Preface

Opioid research is one of the multidisciplinary research areas that involve advanced techniques ranging from molecular genetics to neuropharmacology, and from behavioral neuroscience to clinical medicine. In current opioid research, it has become increasingly important to use multiple approaches at molecular, cellular, and system levels for investigations on a specific opioid-related target system. That often requires understanding and applying cross-field techniques and methods for the success of one's research projects. Through its broad spectrum of coverage, *Opioid Research: Methods and Protocols* provides a comprehensive collection of major laboratory methods and protocols in current opioid research, covering topics from molecular and genetic techniques to behavioral analyses of animal models, and then to clinical practice. It will serve as a convenient reference book from which those involved in opioid research will learn or perfect the necessary cross-field techniques.

The detailed methods and protocols described in *Opioid Research: Methods and Protocols* have each been successfully applied in current opioid research. Part I provides molecular techniques for the cloning and expression of opioid receptors, and for the quantitative characterization of their signaling pathways. Part II includes primary techniques for mapping the distributions and detecting the expression levels of opioid receptors, opioid peptides, and their messages in brain tissues and in individual cells. Part III deals with methods for creating in vitro receptor models and in vivo animal models to study opioid functions. Part IV describes practical applications of opioids in clinical medicine for the treatment of pain and opioid addiction.

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Expression of Opioid Receptors in Mammalian Cell Lines

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1. Introduction

Three major opioid receptors, δ (DOR-1) ***(1,2)***, μ (MOR-1) ***(3–5)***, and κ (KOR-1) ***(6–9)***, and an opioid-like receptor (ORL-1/KOR-3) ***(10–16)*** have been identified by molecular cloning. Although each of the cloned opioid receptors is derived from a single gene, a number of alternatively spliced variants from their own genes have been isolated ***(16–20)***. One extraordinary example is the mouse μ opioid receptor (*Oprm*) gene in which alternative splicing of the fourteen exons generates at least 15 variants ***(21–25)***. It is difficult to study these cloned receptors in vivo. But expressing individual receptors in a particular cell line through transfection of the cloned receptor cDNAs offers a valuable system for exploring their pharmacological and biological properties, as well as their structure and function relationships. To successfully express the cloned receptors, several factors must be considered.

1.1. Choice of Cell Lines

Criteria for choosing a cell line for expression of opioid receptors include no expression of endogenous opioid receptors, easy handling, fast growing, and accessibility for transfections. Several nonneuronal cell lines, such as the Chinese hamster ovary (CHO), the human embryonic kidney (HEK) 293, and the African green monkey kidney (COS-7) cell lines, are commonly used for expressing the cloned opioid receptors. However, differential expression of endogenous G-proteins and other factors involved in the signal transduction pathways among the cell lines may contribute to different pharmacological or biochemical profiles for the same receptors. Therefore, functional comparison

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between two or more receptors should be made in the same cell line with cautious interpretation of the results in terms of the restricted cell environment.

1.2. Choice of Mammalian Expression Vectors

For expression in a mammalian cell line, an opioid receptor cDNA containing its own or a Kozak consensus translation initiation site ***(26)*** has to be subcloned into mammalian expression vectors. Many mammalian expression vectors are available from a variety of sources. All mammalian expression vectors contain components necessary for both their propagation in bacteria and the transcription of the inserted DNA in mammalian cells. A cytomegalovirus (CMV) promoter or a SV40 promoter is commonly used for permitting high-level constitutive transcription of the inserted DNA in various mammalian cell lines, whereas a polyadenylation signal site is always built at the downstream of the inserted DNA for efficient transcription termination and polyadenylation of mRNA. However, choosing a vector mainly relies on the selectivity of its polylinker for efficient cloning and the availability of its antibiotic resistant genes for selection of stable cell clones. Additionally, many inducible vector systems are available for permitting control of transcription level of the inserted DNA. Common inducible systems include the Tet-Off or Tet-On system (ClonTech and Invitrogen) regulated through tetracycline, the Ecdysone-inducible system (Invitrogen) responsive to Muristerone A and the LacSwitch inducible system (Stratagene) induced by isopropylthiogalactose (IPTG). Recently, a Flp-In vector system (Invitrogen) has been developed to generate stable cell lines through Flp recombinase-mediated integration, in which a cDNA is integrated into a specific and transcriptionally active genomic site in the host cells.

1.3. Choice of Transfection Methods

Methods such as diethylaminoethyl (DEAE)-dextran transfection, calcium phosphate transfection, electroporation, and liposome-mediated transfection have been developed to introduce DNA into mammalian cells by using different mechanisms ***(27)***. Choice of a transfection method depends upon the type of cell lines used, the detailed procedures, and overall costs. For a given cell line, different methods with the same DNA may have different transfection efficiencies by severalfold. For instance, the rank order of transfection efficiency in CHO cells from our laboratory is: LipofectAmine (Invitrogen, one type of liposome-mediated transfections) > DEAE-dextran transfection > Calcium phosphate transfection. The procedures in most liposome-mediated transfections are more convenient than those of DEAE-dextran or Calcium phosphate transfection, but the cost of the liposome-mediated transfection is

much higher than those of DEAE-dextran or Calcium phosphate transfection if a large number of cells are used.

1.4. Transient Transfection and Stable Transfection

DNA can be transiently or stably transfected into cell lines, depending upon the type of applications used in the transfected cell lines. A transient transfection allows the transfected genes to be expressed within a short period of time and the cells are usually harvested or analyzed after a 24–72 h transfection. The transient transfection provides a convenient way to obtain results quickly. A stable transfection allows obtaining individual cells in which the transfected DNA is integrated into the active transcription sites of the host genome through an antibiotic selection that is often based upon expression of the antibiotic resistant gene in the same transfected DNA. It takes a relatively long time, usually 2 wk–2 mo, depending on the cell types and the antibiotics, to obtain the stable cells. However, the cells stably expressing the transfected receptors at a relatively constant level are valuable for applications that require a large number of cells, such as receptor binding and G-protein coupling studies.

This chapter describes procedures for cloning the cDNA into the mammalian expression vector. Also presented are both a transient transfection with DEAE-dextran and a stable transfection with LipofectAmine reagent in CHO cells. Finally, methods to verify expression of the transfected cDNAs are briefly discussed.

2. Materials

1. pcDNA3.1 vector series (Invitrogen) (*see* **Note 1**).
2. Restriction enzymes with 10× reaction buffers (New England BioLab) (*see* **Note 2**).
3. DNA Clean and Concentrator (ZYMO Research) (*see* **Note 3**).
4. T4 DNA ligase with 10× ligation buffer (NEB).
5. JM109 competent cells (> 10^8 colony-forming unit (cfu)/µg) (Promega) (*see* **Note 5**).
6. Plasmid Mini and Maxi kits (Qiagen).
7. 1% agarose gel with 0.2 µg/mL ethidium bromide.
8. TBE buffer: 89 m*M* Tris base, 89 m*M* boric acid, and 2 m*M* ethylenediamine tetraacetic acid (EDTA) in H_2O.
9. F12 medium (Invitrogen).
10. Fetal bovine serum (FBS).
11. pCH110 vector (Amersham).
12. Phosphate-buffered saline (PBS): 8 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4, 137 m*M* NaCl, and 27 m*M* KCl in H_2O. Adjust pH to 7.4.
13. DEAE-dextran stock: Dissolve 5 g DEAE-dextran (Amersham) in 100 ml of PBS. Sterilize the solution by filtrating through a 0.22-µm filter and store at –20°C.

14. 0.25 *M* chloroquine. Dissolve 6.45 g chloroquine in 50 mL of H_2O. Sterilize by filtrating a 0.22 μm filter and store in a foil-wrapped tube at –20°C.
15. CHO cells (ATCC).
16. OPTI-MEM I reduced serum medium (Invitrogen).
17. LipofectAmine (Invitrogen).
18. 10% dimethyl sulfoxide (DMSO) solution in PBS. Filtrate the solution through a 0.22-μm filter.
19. Treated-Tris-HCl buffer: 50 m*M* Tris-HCl, pH 7.4 at 25°C, 1 m*M* EDTA, and 100 m*M* NaCl.
20. Water bath.
21. Tissue culture hood.
22. CO_2 cell culture incubator.

3. Methods

3.1. Cloning the cDNA Fragment into pcDNA3.1 (see Note 1)

3.1.1. Digesting the cDNA and pcDNA3.1 with Restriction Enzymes (see Note 2)

1. For digesting with single restriction enzyme, pipet 5–10 μg of DNA, 3 μL of 10× restriction buffer, and appropriate volume of ddH_2O into a sterile microcentrifuge tube. Then add <3 μL of 10–20 U restriction enzyme to bring the final volume to 30 μL. Incubate the tube at the proper temperature (most at 37°C) for >1 h.
2. For digesting with two restriction enzymes, simultaneously cut DNA with the two enzymes in the same reaction if both enzymes are active in the same buffer. However, if one buffer cannot fit two enzymes, digest DNA with one enzyme at a time. Purify the digested DNA with a DNA Clean & Concentrator kit by following the manufactory protocol to remove the buffer and enzyme. Then digest the purified DNA with the second enzyme.

3.1.2. Purifying the Digested DNA and pcDNA3.1 (see Note 3)

1. Run the digested DNA on 1% agarose gel in TBE buffer.
2. Cut off the gel containing the desired DNA band and extract the DNA from the gel by using a Zymoclean Gel DNA Recovery kit by following the manufactory protocol.
3. Purify the digested pcDNA3.1 with the DNA Clean & Concentrator.
4. Analyze a small portion of the purified DNA fragment and pcDNA3.1 on 1% agarose gel to estimate the purity and quantity of the DNA and pcDNA3.1 for next ligation reaction.

3.1.3. Ligating the Digested DNA Fragment into the Digested pcDNA3.1 (see Note 4)

1. Add the digested DNA fragment and the digested pcDNA3.1 at 5:1–10:1 ratio in a sterile 1.5-mL microcentrifuge tube and bring the volume to 17 μL with H_2O.
2. Incubate the tube at 37°C for 5 min and place the tube on ice for 3 min.
3. Add 2 μL of 10× T4 DNA ligase buffer and 1 μL of T4 DNA ligase (400 U), and gently vortex the tube.
4. Incubate the tube at room temperature overnight.

3.1.4. Transformation and Isolation

1. Transform the ligated DNA into JM109 competent cells by following the manufactory protocol (*see* **Note 5**).
2. Isolate individual plasmids from 5–10 colonies by using a Pladmid Miniprep kit (*see* **Note 6**).
3. Digest approx 0.5 µg of the isolated DNA with appropriate restriction enzymes to identify the constructs with right inserts.
4. Further confirm the orientation and sequence of the inserts by sequencing with proper primers.

3.2. Transient Transfection with DEAE-dextran Method in CHO Cells (see Note 7)

3.2.1. Preparation of DNA and CHO Cells

1. Purify DNA with a Plasmid Maxi prep kit. Estimate the DNA concentration and purity by measuring its OD_{260} and ratio of OD_{260}/OD_{280} in a ultraviolet (UV) spectrophotometer, respectively.
2. Thaw a vial of frozen CHO cells (approx 10^7 cells) quickly in a 37°C water bath and transfer the cells into a 100-mm tissue culture dish containing 15 ml of F12 medium with 10% FBS (complete medium).
3. Grow the cells in a humidified culture incubator with 5% CO_2 at 37°C to approx 90% confluence.
4. To expend the cells, aspirate the medium, add 5 mL of PBS containing 1 m*M* EDTA, incubate at 37°C for 5 min, lift the cells by pipetting with a 10-mL pipet, and transfer the lifted cells equally into five 150-mm tissue culture dishes, each containing 25 mL of complete medium.
5. Grow the cells to 85–90% confluence at the time of transfection (*see* **Note 8**).

3.2.2. Preparation of DNA-DEAE-Dextran Complex and Transfection Medium

1. For transfection with five 150 mm dishes, mix 200 µg of DNA with appropriate volume of PBS in a sterile 50-mL conical tube.
2. Add 0.75 mL of DEAE-dextran stock (50 mg/mL) into the tube with a final volume of 3.75 mL and gently swirl the tube.
3. Incubate the tube at room temperature for 5 min.
4. For transfection with five 150-mm dishes, mix 60 mL of serum-free F12 medium with 24 µL of 0.25 *M* Chloroquine stock in a 100-mL sterile glass bottle.
5. Add 3.75 mL of the DNA-DEAE-dextran mixture into the bottle and gently mix.

3.2.3. Incubation and Shocking

1. Aspirate the complete media from the dishes, wash the dishes with 15 mL of serum-free F12 media, and completely remove the F12 medium.
2. Add 12.7 mL of the transfection medium into each dish.
3. Incubate the dishes in the incubator at 37°C for 3 h.

4. Aspirate the transfection medium and add 10 mL of 10% DMSO solution (*see* **Note 9**).
5. Incubate the dishes at room temperature for 90–120 s.
6. Aspirate 10% DMSO solution and wash the cells with 15 ml of serum-free F12 medium once.
7. Add 20 mL of complete medium and incubate the dishes in the incubator with 5% CO_2 at 37°C.
8. Harvest or analyze the cells after 24–72 h.

*3.3. Stable Transfection with LipofectAmine in CHO Cells (***see** ***Note 7 and 10)***

*3.3.1. Determining the Optimum Concentration of Antibiotics for Selection (*see ***Note 11****)*

1. Pass CHO cells as described in **Subheading 3.2.1.** into one 12-well plates with 1:15 dilution.
2. Add 12 different concentrations of antibiotics into individual 12 wells.
3. Replace the medium with fresh medium containing the antibiotic every 3 d.
4. Choose the concentration in which the antibiotic kills 99% cells after 10–14 d selection.

3.3.2. Preparation of DNA, CHO Cells and DNA-LipofectAmine Complex

1. Perform DNA purification as described in **Subheading 3.2.1.** (*see* **Note 12**).
2. Grow and expend the cells as described in **Subheading 3.2.1.**, **steps 2–5** except for using a 6-well tissue culture plate and growing the cells to 80% confluence at the time of transfection.
3. Label two sterile 1.5-mL tubes as A and B. In A tube, mix 1 µg of DNA with 100 µL of OPTI-MEM medium. In B tube, dilute 6 µL of LipofectAmine into 100 µL of OPTI-MEM medium.
4. Transfer 106 µL of the LipofectAmine-containing medium from B tube to A tube containing the DNA and gently vortex.
5. Incubate A tube at room temperature for 30 min.

3.3.3. Incubating DNA-LipofectAmine Complex with CHO Cells

1. Aspirate the medium from the 6-well plate.
2. Wash the cells with serum-free F12 medium once and remove the medium.
3. Add 0.8 mL of serum-free F12 medium into A tube containing the complex, and gently mix.
4. Transfer the diluted solution (approx 1 mL) into the washed six well.
5. Incubate the plate in the incubator at 37°C for 5–8 h (not overnight).
6. After 5–8 h incubation, aspirate the medium containing the complex and add 2 mL of complete medium.
7. Continue incubating the plate for 24–48 h.

3.3.4. Selecting Stably Transfected Cells with an Appropriate Antibiotics

1. After 24–48 h of incubation, aspirate the complete medium and wash the cells with 2 mL of serum-free F12 medium once.
2. Add 0.5 mL of PBS containing 1 m*M* EDTA.
3. Incubate the plate at 37°C for 5 min.
4. Lift the cells with a pipet and transfer the lifted cells into one 150-mm culture dish containing 25 ml of complete medium with the appropriate antibiotics (approx 1:15 pass).
5. Incubate the dish for 10–14 d until individual colonies grow. During the incubation, replace the medium with the fresh selective medium every 3 d.

3.3.5. Isolating Individual Colonies (see **Note 13**)

1. Aspirate the medium and rinse the cells with PBS once.
2. Add 20 mL of PBS.
3. Pick up 10–20 foci one at a time by using a 200 µL pipet under a microscope with 10× objective.
4. Find the colony under microscope, loosen the colony by gently scraping with the pipet tip.
5. Suck out 30 µL of PBS containing the loosened colony into the tip, transfer into a well of the 96-well plate containing 30 µL PBS with 2 m*M* EDTA, and gently mix with the pipet.
6. Incubate the 96 well at room temperature for 5–20 min.
7. Transfer the cell suspension from the 96 well into a six-well plate containing 2 mL of the selective medium.
8. Continue passing the cells from the six well to large plates until appropriate amount of the cells are obtained for further analysis.

3.4. Verification of Opioid Receptor Expression in Transfected Cells

3.4.1. Verification of the Expression by Receptor Binding

1. Prepare cell membranes as in our previous studies ***(23–25,28)***. Rinse the cells with PBS twice and add approx 5–10 mL PBS just to cover the plate.
2. Scrap the cells off the plates with a rubber policeman (*see* **Note 14**).
3. After collecting the cells in a centrifuge tube, spin the tube at 1000 *g*, resuspend the cells in cold Treated-Tris-HCl buffer containing 0.1 m*M* phenylmethanesulfonyl fluoride and homogenize with a polytron homogenizer at 4°C for 30 s.
4. Centrifuge the homogenate at 20,000*g* for 30 min at 4°C, resuspend the membrane pellet in 0.32 *M* sucrose, and store at –80°C.
5. Choose an appropriate radiolabeled ligand for a receptor binding assay: for all types of opioid receptors, [^{3}H]-Diprenorphine and [^{3}H]-Naloxone; for µ opioid

receptors, [^{3}H]-DAMGO; for δ opioid receptors, [^{3}H]-DPDPE; for κ opioid receptors, [^{3}H]-U69593; and for ORL-1/KOR-3, [^{3}H]-OFQ or [^{125}I]-OFQ.
6. Perform binding assays ***(23,28–31)***.

3.4.2. Verification of mRNA Expression by RT-PCR or Northern Blot Analysis

1. To determine transcription level of the transfected receptor DNA, extract total RNA from 10^6 cell (one 6 well) by using a RNeasy mini kit (Qiagen).
2. Perform RT reaction with Superscript II reverse transcriptase (Invitrogen) and random hexamers.
3. Perform PCR by using the first-strand cDNA from the RT reaction as template with appropriate primers derived from the transfected opioid receptor sequences ***(10,19,25)***.
4. Analyze the PCR products on 1% agarose gel.
5. Perform Northern blot analysis with an appropriate probe ***(10,23,25,32)*** .

3.4.3. Verification of Protein Expression by Western Blot or Immunostaining

Perform Western blot analysis or immunostaining with appropriate polyclonal or monoclonal antibodies on the whole cells or the isolated membrane ***(10,33)***.

4. Notes

1. I prefer using the pcDNA3.1 vector series since its (+) and (–) versions offer a polylinker with 16 unique cloning sites in both orientations, providing more choices for cloning. It also offers three sets of different selection markers, neomycin, hygromycin, and zeocin, which allow for selection of double- or triple-stable cells with cotransfection of different cDNA clones. The first step of the cloning is to find unique restriction enzyme sites in both the polylinker of the vector and the cDNA-containing plasmid, so that they can lift out the entire cDNA fragment from the plasmid without cutting its own coding regions. Using the fragments with different cohesive ends can facilitate unidirectional ligation. If no appropriate restriction enzyme sites are available to lift the fragment from its plasmid, the fragment containing proper restriction sites at its both 5'- and 3'-ends can be generated by PCR with the gene-specific sense and antisense primers having appropriate restriction sequences at their 5'-end. It is recommended to use a high-fidelity DNA polymerase in PCR to reduce potential mutations and confirm the amplified sequence after cloning.
2. In general, 1 U of restriction enzyme can digest 1 μg of DNA at its optimum temperature in 1 hour. However, I often add more enzymes to achieve complete digestion. Most enzymes are stored in 50% glycerol, but they are usually less active in >5% glycerol. Therefore, it is not recommended to add more than 1 μL of enzyme in a 10-μL reaction. Although restriction enzymes are available from

many companies, using enzymes from one company makes easy selection of the appropriate buffer for double digestion because most companies already formulate different enzyme activities in different buffers.

3. The desired DNA fragment must be separated and purified from its associated vector sequence, which can be easily done by using a gel extraction procedure. Many DNA cleaning and gel extraction kits are available from various companies. No matter the type of kit used, it is better to elute DNA with water rather than with the elution buffer provided in the kits. Though the yield may be low, elution with water prevents possible inhibition of the following ligation reaction by an elution buffer.
4. The ratio of DNA to vector is critical for efficient ligation. In our experience, the ratio of 5:1–10:1 is suitable for a cohesive-end ligation, whereas a blunt-end ligation requires an even higher ratio ranging from 10:1 to 20:1.
5. Other types of competent cells like XL1-Blue (Stratagene), TOP10F', or DH10 (Invitrogen) can be used. Transformation efficiency for all the competent cells can be greatly reduced by repeating thaw-frozen cycles. Aliquot the unused cells, quickly freeze on dry ice and store at –80°C.
6. Any other kits or protocols for isolating plasmid DNAs can be used. It is highly recommended to confirm the clones through sequencing even if the result from restriction enzyme digestion has been satisfied.
7. The protocols for DEAE-dextran transfection and LipofectAmine transfection described in this chapter have been optimized in our CHO cells. However, if another cell line or a CHO cell line from a different source is used, the protocols may not be useful. It is highly recommended to optimize transfection conditions for each new cell line with a vector containing a reporter gene to determine the transfection efficiency. Luciferase and β-galactosidase (*LacZ*) are commonly used as the reporters. We use pCH110 vector containing a *LacZ* reporter under control of a SV40 promoter for optimization. Transfection efficiency can be easily determined by β-gal staining or by measuring β-gal activity with available kits (Promega and Boehringer Mannheim). The optimized conditions include the ratio and the amount of the DNA and its reactive reagents, the cell density reached before transfection, the incubation time after adding the DNA-reagent mixture, and the additional shock steps in DEAE-dextran transfection. DEAE-dextran transfection is suitable for transiently transfecting a large number of CHO cells, whereas LipofectAmine transfection is mainly used for obtaining stable clones. However, a small number of the cells from the transient transfection can also be used for selecting stable clones.
8. It is crucial to manipulate mammalian cells under strict sterile conditions to prevent contamination by bacteria or fungi. All materials including media, reagents, buffers and glassware should be sterilized by either standard autoclaving or filtering through a 0.22-μm filter. Standard hood operations and incubator maintenance should be strictly followed. The protocol described here is for transfecting 5×150 mm dishes. If more or less dishes are used, all the solutions and volumes can be multiplied or divided based upon their surface areas.

9. DMSO shock can increase transfection efficiency by 2–3 folds in our CHO cells, but it may not be necessary for other cell lines. The shock time, from 90–120 s, but no more than 120 s, should be followed to avoid overshocking the cells.
10. Many types of liposome-mediated transfection reagents are available from same or different companies. There are also several formulas even with the same type of lipid. For instance, LipofectAmine has three different formulas, LipofectAmine 2000, LipofectAmine Plus and LipofectAmine. In our CHO cells, transfection with LipofectAmine is better than that with LipofectAmine Plus or LipofectAmine 2000. However, in our HEK293 cells, transfection with LipofectAmine Plus is more efficient than that with LipofectAmine or LipofectAmine 2000.
11. Cell density can greatly influence the antibiotic sensitivity. If a selection starts with high cell density, cells may be killed by overcrowding rather than by antibiotics. Therefore, the optimum concentration of antibiotics should be selected under the cell density similar to that plated in actual stable selection.
12. Because the stable transfection with a small number of cells needs much less DNA than transient transfection, the DNA isolated from the miniprep is usually enough for the stable transfection. However, if the DNA concentration is too low, it is necessary to increase DNA concentration by either ethanol precipitation or by a DNA clean and concentrator kit.
13. Isolating individual colonies with a pipet is easier and faster than with traditional cloning cylinders. If the cell growth rate is slow, the lifted colony can be transferred into a smaller well (12-well or 24-well plate) so that the cells are not diluted too much.
14. Opioid receptor binding is very sensitive to trypsin. Do not lift the cells with trypsin when the cells are passed for binding.

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