Ammonium persulphate (APS; 10% w/v). Dissolve 1 g APS in 10 mL water.
6. Resolving gel (7.5%). Add 10 mL resolving gel buffer then 10 mL acrylamide-bis to 20 mL water, mix, and add 120 µL APS and 100 µL TEMED (see Note 16).
7. Stacking gel (5%): Prepare as per resolving gel by mixing 2.5 mL stacking gel buffer, 1.5 mL acrylamide-bis, 6 mL water, 30 µL APS, and 100 µL TEMED (see Note 17).
8. First overlay. Add in order 250 µL SDS (2% w/v), 35 µL APS, and 150 µL TEMED to 4.25 mL water.
9. Second overlay. Mix 3 mL resolving gel buffer with 2.7 mL water.
10. Wash buffer: 5 mL stacking gel buffer, 0.5 µL APS, 1 mL TEMED, 3 mL water.
11. TBS containing tween-20 (TTBS; 0.05% v/v): Measure 10 mL 10% (v/v) Tween-20 and make to 2 L with 1× TBS.
12. Blocking solution: 5% (w/v) skim milk in TBS.
13. Antibody buffer: 0.02% (w/v) skim milk in TTBS.

14. Medium binder clips, plastic container.
15. Hoefer (Inc, San Francisco, CA, USA) TE 42 transphor electro-transfer unit.
16. Imidazole buffer (20 mM, pH 7.4). Dissolve 136 mg imidazole in 100 mL water and adjust pH to 7.4 with HCl.
17. Horseradish peroxidase (HRP) development solution: 0.05% (w/v) 3,3'-diaminobenzidine (DAB) and 0.05% (v/v) H₂O₂ in 20 mM imidazole buffer (see Note 18).

2.2.4. Antigens and Conjugates

1. Rabbit anti-human PPAR α polyclonal primary antibody (1:200 dilution; Santa Cruz Biotechnology, Inc, Heidelberg, Germany).
2. Mouse anti-rabbit IgG HRP-conjugated (1:2,000 dilution; Santa Cruz).
3. Pre-stained protein ladder (GibcoBRL).

3. Methods

3.1. Isolation of RNA and cDNA

1. For optimal RNA extraction, fresh tissue is preferable. Alternately snap-frozen tissue can be used. Remove quickly the tissue from the animal at the time of death, immediately freeze in liquid nitrogen, and store at -80°C until required.
2. For RNA extraction and purification (see Note 19), bake all general laboratory glassware at 180°C overnight prior to use. Soak plastic ware and other equipment in DEPC-treated water (0.1% v/v) overnight, then autoclave the glassware and equipment for 15 min at 121°C (see Note 20).
3. Prepare all solutions for the preparation of RNA using Milli-Q grade water to which add 0.1% w/v DEPC, leave overnight at room temperature, then autoclave the solutions for 15 min at 121°C . Use molecular grad reagents and chemicals free of RNAase where possible.
4. Sterilize all solutions used for DNA preparation.

3.1.1. Total RNA Extraction

Prepare according to Chomcynski and Sacchi (42) with modifications (see Note 21).

Carry out centrifugation at $11,000\times g$ at 4°C .

1. Homogenize 1 g tissue with 10 mL denaturing solution in a 20 mL Teflon glass homogenizer. Add 1 mL sodium acetate, 10 mL phenol and 2 mL chloroform:isoamyl, mix vigorously and stored on ice for 15 min.

2. Transfer the contents to a 30 mL centrifuge tube (see Note 22), centrifuge for 20 min, collect the supernatant in a fresh 30 mL centrifuge tube.
3. Add 10 mL denaturing solution and repeat the procedures in steps 1–2.
4. Add 10 mL isopropanol and store at -20°C for at least 1 h to precipitate the RNA.
5. Centrifuge the tube for 20 min, collect the pellet, briefly dry, and then resuspend in 300 μL denaturing solution. Add 300 μL isopropanol and allow the RNA to precipitate at -20°C overnight.
6. Centrifuge for 15 min, recollect the pellet, rinse with ethanol, dry briefly, and then resuspend in 200 μL DEPC-treated water.
7. Store RNA at -80°C in small aliquots as ethanol precipitates (see Note 23) or in DEPC-treated water with RNaseOUT Ribonuclease inhibitor (0.2 I.U./L; Invitrogen).

3.1.2. Purification of mRNA
(Amersham mRNA
Purification Kit)

Prepare in accordance with the manufacturer's protocol. Carry out centrifugation at $350\times g$ for 2 min room temperature otherwise stated.

1. Prepare the spin column by washing the oligo(dT)-cellulose twice with 1 mL high salt buffer.
2. Dissolve 1.3 mg total RNA in 1 mL elution buffer, heat the sample at 65°C for 5 min then place on ice. To the tube, add 200 μL sample buffer, mix then pour into the prepared column and allow the column to drain by gravity. Centrifuge and wash the column twice with 250 μL high salt buffer and three times with 250 μL low salt buffer. Centrifuge the column between each wash.
3. Apply 4×250 μL elution buffer (preheated at 65°C), centrifuge and collect the eluted RNA solution. To the mRNA solution, add 100 μL sample buffer, 10 μL glycogen, and 2 mL ethanol then stored at -20°C overnight. Collect the mRNA pellet by centrifugation at $12,000\times g$ for 20 min at 4°C , and then resuspend in DEPC-treated water. Store mRNA in small aliquots as in previous step (Subheading 3.1.1 step 7).

3.1.3. Synthesis of cDNA
(Amersham First-Strand
cDNA Synthesis Kit)

Prepare in accordance with the manufacturer's protocol. Briefly, heat mRNA (10 ng) in 8 μL RNase-free water at 65°C for 10 min, chill on ice, add 5 μL bulk first-strand cDNA reaction, 1 μL DTT, 1 μL Not 1-d(T)18 primer, mix, and incubate at 37°C for 1 h. Store cDNAs at -20°C .

**3.2. Reverse
Transcription
Polymerase Chain
Reactions (RT-PCR)
and Rapid Amplification
of cDNA Ends (RACE)**

3.2.1. Primer Design

1. RT-PCR degenerative primers: Design degenerative primers based on PPAR α cDNA sequences already been cloned from other species. These sequences are often available in the GenBank database:

PPAR α cDNA	Accession number	Authors
(i) Mouse	NM_011144	Isseman and Green (1990) (6)
(ii) Rat	NM_013196	Göttlicher et al. (1992) (9)
(iii) Guinea-pig	AJ006218	Tugwood et al. (1998) (4)
(iv) Human	NM_005036	Qi et al. (2001) (5)
(v) Koala	AF_463455	Ngo et al. (2007) (10)
(vi) Chicken	NM_001001464	Diot and Douaire (1999) (11)
(vii) <i>Xenopus</i>	NM_001089813	Dreyer et al. (1992); Krey et al. (1993) (7, 8)
(viii) PPAR α cDNA has also been cloned in a number of marine species (12–15).		

2. Gene-specific primers (GSPs): For RACE reactions to obtain 3'- and 5'- cDNA ends in order to clone full-length PPAR α cDNA, design GSPs from already know partial sequences of the full-length cDNA. For a complete RACE protocol, at least two GSPs are required: an antisense for the 5'-RACE PCR and a sense primer for the 3'-RACE PCR. If only 5'- or 3'- RACE is carried out, only one GSP is needed. Design the primers to create overlapping 5'- and 3'-RACE products, which, if a suitable restriction site is located in the region of overlap, can subsequently be joined by restriction digestion and ligation to create the full-length cDNA. The relationship of the primers used in RT-PCR and RACE reactions to the template and resulting PCR, RACE products is shown in Fig. 1.
3. Primer sequence: It is best and where possible degenerative and GSPs should be:
 - (i) 23–28 nt (see Note 24).
 - (ii) $T_m \geq 65^\circ\text{C}$; best results are achieved if $T_m \geq 70^\circ\text{C}$ (43) (enable the use of touchdown PCR if needed) (see Note 25).
 - (iii) 50–70% GC.
 - (iv) The 3'-terminal ends of each primer should not be complementary to each other and should contain a low GC content.
4. Location of primer sequences within the gene: If possible, choose the location of primer sequences within the gene so that the 5'- and 3'-RACE products will be 2 kb or less

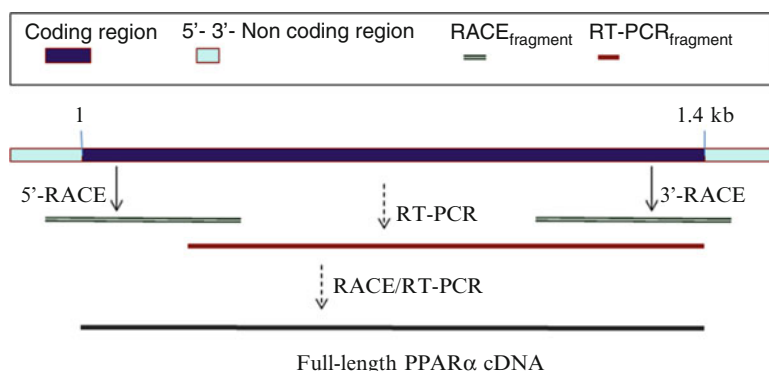


Fig. 1. Schematic diagram of the cloning strategy to obtain full-length PPAR α cDNA by PCR approaches-RT-PCT and RACE. The solid box represents the coding region of PPAR α and the lighter boxes depict the 5'- and 3'- non coding regions. Single and double lines represent PPAR α cDNAs: 5'-cDNA amplified fragment obtained by RACE is double-line; partial PPAR α cDNA obtained by RT-PCR is light solid; full-length PPAR α cDNA is dark solid.

(see Note 26). Design GSPs that produce overlapping 5'- and 3'-RACE products and it is best when the overlap between primers is at least 100–200 bases (see Note 27).

3.2.2. Template Quality

It is important to use high quality and high molecular weight DNA, particularly when amplifying large sequences and/or the highest possible sensitivity is required (see Note 28).

1. Use first-strand cDNA synthesis kit (Amersham) to obtain pure and high quality cDNA template.
2. The longer or more complex the sequence, the more important the template quality is. This is because the number of un-nicked, full-length targets decreases as the target length increases, poor-quality DNA will have very few large un-nicked targets.
3. Poor cDNA template due to incomplete reverse transcription can lead to an absence of product, truncated products, or a mix of truncated and full-length products, resulting in a smeared band on a gel. This problem can be minimized when highest quality template is used.
4. During thermal cycling, some depurination occurs when DNA is denatured which can result in truncated products. This can be minimized by using high quality cDNA template.

3.2.3. PCR Master Mix

The use of PCR master mix can greatly reduce tube-to-tube variation and save time.

1. Assemble PCR master mix by the appropriate volumes of all reagents required for multiple PCR reactions. Vortex gently to

mix (without bubbling) PCR master mix, or to mix by gentle pipetting.

2. To test multiple primer sets with one cDNA template, include the template in PCR master mix. To test the same primers with multiple templates, include the primers in the master mix. For setting up several sets of parallel samples, prepare multiple PCR master mixes, i.e., each with a different set of primers or templates.

3.2.4. Touchdown PCR

Touchdown PCR approach can significantly improve the specificity of RT-PCR and RACE reactions (44, 45). It involves the use of an annealing/extension temperature that is several degrees higher than the T_m of the primer ($>3-10^\circ\text{C}$) during the initial PCR cycles (5–10 cycles). The annealing/extension temperature is then reduced to the primer T_m for the remaining PCR cycles (see Note 29).

3.2.5. Recommended Cycling Parameters

1. Optimal RT-PCR conditions (see Note 30):
 - (i) Denaturing at 94°C for 3 min.
 - (ii) 30 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min.
 - (iii) Final extension at 72°C for 10 min.
2. Touchdown PCR conditions for 5'- and 3'-RACE reactions (see Note 31):
 - (i) 5 cycles of 94°C for 5 s and 72°C for 2 min.
 - (ii) 5 cycles of 94°C for 5 s, 70°C for 10 s, and 72°C for 2 min.
 - (iii) 32 cycles of 94°C for 5 s, 68°C for 10 s, and 72°C for 2 min.

3.2.6. RT-PCR Procedure

1. Before preparing the RT-PCR master mix: Place all components on ice and allow the reagents to thaw completely. Mix each component thoroughly by vortexing on low, gentle speed, or by gentle pipetting.
2. PCR reactions: $1\times$ PCR reaction buffer, $200\text{ }\mu\text{M}$ of each dNTP, $0.1\text{ }\mu\text{g}$ of each forward and reverse primer, 1.5 I.U. Tag DNA polymerase, magnesium (Mg^{++} ; $0.5-2.0\text{ mM}$) in a final volume of $20\text{ }\mu\text{L}$ with PCR water.
3. Master mix: Prepare enough PCR master mix required for all PCR reactions plus one additional tube (see Note 32). Mix well, spin briefly using a microcentrifuge, and transfer the master mix into each PCR reaction tube.
4. Add each of the remaining reagents, mix gently, then overlay the contents of each tube with a few drops of mineral oil, and place cap on firmly.

5. It is best to include a negative and/or positive control PCR reaction (see Note 33).
6. Commence thermal cycling using a standard or touchdown PCR program.

3.2.7. RACE Procedure
(Clontech Smart RACE
cDNA Amplification Kit)

1. RACE reactions: Prepare PCR master mix as in previous step (Subheading 3.2.6 step 3), however using a 50 μL RACE reaction (see Note 34). The same master mix can be used for both 5'- and 3'- RACE reactions.
2. To prepare 5'- and 3'-RACE reactions, add the components in order in a 0.5 mL PCR tube:

PCR reactions	5'-RACE	3'-RACE
(i) Test cDNA template	5 μL	5 μL
(ii) RACE AP (10 μM)	1 μL	1 μL
(iii) 5'-GSP (antisense, 10 μM)	1 μL	–
(iv) 3'-GSP (sense, 10 μM)	–	1 μL
(v) Master mix	43 μL	43 μL
Final volume	50 μL	50 μL

3. Mix, overlay the contents with a few drops of mineral oil, and commence thermal cycling using a touchdown PCR program.

3.2.8. Isolation of
Full-length PPAR α by PCR

1. Design 5'- GSP and 3'- GSP based on the sequence obtained from 5'- and/or 3'-RACE products. These primers are derived from the actual 5'- and/or 3'- end of the cloned PPAR α cDNA sequences (Subheading 3.2.1 steps 2, 3 and 4).
2. Assemble PCR master mix as in previous step (Subheading 3.2.7 step 1).
3. To prepare PCR reactions, add the components in order in a 0.5 mL PCR tube (can include 5'- and/or 3'-RACE reaction as optional controls):

PCR reactions	Full-length	5'-RACE	3'-RACE
(i) Test cDNA template	5 μL	5 μL	5 μL
(ii) 5'-RACE GSP (antisense, 10 μM)	1 μL	1 μL	–
(iii) 3'-RACE GSP (sense, 10 μM)	1 μL	–	1 μL
(iv) 5'-GSP (antisense, 10 μM)		1 μL	–
(v) 3'-GSP (sense, 10 μM)		–	1 μL
(vi) Master mix	43 μL	43 μL	43 μL
Final volume	50 μL	50 μL	50 μL

4. Mix, overlay the contents with a few drops of mineral oil, and commence thermal cycling.
5. Optimal PCR conditions for full-length PPAR α amplification:
 - (i) 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min.
 - (ii) Final extension at 72°C for 10 min (see Note 35).

3.3. Cloning and Sequencing of PPAR α cDNA RT-PCR and RACE Products

3.3.1. DNA Electrophoresis and Extraction of DNA from Agarose Gel

1. When cycling is completed, transfer 5 μ L of the PCR reaction to a fresh tube (see Note 36), and add 1 μ L 5 \times loading or stop buffer. Analyze PCR sample(s) along with suitable DNA size markers (see Note 37) on a 1% (w/v) agarose gel containing EtBr. A 0.5% (w/v) gel can be used for cDNA fragments size \geq 1 kb. For resolution of amplified PCR products, low-melt agarose gel is used. View the gel under UV illumination.
2. Excise portions of the agarose gel containing the desired insert using a sterile scalpel, subdivide into 250 μ g portions, and place in sterile eppendorf tubes. Purify the PCR products (QIAEX II protocol) and store DNA at 4°C.

3.3.2. Sub-cloning of PCR Products into a Plasmid Vector

Clone the purified PCR product directly into a T/A-type PCR cloning vector. Alternately, clone the product into a conventional vector using restriction enzymes and introduce restriction enzymes into the GSP (see Note 38).

1. Ligation reactions (see Note 39):
 - (i) Ligation into pGEM-T[®] or pCI-neo: 150 ng cDNA insert, 1 μ L T4 DNA ligase 10 \times buffer, 1 μ L T4 DNA ligase (3 I.U./ μ L), 1 μ L pGEM-T[®], or pCI-neo (10 ng/ μ L) in a final volume of 10 μ L with sterile water.
 - (ii) Ligation into pCMV-Script XR[®]: 150 ng cDNA insert, 0.5 μ L T4 DNA ligase 10 \times buffer, 0.5 μ L rATP, 0.5 μ L T4 DNA ligase (4 I.U./ μ L), 1 μ L pCMV-Script XR[®] (10 ng/ μ L) in a final volume of 5 μ L with sterile water.
 - (iii) Set up ligation reactions, mix gently by pipetting, and incubate at 4°C overnight.
2. Bacterial cell culture preparation:
 - (i) Cell culture and growth. Sterile all media for bacterial cell culture by autoclaving at 121°C for 15 min. Grow cells in LB containing an appropriate antibiotic (see Note 40) in a mixer incubator at 37°C overnight with vigorous shaking.
 - (ii) LB plates. Add 1% (w/v) agar to the LB broth, autoclave, and cool the media to 50°C then add an appropriate antibiotic (steriled by filtration through a 0.22 μ m filter) and pour into 90 mm Petri dishes.

3. Competent cell preparation (see Note 41):

- (i) Inoculate 0.5 mL of an overnight culture in 30 mL LB broth containing an appropriate antibiotic and incubate at 37°C for about 3 h (see Note 42).
- (ii) Centrifuge the suspension at $3,000\times g$ at 4°C for 15 min, collect the cell pellet, and suspend in 15 mL cold CaCl_2 . Centrifuge the suspension, recollect the cells, then resuspend in 3 mL cold CaCl_2 , and place on ice for 45 min. Store cells in 25% (v/v) sterile glycerol at -80°C.

4. Transformation:

- (i) Mix 2 μL (up to 5 μL) ligation reaction with 100 μL competent cells and place on ice for 45 min. Heat shock the cells at 42°C for 90 s, place on ice for 2 min, then suspend in 1 mL LB broth at 37°C for 90 min with gentle agitation to recover the cells. Centrifuge briefly, resuspend in 100 μL LB, then plate the cells onto LB plates containing a suitable antibiotic, and incubate at 37°C overnight.
- (ii) For screening of cDNA insert cloned in pGEM-T® or pCMV-Script XR®, add 20 μL IPTG and 20 μL X-gal to the cell suspension prior to plating. This enables color selection of the plasmid. Select the bacterial cells harboring recombinant plasmid DNA which will appear as white colonies.

3.3.3. Plasmid DNA Preparation

1. Small scale purification (alkaline lysis method):

- (i) Inoculate a single isolated bacterial colony containing the recombinant plasmid DNA in 5 mL LB containing an appropriate antibiotic and incubate at 37°C overnight with vigorous shaking. Pour 1.5 mL of the culture into an eppendorf tube, centrifuge at $1,000\times g$ for 1 min, remove the medium by aspiration, and leave the bacterial pellet as dry as possible. Resuspend (by vortexing) the pellet in 100 μL ice-cold re-suspending buffer and store at room temperature for 5 min (with the cap of the tube opened).
- (ii) Add 200 μL cell lysis solution, close the cap, and invert the tube rapidly up and down three times to mix the contents (do not vortex). Store the sample mixture on ice for 5 min. Add 150 μL ice-cold neutralizing solution (see Note 43), mix gently, and place on ice for 5 min. Centrifuge for 5 min, transfer the supernatant to a new tube, add equal volume of calibrated phenol/chloroform, mix, and centrifuge for 2 min. To the supernatant, add two volume of ethanol, mix, incubate at room temperature for 2 min, then centrifuge for 10 min to collect the DNA pellet. Allow the DNA to dry.

- (iii) Wash the DNA pellet with 1 ml 70% (v/v) ethanol, vortex briefly, recover the pellet by centrifugation for 10 min, dry briefly, and resuspend in 20 μ L sterile water. Store DNA at 4°C.
2. Qiaquick spin mini-prep (Qiagen):
 - (i) Carry out at room temperature essentially as per manufacturer's protocol.
 - (ii) It is best to use Qiaquick protocol to obtain high quality plasmid DNA for sequencing.
3. Large scale purification (Qiagen maxi plasmid purification kit):
 - (i) Perform essentially as per manufacturer's protocol.
 - (ii) Once the pellet DNA has been obtained, wash DNA with ice-cold 70% (v/v) ethanol, dry briefly, then resuspend in appropriate amount of sterile water. Stored DNA at 4°C.
4. Purification of plasmid DNA for transfection (Qiagen QIAfilter midiprep spin kit): For transfection of cloned cDNA into a mammalian expression cell system (e.g., Cos-7 cells), it is best to use high quality plasmid DNA, i.e., DNA prepared by this kit.
5. Quantification of DNA:
 - (i) Prepare a 1 in 500 dilution of DNA solution in Milli-Q or sterile water.
 - (ii) Measure the absorbance of the solution at 260–280 nm (using a UV spectrophotometer) to determine the DNA concentration.

3.4. Sequencing

1. Sequencing primers (see Note 44):

Name	Nucleotide sequence (5'-3')
M13 universal sequencing primer	GTA AAA CGA CGG CCA GT
M13 reverse sequencing primer	CAC ACA GGA AAC AGC TAT GAC CAT G
T3 primer	AAT TAA CCC TCA CTA AAG GG
T7 primer	GTA ATA CGA CTC ACT ATA GGG C
T7 _{PCI} primer	TTA ATA CGA CTC ACT ATA GGC

2. Sequencing reactions: 400 ng DNA (1–2 μ L), 8 μ L ABI Prism™ Big-Dye reaction mix, and 3.2 pmole primer (1–2 μ L each primer) in a final volume of 20 μ L with water.
3. Thermal cycling conditions: 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min.

4. To each reaction, add 80 μL 75% (v/v) isopropanol, mix, incubate for 15 min, and centrifuge at $10,000\times g$ at room temperature for 25 min to obtain the DNA pellet. Before subjected to analysis by electrophoresis in a sequencer, wash the DNA pellet with 200 μL 75% isopropanol and dry briefly (see Note 45).
5. Use a computer program, i.e., MacVector™ (Oxford Molecular Lt.) to analyze the data obtained from the sequencing reactions. To identify the cloned cDNAs, accomplish ClustalW alignment of cDNA cloned sequences against sequences from other species (GenBank database, <http://www.ncbi.nlm.nih.gov/blast>).

3.5. Characterization of PPAR α in Cytosolic and Nuclear Extracts and cDNA-Expressed PPAR α Protein

3.5.1. Preparation of Cytosolic and Nuclear Extracts

Use a Teflon/glass Potter homogenizer.

1. Cytosolic extract: Carry out centrifugation at 4°C. Homogenize 1 g tissue in 10 mL potassium phosphate buffer, transfer the contents into a 30 mL ultracentrifuge tube (see Note 22), and centrifuge at $20,000\times g$ for 20 min. Transfer the supernatant into a new tube, centrifuge at $280,000\times g$ for 1 h, and collect the supernatant. Store the cytosol at 4°C.
2. Nuclear extract (46): Carry out centrifugation at $1,000\times g$. Homogenize 1 g tissue in three volumes of homogenization buffer, centrifuge the contents in a 30 mL tube for 5 min, collect the pellet, and suspend in three volumes of homogenization buffer. Centrifuge the suspension for 5 min (see Note 46), collect the pellet, and resuspend in two volumes of homogenization buffer containing 0.4 M NaCl. Mix the suspension gently at 4°C for 20 min (by stirring with a magnetic stir bar), centrifuge for 60 min, and collect the supernatant. Store the nuclear extract at -80°C.

3.5.2. Expression of PPAR α in Cos-7 Cells (see Note 47)

1. Grown Cos-7 cells in a monolayer in supplemented DMEM at 37°C and 5% (v/v) CO₂. Seed the cells at 6×10^5 cells per well, i.e., using a 6-well 33-mm tissue culture plate, then allow the cells to grow in DMEM containing 10% (v/v) FBS until 90% confluent. Immediately prior to transfection, wash the cells gently with DMEM with no serum, and remove the media.
2. Use 10 μL LipofectAMINE reagent (GibcoBRL) to transfect full-length PPAR α cDNA (cloned in an appropriate mammalian expression vector) into Cos-7 cells as per manufacturer's protocol. Before overlaying the mixture sample on the cells, incubate the sample for 30 min at room temperature to allow the LipofectAMINE-DNA complexes to form. Incubate for 16 h, replace with fresh supplemented DMEM, and harvest the cells 3 days following transfection. Rinse cells briefly in 0.1 M phosphate buffer (pH 7.4), homogenize using a

hand-held eppendorf homogeniser, and use this crude cell lysate directly for further analyses.

3.5.3. Measurement of Protein Concentrations (47)

1. Dilute the protein sample (cytosol, nuclear extract, or cell lysate) 1 in 20 to a final volume of 300 μ L with water in a 10 mL glass tube. Similarly, prepare a range of BSA standard samples, i.e., from 0 to 1 mg/mL in 300 μ final volume in each 10 mL glass tube, for constructing the standard absorbance curve (see Note 48).
2. To each sample and standard tube, add 5 mL protein measurement solution, incubate at room temperature for 15 min. Add 500 μ L Folin reagent, mix briefly by vortexing, and incubate for 15 min.
3. Measure absorbance at 660 nm and calculate the protein concentration based on the standard absorbance curve constructed from a range of known BSA concentrations.

3.5.4. Immunoblot Analysis of PPAR α from Cytosolic, Nuclear Fractions, and cDNA-Expressed PPAR α Proteins (48)

1. 12.5% SDS polyacrylamide gel electrophoresis (see Note 49):
 - (i) Prepare the resolving gel by mixing the reagents (Subheading 2.2.3 step 6) in a 50 mL conical flask and pour into the apparatus. Add the first overlay and allow the gel to set. Remove the first overlay (by pouring off), add the second overlay, and leave overnight (covered with aluminum foil) (see Note 50).
 - (ii) Remove the second overlay, add the wash, then remove the wash (use blotting paper to remove excess wash if needed).
 - (iii) Prepare the stacking gel by mixing the reagents as in previous step (Subheading 3.5.4 step 1 and i) and insert the gel combs (10-well) immediately without introducing air bubbles (by sliding in slowly and pushing down hard). Allow the gel to set for 1 h.
 - (iv) Solubilize the protein (50 μ g/lane, in 10 μ L) with an equal volume of solubilising buffer and heat the samples to 100°C for 10 min, then centrifuge the heated sample at 3,000 $\times g$ for 30 s to bring down condensate (see Note 51).
 - (v) Load the samples starting from the second lane and add the pre-stained protein ladder in the first lane. Do not touch the bottom of well or sides when loading samples. Stack and run the gel (see Note 52).
 - (vi) Immediately following SDS-PAGE, when the dye front reaches the end of the gel, turn off the power supply. Separate the gel plates with the help of a spatula or a similar tool, with the gel to remain on one of the glass plates. Remove the stacking gel. Rinse the gel with water and transfer carefully to a container with transblotting buffer.

2. Immunoblot:

- (i) Cut a nitrocellulose membrane to the shape of the gel. Rinse twice in distilled water and immerse in transblotting buffer. In another dish, immerse the blotting paper and the sponge pads.
- (ii) After rinsing the gel (still supported by the bottom glass plate) in deionized water for 10 min (to remove traces of SDS-PAGE running buffer), excise the gel and leave to air-dry for 5 min. Gently lay the nitrocellulose membrane on top of the gel.
- (iii) Gently lift the gel-membrane sandwich from the glass plate and place the sandwich on two sheets of Whatman no. 3 filter paper (place membrane side directly on the filter paper and the exposed gel side on top), cut to the size of the gel. Place another two sheets of Whatman no. 3 filter paper on top of the gel, cut to the shape of the gel.
- (iv) Place the nitrocellulose-gel-filter paper sandwich between the two sponges, then place the sandwich-sponge between the two white racks and secure with clamps.
- (v) Add in transblotting buffer to the mesh tank (3/4 of the way up), place this assembly in (by sliding in), and arrange so that the nitrocellulose faces the positive anode (red).
- (vi) Allow the gel to electrophorese at 50 mA overnight.
- (vii) Remove the membrane for immunoblotting.
Use a shaking water bath at 37°C from now on:
- (viii) Wash the membranes in TBS for 5 min to remove the blotting buffer. Block the membranes with blocking solution for 1 h.
- (ix) Wash twice in TTBS, 5 min each wash.
- (x) Add the primary antibody and incubate for 3 h.
- (xi) Wash as in step ix of this section.
- (xii) Transfer the membrane to a glass plate (facing upwards) and add 15 mL secondary antibody. Use a plastic dispensing pipette to keep topping up the secondary antibody to ensure the nitrocellulose membrane is not left dry.
- (xiii) Wash as in step ix of this section, then wash twice in TBS, 5 min each time.
- (xiv) Add 20 mL HRP development solution and let bands develop.
- (xv) Rinse two to three times with deionized water.

4. Notes

1. Guanidium thiocyanate is toxic; minimize handling as much as possible. Handle the 2-mercaptoethanol under a fume hood.
2. Make up a stock solution minus the 2-ME and store up to 3 months at room temperature, wrapped in foil. The working Denaturing solution should be prepared just before use by adding in the 2-ME and can be stored up to 1 month at room temperature.
3. The solution can be stored up to 1 year at room temperature. Handle the glacial acetic acid in a fume hood.
4. Use nucleic acid grade phenol. The saturated phenol can be stored up to 1 month at 4°C. Handle the phenol under a fume hood.
5. Prepare the solution under the fume hood.
6. The Amersham mRNA Purification kit utilizes oligo(dT)-cellulose pre-packed spun columns and suitable for mRNA purification from total RNA extracted from 25 mg up to 1 g tissue.
7. Autoclave the solution to inactivate DEPC. Handle the DEPC under a fume hood.
8. For optimal DNA amplification, we custom-make oligonucleotide primers (both degenerate- and gene-specific) so that they are within 20–30 base pairs length.
9. Having the cylinder prefilled with 100 mL water helps to dissolve Tris and SDS more easily, allowing the magnetic bar to go to work immediately. If using a glass beaker, Tris can be dissolved faster given that water is warmed to about 37°C. However, the solution should be brought to room temperature before adjusting pH.
10. Care should be taken to mix and dissolve SDS since it makes bubbles.
11. Can use 12 M HCl to adjust pH. Care should be taken to avoid sudden drop in pH below the required pH. Alternately, use 12 M HCl at first to narrow the gap from the starting pH to the required pH, and from then use a series of lower concentrations (e.g., 6 and 1 M) to get to pH 8.8.
12. SDS precipitates at 4°C. Thus, the buffer needs to be warmed to dissolve SDS prior to use.
13. Acrylamide is highly toxic. It is a neurotoxin, thus care should be exercised to avoid skin contact and exposing the chemical to yourself and coworkers as much as possible. Handle all weighing, transfer, mixing of acrylamide in a fume hood. Wear a mask when weighing.

14. The acrylamide solution can be stored at 4°C up to 1 month. It is best to prepare this fresh each month when the gels are prepared.
15. Dilute 400 mL 5× TBS to 2 L with water to obtain 1× TBS.
16. Add TEMED last.
17. Once the resolving gel has set and been ready, prepare the stacking gel.
18. Freshly prepare the development solution just before use. Dissolve DAB in the buffer then add to the plastic container. Add H₂O₂ last.
19. Should take maximum care not to contaminate RNA samples with RNase. Appropriate treatment of glass/plastic wares and other equipment by DEPC and autoclaving is critically important as this will help to minimize the activity of RNase deliberated during cell lysis and to avoid the accidental introduction of trace amount of RNase from other potential sources in the laboratory.
20. After soaking, it is important to autoclave to remove traces of DEPC that might modify purine residues in RNA by carboxymethylation.
21. Our laboratory uses this method, however we repeat the phenol-chloroform extraction step twice in order to obtain cleaner RNA.
22. We use sterile and DEPC-treated compatible plastic centrifuge tubes for RNA preparation and keep these tubes only for RNA work to avoid contamination with RNase. For preparation of cellular fractions, we use plastic centrifuge tubes which are compatible for high speed centrifugation.
23. After the RNA pellet is suspended in 200 µL DEPC-treated water, add three volumes of ethanol, mix, pipette 200 µL into each sterile DEPC-treated eppendorf tube, and store at -80°C. When the RNA is required, centrifuge the tube for 5 min to recover the pellet, wash briefly with 80% isopropanol and resuspend in DEPC-treated water.
24. Generally there is no advantage to using primers longer than 30 nucleotides (nt). The T_m should be 70°C or higher to achieve optimal PCR results as determined by nearest neighbor analysis (43). This also allows using touchdown PCR. The T_m of primers can be calculated or determined experimentally by doing PCR at different temperatures. Do not use self-complementary primer sequences which can fold back and form intra-molecular hydrogen bands or primers that have complementarity to the provided RACE primers, in particular in the 3'- ends.
25. We find that longer primers with T_m ≥ 70°C give more robust amplification in RT-PCR and RACE, particularly from difficult samples.

26. PPAR α full-length cDNA is about 1.5 kb, we have had good success using degenerative primers to amplify partial cDNA fragments of ≥ 1 kb and amplifying 5'-cDNA fragments that extend up to 800 bp from the GSP sites by RT-PCR and RACE, respectively.
27. The overlap 100–200 bases between primers will allow a stretch of known sequence incorporated into the amplified RT-PCR and RACE 5'- and 3'- fragments and can be used to verify that the correct gene was amplified.
28. Although most PCR applications work well with templates of average or even low quality (as PCR amplification proceeds exponentially), we find that fewer problems are encountered and best results achieved in RT-PCR and RACE protocols when the starting DNA material is of highest quality.
29. Often use touchdown PCR approach to achieve high specificity, particularly in 5'-, 3'- RACE PCRs. If the T_m of GSP $> 70^\circ\text{C}$ and T_m of RACE adapted primer (AP) is 71°C , only gene-specific synthesis occurs during these initial cycles, enabling a critical amount of gene-specific product to accumulate. The annealing/extension temperature is then decreased to the AP primer T_m for remaining PCR cycles, allowing efficient, exponential amplification of the gene-specific template.
30. We find these parameters are optimal conditions for PCR amplifications to obtain PPAR α cDNAs of ~ 1 kb. Manufacturers' protocols often recommend different parameters and a shorter denaturing time, e.g., initial denaturing at 95°C for 1 min; 25–35 cycles of 95°C for 1 min and 68 for 1 min (< 1 kb) or 3 min (1–5 kb); final extension 68°C for 1 min.
31. We have had good success amplifying 5' PPAR α cDNA fragments of 500–800 bp using these touchdown PCR parameters.
32. In our laboratory, we prepare a master mix for 10 PCR reactions testing the same primers:

Master mix	10 reactions ($\times 11$)	Each 20 μL reaction ($\times 1$)
(i) PCR water:	121 μL	11 μL
(ii) 10 \times PCR buffer	22 μL	2 μL
(iii) 50 \times dNTP mix (10 mM each)	4.4 μL	0.4 μL
(iv) Forward primer	11 μL	1 μL
(v) Reverse primer	11 μL	1 μL
(vi) Tag DNA polymerase	11 μL	1 μL
Final volume	180.4 μL	16.4 μL

Add the components in order in a 0.5 mL PCR tube. Use 1 μL cDNA synthesis reaction (cDNA template) and 2.6 μL MgCl_2 (testing a range of concentrations).

33. For negative control, we use a PCR reaction without the addition of the cDNA template.

For positive control, we include a cDNA template that we have previously used successfully to generate a known PCR product.

34. We prepare a master mix for three RACE reactions:

Master mix	3 reactions ($\times 4$)	Each 50 μL reaction ($\times 1$)
(i) PCR water:	144 μL	36 μL
(ii) 10 \times PCR buffer	20 μL	5 μL
(iii) 50 \times dNTP mix (10 mM each)	4 μL	1 μL
(vi) Advantage 2 Polymerase Mix	4 μL	1 μL
Final volume	172 μL	43 μL

35. We have had good success amplifying full-length PPAR α cDNA using this PCR protocol. The final extension is optional; however, it may reduce background in some cases. We also find that better results are obtained by reducing the denaturation step (as exposure of DNA to high temperatures causes some depurination of single-stranded DNA during denaturation, which eventually leads to strand scission). High temperature also causes gradually loss of Tag DNA polymerases enzyme activity. In experiments with larger template, it is best and particularly important to minimize the denaturation time.
36. Store the remaining 45 μL of the reaction mixture on ice so that it can be subjected to further cycling if needed.
37. For 0.5–2 kb insert, we use 1 kb DNA ladder. For larger size DNA fragment, we use *Hind III*. Carry out electrophoresis at 10 V/cm of the gel and use lower voltage, e.g., 5 V/cm for low-melt agarose gel to minimize the amount of heat generated in the tank.
38. For directional cloning of full-length PPAR α cDNA into pCMV-Script XR[®] expression vector, we include restriction sites *EcoRI* and *XhoI* at the 5'- and 3'- ends of the forward and reverse GSP respectively. For sequencing of PCR, RACE products, we subclone directly into pGEM-T[®] vector.
39. In our laboratory, we set up a series of control ligation reactions to check the efficiency of ligation into a vector (as described below). For optimization of insert:vector molar ratios, we perform ligation using 1:3, 1:1, and 3:1 ratio.

Testing	Reaction set up with
DNA ligase is active	<i>Hind III</i> restricted lambda DNA
Ligation efficiency	control insert DNA (supplied)
Number of background colonies	cut plasmid
Transformation efficiency	un-cut plasmid

40. We add an appropriate antibiotic (as describe below) when carrying out transformation of plasmid DNA into cells and competent cells preparation.

(i) Preparation of competent cells		
	Media	Working concentration
DH5 α	LB with no antibiotic	–
XLI-blue <i>E. coli</i>	LB tetracycline	50 μ g/mL (in 50% ethanol)
(ii) Transformation		
pGEM-T [®] , pCI-neo	Ampicillin	100 μ g/mL (in water)
pCMV-Script X [®]	Kanamycin	50 μ g/mL (water)

w

41. We have had good success using in-house prepared XLI-blue *E. coli* and DH5 α competent cells for transformation of plasmid DNA containing PPAR α cDNA.
42. After incubation of the cells for about 3 h, we start to take a small aliquot of the culture and measure the OD (optical density) at 600 nm (using a spectrophotometer). We stop incubating the cells when the OD is between 0.3 and 0.4 (log phase).
43. By adding potassium acetate solution, it neutralizes the solution and precipitates cell debris and chromosomal DNA.
44. In our laboratory, we sequence PPAR α cDNA cloned in pGEM-T[®], pCMV-Script XR[®], and pCI-neo vectors using the M13, T3, T7, T7_{PCI} obtained desalted (Geneworks) and internal primers derived from known PPAR α cDNA sequences. We modify the T7 primer sequence to obtain T7_{PCI} in order to sequence cDNA cloned in pCI-neo vector.
45. We dry the DNA pellet on a heating block for about 1–2 min at 55°C.
46. This step helps to remove the remainder of cytosol.
47. In our laboratory, we use Cos-7 cells for expression of cloned PPAR α cDNAs. Cos-7 cells are able to amplify and over express a transfected cDNA under the control of the SV40 promoter such as cDNA clone in pCMV-Script XR[®] or pCI-neo vector.

48. We prepare the cytosolic/nuclear extract and BSA standard samples by adding:

Tube	Conc (mg/mL)	Cytosol/nuclear	BSA (1 mg/mL, μ L)	Water (μ L)
Sample	–	10 μ L	–	290
1 (BSA)	0.0		–	300
2	0.16		50	250
3	0.332		100	200
4	0.500		150	150
5	0.667		200	100
6	0.833		250	50
7	1.0		300	–

49. Must wash the gel glass plates separately to avoid scratching, dry with a Chux cloth, and wipe over with methanol. Apply the Cello-seal (supplied as part of the Hoefer electro-transfer unit) onto the plastic lining and put the apparatus together. The clamps and the lower glass plate should face inwards.
50. Make the resolving gel and the first overlay together. The first overlay must be added immediately after casting gel within the gel cassette. Allow space for stacking the gel.
51. While the gel is setting, we solubilize and heat the protein samples. Fill the gel tank with gel running buffer 1/3 of the way up. Once the gel is set, place the gel apparatus in the tank. Remove the comb slowly and quickly fill the tank with gel running buffer. Remove air bubbles along the bottom of the apparatus.
52. We stack the gel at 100 V for 1 h and run at 200 V for 1 h 45 min. It is stacked when it reaches the interphase, i.e., top of the lower gel.

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