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## Preface

By the end of the 1980s only two microtubule-dependent motors, the plus end-directed kinesin and the minus end-directed cytoplasmic dynein, had been identified. At the time, these two motors seemed almost sufficient to explain directional motility events on polar microtubule tracks in the cell. Nonetheless, shortly after, the tip of the iceberg began to emerge with the identification of proteins containing in their sequences a domain found in kinesin. This domain, called the “motor domain,” conferred on these proteins the essential property of moving on microtubules, using the energy derived from ATP hydrolysis. Since then, the identification of new proteins belonging to the kinesin superfamily of microtubule-dependent motors has gone at such a pace that nowadays more than 200 entries with motor domain sequences are deposited in the database. Kinesin family members are found in all eukaryotic organisms tested. They present a wide range of domain organizations with a motor domain located at different positions in the molecule. Their motility properties are also variable in directionality, velocity, and such other characteristics as bundling activity and processivity. Finally, and most important, they participate in a multitude of cellular functions. Our understanding of many cellular events, such as mitotic spindle assembly and neuronal transport, to cite only two, has progressed substantially in the last few years thanks to the identification of these motors. Kinesin-like proteins (KLP) appear now to be involved in so many different cellular events that it is no longer a surprise to see researchers finding a KLP involved in their favorite cellular process. The general interest in this field is thus growing and many researchers are now faced with the task of identifying and/or characterizing a KLP to understand the process they are studying.

The aim of *Kinesin Protocols* is to provide a set of clear and concise protocols for the identification and characterization of members of the kinesin superfamily of microtubule-dependent motors. These protocols should prove to be especially useful for nonspecialists in the field, but others are actually up-to-date protocols based on recent findings that may turn out to be of interest for specialists as well. As in other books in the *Methods in Molecular Biology* series, a special emphasis has been put on the “Notes” section. There, the reader will find all the little tricks—gathered through experience by the authors—that make the difference between a successful and an unsuccessful experiment.

The protocols presented in *Kinesin Protocols* cover many different aspects of kinesin identification and characterization. The volume opens with a few chapters dealing with various approaches to the identification and purification of kinesins from different sources (Chaps. 1–6). These include methods to express and purify kinesins in different systems. Purified proteins or fragments are used to determine some of the properties intrinsic to members of this family. Methods presented in the following chapters are aimed at characterizing microtubule-enhanced ATPase activity (Chap. 7) and motility properties at different levels (Chaps. 8 and 9). Although most KLPs indeed have the property to move directionally on microtubules, new types of activities have recently been found for some members of the class. Several ways to test their microtubule destabilizing activity are presented in Chap. 10. Some examples of how to address functional studies are presented in the following few chapters (Chaps. 11–17). Although the function of KLPs is generally thought to be the transport of cargoes, it has become clear that they also play an important role in the organization of microtubules in three dimensions. Chapter 18 describes some very new methods to address this aspect of KLP function. Finally, two chapters present technically more demanding protocols for the study of kinesins at the structural level. One chapter describes methods that have been successful in crystallizing kinesin motor domains (Chap. 19) and the other chapter presents protocols to study the structure of the kinesin–microtubule complex (Chap. 20).

Obviously, every reader will select and read first the chapter(s) of interest in *Kinesin Protocols*. Nonetheless, the content of the entire volume is recommended to everyone working in the field as a means to gain a better understanding of how best to handle kinesins experimentally. Some redundancy or overlap among chapters has proved unavoidable because certain basic experimental principles may be used for different purposes. We are confident that our book will both help the reader solve current research problems and stimulate the design of new experimental approaches adapted to the kinesin of interest in their work.

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## RT-PCR for the Identification of Developmentally Regulated Novel Members of the Kinesin-like Superfamily

Niovi Santama

### 1. Introduction

The kinesin-like motor protein superfamily comprises to date about 100 members present in essentially all the major eukaryotic phyla (reviewed in **ref. 1**), including over 30 in the mouse alone (**2**). The common feature of the kinesin-like proteins is a highly conserved 350-amino-acid “motor domain” (30–40% sequence identity across species) that includes even more highly conserved ATP- and microtubule-binding sites (**3**).

It is exactly the presence of the conserved motor domain and motor-domain “signature” sequences that has facilitated enormously the cloning and identification of further novel kinesin-like proteins both by conventional homology screening of cDNA libraries and by the application of reverse transcription–polymerase chain reaction (RT-PCR) methodology. In the later, oligonucleotide probes complementary to consensus motor motifs are used to coamplify motor-domain sequences from multiple kinesin-like proteins. Subsequent characterization of the product mixture can reveal the existence of novel motor cDNAs in a cell type, tissue, or organ of choice of a given organism (examples included in **refs. 4–7**, among others).

The RT-PCR strategy detailed in this chapter describes successive steps of RNA isolation and cDNA production, use of cDNA in PCR using degenerate oligonucleotide primers to coamplify kinesin-like motor sequences, and a method for fast and easy subcloning of the mixed PCR product. Protocols (and an experimental example) for the selection of developmentally regulated kinesin-like proteins from mouse hippocampus are also included. The source of RNA extraction and cDNA preparation in our experiments was mouse hip-

pocampal cells in a primary culture, a well characterized system in which mostly synchronized embryonic neurons develop *in vitro* through distinct stages of differentiation (8). Nevertheless, the methods described are readily applicable and adaptable to any other material (i.e., cell lines, tissues, or organs from any organism of interest).

It must be highlighted that once the subcloning of PCR products is achieved in this or similar protocols, the question that needs to be addressed and resolved will determine subsequent strategy. There are two approaches:

1. If the aim is to identify as many new members of the kinesin-like superfamily as possible, then this can be achieved by extensive sequencing of different selected clones. These clones, which are partial because they only contain the motor domain, can then be used to fish out full-length cDNAs either by screening appropriate cDNA libraries or by 5' and 3' rapid amplification of cDNA ends (RACE).
2. Alternatively, or in addition, it may be desirable to identify from the pool of clones those subject to developmental or cell-specific regulation. The relevant protocol offered in this chapter is one approach devised to explore this question. In this case, pools of kinesin-like cDNAs must be prepared independently from the two developmental stages or cell types that are to be compared. One pool will be subcloned and the second pool can be labeled and used as a mixed probe to screen by Southern blotting the series of clones of the first and vice versa. This is a first-step approach which is more reliable for revealing qualitative differences, but even quantitative differences can be detected if results are interpreted carefully. As such, it must always be complemented and confirmed by alternative methods of investigation such as comparative PCR of the two sources of RNA using cDNA-specific primers (9) or *in situ* hybridization (10).

## 2. Materials

### 2.1. Design and Preparation of Degenerate Primers

1. Custom-made, degenerate, synthetic oligonucleotides (usually 20–25 nucleotides), cleaved from the solid matrix, deprotected, and dialysed against distilled water (see **Note 1**).
2. Sephadex G25 (Amersham Pharmacia Biotech) prespun and packed in 1-mL disposable sterile plugged syringes.
3. TEN buffer: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 100 mM NaCl.

### 2.2. RT-PCR

#### 2.2.1. Poly-A<sup>+</sup> RNA Isolation and Construction of cDNA

To avoid the action of RNases, double-distilled sterile DEPC-treated water (treated with diethyl pyrocarbonate at 1:1000 dilution and autoclaved), sterile plasticware and glassware baked at 150°C overnight should be used for the

preparation of all solutions. All handling is carried out with gloves. Specific RNase inhibitors should be included as indicated in the protocol.

1. QuickPrep micro mRNA purification kit (Amersham Pharmacia Biotech, Sweden) for the direct isolation of poly-A<sup>+</sup> RNA from hippocampal cells in vitro. The kit includes the following:
  - a. Oligo(dT)<sub>18</sub>-cellulose at 25 mg/mL in storage buffer.
  - b. Extraction buffer (guanidium thiocyanate/N-lauroyl sarcosine).
  - c. High-salt buffer: 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5 M NaCl.
  - d. Low-salt buffer: 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 M NaCl.
  - e. Elution buffer: 10 mM Tris-HCl (pH 7.5), 1 mM EDTA.
  - f. Microspin columns.
2. Advantage RT-for-PCR kit (Clontech Laboratories, Inc. Palo Alto, CA) for the reverse transcription of poly-A<sup>+</sup> RNA to cDNA. The kit contains the following:
  - a. MMLV reverse transcriptase at 200 U/μL, oligo.
  - b. (dT)<sub>18</sub> primer at 20 μM.
  - c. 5X conc. reaction buffer: 250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl<sub>2</sub>.
  - d. dNTP mix at 10 mM for each deoxynucleotide.
  - e. RNase inhibitor at 40 U/μL.

### 2.2.2. PCR

1. Expand Long Template PCR polymerase mix (Boehringer LaRoche, Germany).
2. 10X conc. polymerase buffer: 500 mM Tris-HCl (pH 9.2), 160 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 22.5 mM MgCl<sub>2</sub> (supplied with the polymerase by the manufacturer).
3. Custom-made oligonucleotide primers (as in **Subheading 2.1**) each to be used at a final concentration of 20 pmole per reaction.
4. dNTP mix at 10 mM for each deoxynucleotide (Amersham Pharmacia Biotech).
5. cDNA template, prepared as in **Subheading 3.2.1**.
6. Thin-walled micro PCR tubes (0.2 mL) (Perkin-Elmer GmbH, Germany).

### 2.3. T/A Cloning of PCR Products

1. T/A cloning kit (InVitrogen, The Netherlands), containing:
  - a. Linearized T/A cloning vector pCR2.1.
  - b. T4 DNA ligase (4 Weiss U/μL).
  - c. 10X conc. ligation buffer: 60 mM Tris-HCl (pH 7.5), 60 mM MgCl<sub>2</sub>, 50 mM NaCl, 1 mg/mL bovine serum albumin (BSA), 70 mM β-mercaptoethanol, 1 mM ATP, 20 mM dithiothreitol, 10 mM spermidine.
  - d. Ready-to-use 50-μL aliquots of strain TOP10F' competent *Escherichia coli* bacteria for transformation.
2. SOC medium: 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.4 mM KCl, 10 mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub>, 20 mM glucose.
3. LB-agar plates containing 50 μg/mL ampicillin, 100 μM iso-propyl-thio-galactopyranoside (IPTG), 40 μg/mL X-gal.

## 2.4. Labeling of Mixed Probe and Southern Hybridization

1. Expand DNA polymerase, 10X conc. reaction buffer and oligo primers as in **Subheading 2.2.2**.
2. dNTP mix containing 10 mM of dATP, dGTP, dTTP, 5 mM dCTP and additionally 2  $\mu\text{Ci}$  (or 0.3 pmol) of  $\alpha\text{-}^{32}\text{P}$  CTP (specific activity 3000Ci/mmol).
3. “Gene Screen” hybridization transfer membrane (NEN Research Products, Du Pont).
4. 10X conc. SSC: 1.5 M sodium chloride, 150 mM sodium citrate (pH 7.0).
5. 1 M  $\text{Na}_2\text{HPO}_4$  buffer (pH 7.2) (“sodium phosphate buffer”).
6. Church buffer: 0.5 M sodium phosphate buffer, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA.

## 3. Methods

### 3.1. Design and Preparation of Degenerate Primers

1. Prior to probe labeling, purify the crude oligonucleotide by applying it, in a total volume of 100  $\mu\text{L}$ , to the top of a prespun and packed Sephadex G25 1-mL column that has been washed several times with sterile TEN buffer. Hold the spun column in a small Falcon tube containing a decapped Eppendorf tube at the bottom and centrifuge at 1600g for 3 min (*see Note 2*).
2. Collect the effluent, containing the oligonucleotide probe, in the decapped tube and then transfer to a fresh tube. Determine the concentration spectrophotometrically at 260 nm (*see Note 3*).
3. Make working dilutions of each oligo at 20 pmol/ $\mu\text{L}$  in water and store at  $-20^\circ\text{C}$ .

### 3.2. RT-PCR

#### 3.2.1. Poly-A<sup>+</sup> RNA Isolation and Construction of cDNA

1. To prepare poly-A<sup>+</sup> RNA, scrape up to  $10^7$  cells with a rubber policeman in 0.4 mL extraction buffer, vortex well, add 0.8 mL elution buffer, and mix again by vortexing (*see Note 4*).
2. After centrifuging at 10,000g for 1 min, place this cleared homogenate on top of the pellet of 1 mL of oligo(dT)–cellulose.
3. Mix gently, incubate slurry for 3 min on a rocking table and then centrifuge at 10,000g for 10 s.
4. Remove supernatant by aspiration, resuspend resin in 1 mL of high-salt buffer, centrifuge as in **step 3**, and repeat this wash for a total of five times.
5. Wash twice using low-salt buffer.
6. Resuspend the resin in 0.3 mL of low-salt buffer, transfer to a MicroSpin column, centrifuge at 10,000g for 5 s, and wash column three more times with 0.5 mL low-salt buffer.
7. Place column in a clean sterile tube and elute poly-A<sup>+</sup> RNA with 0.2 mL of elution buffer, prewarmed at  $65^\circ\text{C}$ . This fraction will contain about 80–90% of the recoverable mRNA and a second elution step with a further 0.2 mL of warm elution buffer is optional.

- Determine the concentration of RNA in the final eluate spectrophotometrically (*see Note 5*) and, if necessary, precipitate to concentrate solution (*see Note 6*).
- To prepare the first strand of cDNA using the stock solutions of **Subheading 2.2.1.**, place 10 ng of purified poly-A<sup>+</sup> RNA into a sterile 0.5- $\mu$ L Eppendorf tube and make up to 12.5  $\mu$ L with sterile DEPC-treated water. Perform this and all subsequent steps on ice unless otherwise specified.
- Add 1  $\mu$ L of the oligo(dT)<sub>18</sub> primer, heat the mix at 70°C for 2 min and quench rapidly on ice.
- Add in order 4  $\mu$ L of 5X conc. reaction buffer, 1  $\mu$ L dNTPs, 0.5  $\mu$ L RNase inhibitor; mix well and incubate for 42°C for 1 h (*see Note 7*).
- To stop cDNA synthesis and destroy any DNase activity, heat at 94°C for 5 min and then dilute to a final volume of 100  $\mu$ L with DEPC-treated water. Aliquot cDNA in convenient volumes (20  $\mu$ L) and store at -80°C, avoiding repeated freezing and thawing. For a typical 50- $\mu$ L PCR reaction, it is recommended to use 5–10  $\mu$ L of this cDNA.

### 3.2.2. PCR

- The recommended amplification protocol is as follows (*see Note 8*):
  - Denaturation at 94°C for 2 min.
  - 33 cycles of denaturation at 94°C for 10 s/annealing at 50°C for 30 s/amplification at 68°C for 1 min.
  - Final amplification at 68°C for 5 min.
  - Cooling at 4°C until sample retrieval.

- For each PCR reaction, prepare on ice two separate master mixes and combine them just prior to inserting the tubes in the PCR thermocycler (*see Note 7*).

Mix 1 contains the following added in order from the stocks solutions (**Subheading 2.2.2.**): sterile distilled H<sub>2</sub>O up to 25  $\mu$ L, 2.5  $\mu$ L of dNTP mix, 1  $\mu$ L of each of the appropriate pair of primers, 5–10  $\mu$ L of template cDNA (prepared as described in **Subheading 3.2.1.**).

Mix 2 contains: sterile distilled H<sub>2</sub>O up to 25  $\mu$ L, 5  $\mu$ L of 10X conc. buffer, and 0.75  $\mu$ L Expand polymerase enzyme mix.

Combine mixes 1 and 2, mix and place tube in PCR thermocycler already set to 94°C (hot start) and commence program. No mineral oil overlay is necessary if the thermocycler model allows use of a heated lid.

- Check 5–10  $\mu$ L of each reaction by 1% agarose gel electrophoresis and maintain reaction tubes on ice for <24 h if subcloning of PCR products by T/A cloning will follow (*see Note 9*). A characteristic example of the amplification results using this protocol is shown in **Fig. 1A**.

### 3.3. T/A Cloning of PCR Products

- From each PCR, set up a ligation reaction by mixing in order sterile distilled water to 10  $\mu$ L, 50–150 ng of the PCR product (*see Note 10*), 1  $\mu$ L of 10X conc. ligation buffer, 50 ng of the pCR2.1 vector, and 1  $\mu$ L T4 DNA ligase.

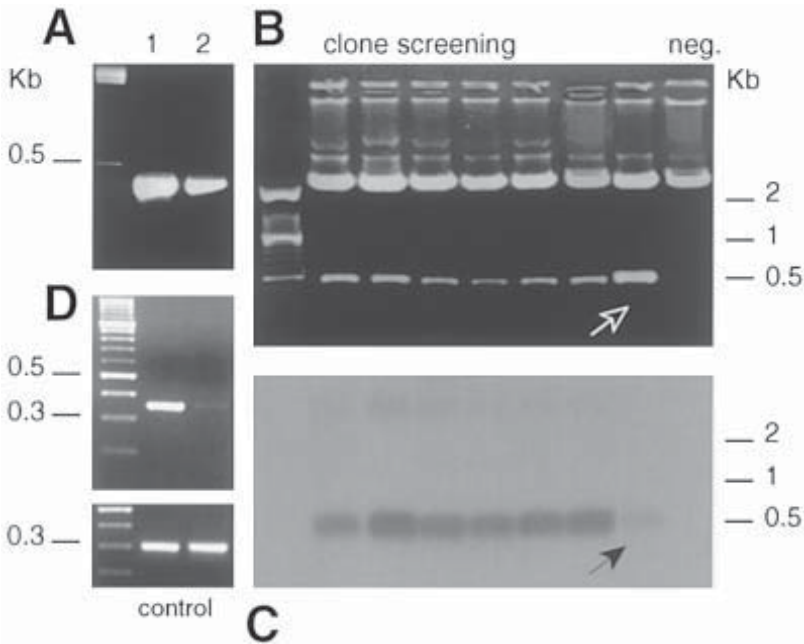


Fig. 1. An example of identification of a novel kinesin-like protein in mouse hippocampus by the concerted methodology described in this chapter. **(A)** RT-PCR using the degenerate primers described in **Note 1** and cDNA derived from stage 3 (immature; lane 1) or stage 5 (mature) hippocampal cultures (lane 2) as a template. The mixed PCR products of each stage were subcloned in vector pCR2.1. **(B)** Agarose gel showing a collection of some of the stage 3-bearing, *EcoRI*-digested plasmids which were subjected to Southern blotting using labeled, stage 5, mixed PCR product as a probe. **(C)** Corresponding autoradiograph of gel in B. The clone indicated by the arrow (M8.7) is shown to hybridize less strongly, suggesting that it is a rarer transcript in stage 5, relative to the other clones shown on this gel. This clone was then sequenced. **(D)** Confirmation of the result of C by RT-PCR, using specific primers for the unique amplification of the M8.7 clone and, as template, stage 3 (lane 1) or stage 5 cDNA (lane 2). This showed that, indeed, M8.7 cDNA is drastically downregulated during the later stages of neuronal differentiation, confirming the result in C. Bottom panel shows equivalent reactions, amplifying mouse GAPDH, carried out in parallel with the same amounts of cDNA as test reactions and used as internal standards. Sequencing of the partial clone M8.7 had revealed a novel kinesin-like protein, a full-length sequence of which would shortly appear as KIFC1 (**II**).

2. Incubate the ligation reaction at 14°C, preferably overnight (or at least for 6 h) and proceed immediately to bacterial transformation or store reaction at -20°C.
3. For each bacterial transformation thaw one 50- $\mu$ L vial of frozen competent cells (supplied by the T/A kit manufacturer), add 2  $\mu$ L of 0.5 M  $\beta$ -mercaptoethanol and 2  $\mu$ L of the ligation mix, and keep on ice for 30 min.



4. Heat shock bacteria for 30 s at 42°C, place on ice for 2 min, add 250  $\mu$ L of SOC medium to each tube, and incubate at 37°C with rotary shaking for 1 h.
5. Spread 50  $\mu$ L and 200  $\mu$ L from each transformation vial on separate LB-agar plates containing 50  $\mu$ g/mL ampicillin, 100  $\mu$ M IPTG, and 40  $\mu$ g/mL X-gal and incubate at 37°C for 18 h or more for blue/white selection (*see Note 11*).
6. Select white colonies, each expected to be recombinants containing an insert from the PCR product, mix and grow them in small-scale, overnight liquid LB cultures (*see Note 12*). Clones can be analyzed for insert size with small-scale plasmid preps and subsequent *Eco*RI restriction digests as *Eco*RI sites flank the insertion site in the pCR2.1 vector (**Fig. 1B**). Sequencing for the identification of inserts at this stage can be carried out using M13 forward and reverse primers whose sequences also flank the insertion site in the vector (*see Note 13*).

### 3.4. Labeling of Mixed Probe and Southern Hybridization

1. To use the mixed PCR product of one developmental stage (in our hands, “stage 3” of mouse primary hippocampal cultures) as a probe for Southern blotting, a collection of plasmids containing products of another developmental stage (“stage 5”), label it with  $\alpha$ -<sup>32</sup>P CTP by a further PCR. Repeat the PCR reaction under exactly the same conditions as in **Subheading 3.2.2.**, but this time using 10–50 ng of the mixed PCR product obtained previously (**Subheading 3.2.2.**) as a template and the “spiked” dNTP mix containing  $\alpha$ -<sup>32</sup>P CTP (described in **Subheading 2.4.**). The labeled mixed product, separated from unincorporated nucleotides by ethanol precipitation, is resuspended in 20  $\mu$ L TE and kept at –20°C to be used the same day. One microliter of the probe is measured by  $\beta$ -scintillation counting to check incorporation.
2. Run the collection of *Eco*RI-digested pCR2.1 plasmids, containing inserts that are derived from the PCR product mix (**Subheading 3.2.2.**), on a 0.8% agarose gel.
3. Denature gel with 0.5 M NaOH/1.5 M NaCl for 30 min and neutralize with 1 M Tris-HCl (pH 7.4), and 1.5 M NaCl.
4. Blot DNA onto a sheet of GeneScreen, presoaked in 10X conc. SSC, by capillary transfer overnight.
5. Remove GeneScreen membrane, float it on 50 mM sodium phosphate buffer for 20 min, bake it in a –80°C vacuum oven for 1 h and crosslink with ultraviolet (254 nm) at 0.15 J/cm<sup>2</sup> for 30 s.
6. Prehybridize blot with Church’s buffer at 65°C for 1 h, denature probe at 94°C for 5 min and immediately dilute it to 1–2  $\times$  10<sup>6</sup> cpm/mL in Church buffer for hybridization.
7. Hybridize blot with the mixed probe at 65°C overnight.
8. Wash blot with 40 mM sodium phosphate/1% SDS twice for 5 min at 55°C, four times for 15 min at 65°C, dry, and expose to X-ray film for 2–6 h or overnight, as required.
9. The autoradiograph will identify clones that do not hybridize to the labeled mixed probe and are likely candidates of stage-specific motor proteins (*see Note 14*; for

an example, *see* **Fig. 1B,C**). Clones can be sequenced (*see* **Subheading 3.3.**) and the partial sequences obtained (corresponding to the motor domain) can be used for pulling out full-length clones by cDNA library screening or by 5' and 3' RACE.

#### 4. Notes

1. The aim of the RT-PCR is to amplify many known and, more interestingly, unknown members of the kinesin-like gene family and, therefore, one has to design a degenerate (mixed) oligonucleotide that incorporates sufficient sequence variation that would be complementary to as many cDNAs as possible. Primers can be designed by including base redundancies representative of all known peptide sequences, but to avoid unnecessary redundancy likely to increase the amplification of nonspecific sequences, it is very useful to carry out nucleotide multiple sequence alignments of all known kinesin-like cDNAs from the given organism and take codon usage bias into account. Some useful empirical rules for the design are the following:
  - As much as it is possible, try to avoid amino acids with six codon choices and instead select those that are specified by one or two codons.
  - At positions where there is a choice of more than two bases (three- or four-fold ambiguity), inosine (I) is recommended. Inosine can form equal-strength stable pairs with all four bases.
  - Inosine can also be used in the third position of synonymous codons.
  - The three bases at the 3' end of the primer must be perfectly matched with the target sequence (choose a single codon amino acid for the 3' end) and should not include inosine.
  - Perfect matching at the 5' is not critical and extra sequences (i.e. restriction sites) can be added if desirable.

For the amplification of kinesin-like cDNAs from the mouse, the following primers were designed to consensus amino acid sequences that are located within the conserved motor domain. A 5' *Cla*I site was added in the upstream primer.

upstream primer: GGATCGA TTT GCI TAC/T GGA/G CAA/G ACN GG  
(for amino acid sequence *FAYGQT*).

downstream primer: CG TTC A/T G/CA C/G/TCC T/AGC NAG G/ATC  
(for amino acid sequence *DLAGSE*).

2. An alternative, simple butanol extraction of oligonucleotides can be used instead of purification with Sephadex G25. In this case, dissolve the crude oligonucleotide in 1 mL distilled water and extract two or three successive times with 400  $\mu$ L of 1-butanol. Discard the upper (organic) phase after each extraction; after the last extraction, centrifuge for 3 min at 15,000g and rinse pellet in 70% ethanol before redissolving in TE buffer (10 mM Tris-HCl [pH 7.4], 1 mM EDTA [pH 8.0]).
3. For the determination of the oligonucleotide concentration, mix 1–5  $\mu$ L of the oligonucleotide solution with 500  $\mu$ L of water in a quartz cuvet and measure the absorbance at 260 nm, using water as a blank sample. To calculate the concentration, first calculate the average molarity of the oligo in millimolar (mM) given by

the ratio  $OD_{260}/E_0$ .  $E_0$  is the millimolar extinction coefficient, determined as the sum of the contribution of the known extinction coefficient of each base in the oligo (i.e., T = 8.8, C = 7.3, G = 11.7, and A = 15.4 mL/ $\mu$ mol). The concentration of the oligo in nanograms per microliter is determined by multiplying the calculated average molarity by 330 (the average molecular mass of a nucleotide) and by the number of nucleotides present in the sequence. (For example, for the 18-mer oligo TCGACCTGGATCCAAGGA, the  $E_0 = [3 \times 8.8] + [5 \times 7.3] + [5 \times 11.7] + [5 \times 15.4] = 198.4$ . If the measured  $OD_{260}$  is 10.65, then  $OD_{260}/E_0 = 0.054$  mM and the oligo concentration is  $0.054 \times 330 \times 18 = 320.8$  ng/ $\mu$ L).

4. The maximum capacity of the Amersham Pharmacia micro mRNA purification kit is  $10^7$  cells or 100 mg of tissue per run. In our experience with hippocampal primary cultures, the starting material was typically  $2 \times 10^6$  cells. When working with primary cells, extracted aliquots can be pooled or extraction buffer can be transferred from dish to dish until the desired amount of cells are harvested, up to the maximum column capacity. If cell lines or tissue is used and more material is available, the macro version of this kit can alternatively be used for larger-scale mRNA purification.
5. A good approximation for determining the concentration of mRNA is given by the formula  $[RNA] = A_{260} \times 40$   $\mu$ g/mL. The minimum measured absorbance that is reliable to be accurate is 0.05, equivalent to 2  $\mu$ g/mL. The use of good quality, undegraded and pure RNA is critical for the production of representative cDNA. RNA should have an  $A_{260}/A_{280}$  ratio of  $>1.7$  and its integrity can be evaluated on a denaturing formaldehyde/agarose gel (12).
6. To concentrate RNA solutions, precipitate RNA with the addition of 1/40 vol of 10 mg/mL glycogen and 1/10 vol of 2.5 M potassium acetate (pH 5.0). Add 2.5 vol of 95% chilled ethanol and keep at  $-20^\circ\text{C}$  for at least 30 min. Collect the precipitated RNA by centrifugation at 13,000g at  $4^\circ\text{C}$  for 5 min. Resuspend in DEPC-treated water and store at  $-80^\circ\text{C}$ .
7. Both for cDNA generation and PCR (**Subheading 3.2.2.**) it is advisable to prepare appropriate reagent "master mixes," especially if a large number of samples are being handled. The appropriate volume is then transferred from the master mix to each tube. This minimizes pipetting errors of small volumes, reduces sample-to-sample variation, and speeds up the procedure. Always prepare a slightly larger volume of master mix to ensure that there will be enough solution, even for the last tube!
8. The importance of selecting the appropriate hybridization temperature in the PCR protocol, especially when using degenerate primers, cannot be overstressed. A balance has to be struck between avoiding too permissive conditions that will increase nonspecific amplification of unrelated sequences and using too stringent temperatures that will decrease yield or fail to amplify simultaneously the whole range of desired, related sequences. The hybridization temperature can be calculated with the following rule of thumb:  $T_H = [(2 \times \text{No. of AT pairs}) + (4 \times \text{No. of GC pairs})]^\circ\text{C} - (2-5^\circ\text{C})$ . If inosine is present multiply the number of pairs by 2. The value of  $T_H$  should be in the range of  $50-65^\circ\text{C}$  for probes of 20-25 nucle-

otides in length. Where degeneracy occurs, take into account the lowest- $T_H$  value for setting the hybridization temperature in the PCR protocol.

9. The principle of T/A cloning is based on the fact that most thermostable polymerases (but not those that exhibit 3' to 5' exonuclease activity like *Vent* and *Pfu*, which should not be used in this method) add single deoxyadenosines (A) to the 3' ends of PCR products. The linearized pCR2.1 vector supplied in the kit possesses 3' deoxythymidine (T) overhangs and this makes it possible for PCR products to ligate directly with the vector. It is critical to protect the integrity of cohesive ends in both the PCR product and vector for efficient ligation; thus, it is recommended to use fresh (less than 24 h old) products, which should be kept on ice and *not* frozen, and to avoid freeze–thaw cycles for linearized vector aliquots (use whole vector aliquot and do not refreeze or refreeze not more than twice).
10. For setting up the ligation reaction, the recommended vector:insert ratio is 1:1, but you may try ratios up to 1:3, provided you do not use more than 2–3  $\mu\text{L}$  of the PCR sample as its salts may inhibit T4 DNA ligase. In my experience, the best results are obtained with the use of 0.5–1  $\mu\text{L}$  of a typical PCR sample in which the product is estimated to be at least 50 ng/ $\mu\text{L}$  on an agarose gel.
11. The manufacturers of the T/A kit advise that about 5% of white colonies will not be recombinants but will be false positives resulting from either frame shifts of the *lacZ* gene or blunt-end self-ligation of the vector caused by degradation of 3'T overhangs. Often this percentage is higher and an advisable step is to always set up a routine control ligation reaction in which the addition of PCR product is omitted. By counting the number of white clones as a percentage of the total number of clones, this “background” can be evaluated, and if it is higher than 30–35%, it may be better to repeat the experiment with a new batch of vector.
12. The number of clones necessary to analyze depends on the complexity of the original PCR product, the ligation efficiency of your experiment (not all white clones will turn out to be recombinants; *see Note 11*), and the aim of the experiment. If the aim is to identify as many new motor cDNAs as possible or to compare the expression pattern between two developmental stages or different tissues, then one should aim to analyze about 100 clones. Start with the analysis of a batch of 20–25 clones to get an idea about ligation efficiency and insert variability and then continue with the analysis of further clones, accordingly.
13. Vector sequence and manuals can be viewed and downloaded from the Invitrogen Web Resource at <http://www.invitrogen.com>.
14. The principle of this protocol is to compare the expression of the set of kinesin-like motor proteins expressed in two distinct developmental (differentiation) stages or cell types, aiming to identify those that are uniquely expressed in each stage or that are subject to downregulation. Technically, this is detectable by the lack of hybridization of specific stage-specific clones to the mixed probe derived from the other stage being compared (this experiment can be conducted in both combinations [i.e., screening clones of one stage using the other stage cDNA pool as mixed probe and vice versa]). Those nonhybridizing clones will be good candidates for stage-specific expression (*see* example in **Fig. 1B,C**). Furthermore,

this technique can also reveal clones that are developmentally downregulated resulting in weak hybridization, although such results must be interpreted with more caution because they may be artifacts resulting from inadequate probe labeling or they may simply reflect the relative abundance of a specific cDNA in the mixed pool of cDNAs used as a probe. For these reasons, the results must always be confirmed by alternative methods such as RT-PCR of the two sources of RNA that are being compared, using primers specific for the candidate clones (see example in **Fig. 1D**) or *in situ* hybridization.

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