Published in final edited form as:

Methods Mol Biol. 2011 ; 789: 89–110. doi:10.1007/978-1-61779-310-3_5.

Intron-Specific Neuropeptide Probes

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Abstract

Measurements of changes in pre-mRNA levels by intron-specific probes are generally accepted as more closely reflecting changes in gene transcription rates than are measurements of mRNA levels by exonic probes. This is, in part, because the pre-mRNAs, which include the primary transcript and various splicing intermediates located in the nucleus (also referred to as heteronuclear RNAs, or hnRNAs), are processed rapidly (with half-lives <60 min) as compared to neuropeptide mRNAs, which are then transferred to the cytoplasm and which have much longer half-lives (often over days). In this chapter, we describe the use of exon-and intron-specific probes to evaluate oxytocin (OT) and vasopressin (VP) neuropeptide gene expression by analyses of their mRNAs and hnRNAs by quantitative in situ hybridization (qISH) and also by using specific PCR primers in quantitative, real-time PCR (qPCR) procedures.

Keywords

Pre-mRNA; Heteronuclear RNA; hn RNA; Real-time qPCR; Oxytocin; Vasopressin; Gene expression

1. Introduction

The idea that measurements of changes in pre-mRNA levels by intron-specific probes more closely reflect changes in gene transcription rates than measurement of mRNA levels by exonic probes is based on the observation that excision of the intron from the primary transcript and the subsequent degradation of the intron is very rapid, typically with half-times <30–60 min (1–4). By contrast, neuropeptide mRNAs can have turnover rates over days, and hence changes in their mRNA levels are as likely to reflect degradation rates as well as transcription rates. The first evaluation of neuropeptide gene expression by the use of intron sequence-specific probes and quantitative in situ hybridization (qISH) procedures was reported by Roberts and coworkers (5, 6) for the study of the proopiomelanocortin (POMC) gene primary transcript in individual neurons of the hypothalamus. Consequently, many assays of gene transcription changes using intron-specific probes and qISH have been used in studies of a wide variety of genes in the CNS (7–15).

The use of intron-specific probes to study neurohypophysial peptide gene expression in the hypothalamus by qISH was first accomplished for vasopressin (VP), and clearly showed that the intron 1-directed riboprobe that was used detected changes in the VP pre-RNA with much more rapid kinetics than exonic probes did for changes in mRNA (9). Experiments

have also been done using intron-specific probes and qISH to evaluate oxytocin (OT) gene expression in the hypothalamus with similar success (16–19). More recently, intronic and exonic primers have also been successfully utilized in quantitative, real-time PCR assays for the study of OT gene expression in the rat hypothalamus (20). In this chapter, we illustrate the use of exon- and intron-specific riboprobes (see Notes 2 and 3) or PCR primers to evaluate oxytocin and vasopressin neuropeptide gene expression by both qISH and quantitative, real-time PCR procedures, respectively (18–22).

2. Materials

2.1. Quantitative In Situ Hybridization: see Note 1

2.1.1. Preparation of Intronic and Exonic OT and VP Riboprobes for qISH—

1. DNA polymerase, platinum Taq polymerase (Invitrogen, Carlsbad, CA), which includes 10×PCR buffer containing Tris–Cl (pH 8.4), KCl, 50 mM MgCl₂.
2. 100 mM dNTP set (PCR grade, dATP, dGTP, dCTP, dGTP, 100 mM each, Invitrogen).
3. Primers (custom-ordered from Sigma Genosys, St. Louis, MO).
T3 CGCGCAATTAACCCTCACTAAAGG
T7 GCGCGTAATACGACTCACTATAGGG
SP6 CATACGATTTAGGTGACACTATAG
4. PCR tubes, MicroAmp Autoclaved Reaction Tube with cap (Perkin Elmer Life Sciences, Waltham, MA).
5. PCR thermocycler (e.g., Gene Amp PCR system 9700, Applied Biosystems, Foster City, CA).
6. Agarose for electrophoresis (available from Invitrogen).
7. Tris–Cl Borate EDTA (available from Quality Biological Inc., Gaithersburg, MD).
8. Ethidium bromide.

²DNA sequences in intron and exons of the rat genes are used to design the PCR primers for the intronic and exonic probes using DNASIS-Mac v3.5 software (Hitachi Software Engineering Co., Ltd. Tokyo, Japan). In all cases, BLAST searches of the nucleotide sequences in GenBank™ are done to reveal any nonspecific matches for these probes, and to confirm that the primers are specific for the gene. In addition, it is advisable to sequence the resultant PCR amplicons to confirm the annotation. The PCR products are used to prepare both sense and the antisense probes ranging in size preferably from 500 to 650 bp in length. Hybridization of the sense probes were performed as negative controls.

³The rat VP intronic riboprobe used here was obtained from Dr. Thomas Sherman, Georgetown University, Washington, DC and was a 735 bp fragment of intron 1 of rat VP gene subcloned into pGEM-3 vector (Promega, Madison, WI) (9). The rat VP exonic probe was a 229 bp fragment of the rat VP gene and the rat OT exonic probe was a 487 bp fragment obtained from Dr. W. Scott Young (NIMH). Both were subcloned into a pGEM-3 vector. Modified T7 and SP6 primers were used to PCR amplify all of three fragments to make templates that were used to synthesize the riboprobes. The T7 RNA polymerase was used to synthesize the antisense of hnVP and exonic VP probes and the SP6 the antisense of the OT exonic riboprobe.

¹The materials described here are directed at studies using RNA probes (riboprobes). These probes provide the greatest sensitivity for ISH. The typical low abundance of intron-containing RNAs in cells usually requires the most sensitive ISH methods for detection and quantification.

9. Ready-load 100 bp DNA ladder (available from Invitrogen).
10. QIA quick gel extraction kit – contains columns, binding buffer, and washing buffer (Qiagen, Hilden, Germany).
11. TE buffer pH 8.0 (Quality Biological Inc.).
12. Tris EDTA buffer pH 8.0 (Quality Biological Inc.).
13. PCR primers to generate the OT intronic riboprobes (see Note 4). For intron 1: 5'-ATGAAGCTTCCGTTCTGGCAAGGCTAAG-3' (sense), and 5'-ATCTGCAGCACAAATCCATATCGGGACTG-3' (antisense). For OT intron 2: 5'-ATGAAGCTTGTGAGCAGGAGGGGGCCTA-3' (sense), and 5'-ATGCTGCAGCTGCAAGAGAAATGGGTCACT-3' (antisense).
14. 1× PCR buffer: 20 mM Tris-HCl, pH 8.4, 50 mM KCl.
15. dNTPs.
16. Platinum Taq DNA polymerase (Invitrogen).
17. PCR thermocycler (e.g., GeneAmp PCR System 9700 – Applied Biosystems).
18. Agarose gel.
19. DNA electrophoresis apparatus.
20. Spectrophotometer (e.g., Ultrospec2100 pro, Amersham Pharmacia Biotech., Piscataway, NJ).
21. MinElute Gel Extraction Kit (Qiagen).
22. pBlueScript II SK (+) for subcloning PCR fragments (Stratagene, La Jolla, CA).
23. Modified T7 (GCGCGTAATACGACTCACTATAGGG) and T3 (CGCGCAATTAACCTCACTAAAGG) primers (see Note 5).

2.1.2. ³⁵S Labeling and Purification of Riboprobes—

1. MAXIscript T7/T3 or MAXIscript SP6/T7 labeling kit, [contains 10× buffer, dNTP (mixture of dATP, dCTP, dGTP), RNA polymerase (T7, T3, SP6), DNaseI, nuclease-free water] (Ambion, Austin, TX).

⁴The PCR primers used to generate the OT intronic riboprobe for intron 1 of OT are: 5'-ATGAAGCTTCCGTTCTGGCAAGGCTAAG-3' (sense), and 5'-ATCTGCAGCACAAATCCATATCGGGACTG-3' (antisense). Using these primers, one obtains a 162 bp fragment of OT intron 1 after PCR. The primers used to obtain an OT intronic probe for intron 2 of OT are: 5'-ATGAAGCTTGTGAGCAGGAGGGGGCCTA-3' (sense), and 5'-ATGCTGCAGCTGCAAGAGAAATGGGTCACT-3' (antisense). Using these primers, one obtains a 84-bp fragment of OT intron 2 after PCR. Because both OT introns are very short, the resulting OT riboprobes are below optimal length (around 500 bp) for ISH. This can be compensated for, in part, by using more than one radioactive nucleotide for the labeling of the riboprobes (see Subheading 3).

⁵These primers are used to PCR amplify the fragments and the resulting PCR products are used as templates for the synthesis of riboprobes. Here, T7 and T3 RNA polymerases are used to obtain both sense and antisense labeled probes, respectively. As for the VP probes, hybridization of sense probes is performed as negative controls.

2. [α - ^{35}S]-uridine 5'-triphosphate (^{35}S UTP) and [α - ^{35}S]-cytosine 5'-triphosphate (^{35}S CTP) (Perkin Elmer Life Sciences).
3. Dithiothreitol (DTT).
4. RNeasy mini kit (for RNA purification: contains columns, lysis buffer, washing buffer, Qiagen).
5. ETOH 100% (Quality Biological Inc.).
6. TE buffer pH 8.0 (Quality Biological Inc.).
7. Tris EDTA buffer pH 8.0 (Quality Biological Inc.).
8. LS6500 Multipurpose Scintillation counter (Beckman-Coulter Co, Fullerton, CA).

2.1.3. Tissue Preparation and Hybridization—

1. Cryostat.
2. Poly-L-lysine coated slides (Fisher Scientific Company, Newark, DE).
3. Slide warmer.
4. 4% formaldehyde in 1×PBS (to fix frozen sections).
5. 1×PBS: 0.017 M KH_2PO_4 , 0.05M Na_2HPO_4 , 1.5 M NaCl, pH 7.4.
6. Solution for hybridization sheet, 4×SSC 50% formamide.
7. For TEA buffers: acetic anhydride (Sigma Chemicals), TEA [triethanolamine (98%), Sigma Chemicals].
8. For nucleic acid mix: salmon sperm DNA (available from Sigma Chemicals or Invitrogen), Yeast total RNA type XI (ribonucleic acid – Sigma Chemicals), Yeast tRNA (Invitrogen).
9. Hybridization buffers: Tris–Cl, pH 7.4, EDTA pH 8.0, NaCl, Formamide, Dextran sulfate, Denhardt's solution, DTT, sodium dodecyl sulfate (SDS), sodium thiosulfate (NTS – Sigma Chemicals), nuclease-free water.
10. SSC buffer: 0.3 M sodium citrate, 3 M NaCl, pH 7.0.
11. TNE buffer [10 mM Tris–Cl pH 8.0, 0.5 M NaCl, 0.25 mM EDTA pH 8.0] containing 20 $\mu\text{g}/\text{mL}$ ribonuclease A (Sigma Chemicals).
12. Ethanol wash solutions (EtOH: 70, 80, 90, 95, and 100%).
13. Dry incubator.
14. Coverslips.
15. X-ray Cassettes.
16. Microscope Slide Holder, Peel-A-Way (Polysciences Inc., Warrington, PA).

17. NTB-3 autoradiography emulsion (Kodak, Rochester, NY).
18. Ilford Nuclear research Emulsions K5D (Polysciences Inc.).
19. Rack and dish for dark storage (Research Products International Corp., Mt. Prospect, IL).
20. Dektol (developer for normal dipping – Kodak).
21. D-19 (developer for digoxigenin double label – Kodak).
22. Fixer (VWR International, West Chester, PE).

2.1.4. Quantitation of Hybridization—

1. Storm 860 Phosphoimager (Amersham Bioscience, Amersham, UK).
2. Image Quant software version 5.2 (Amersham Biosciences).
3. Phosphorscreen, low energy (LE), unmounted (Amersham Biosciences).

2.2. Quantitative Real-Time PCR

1. Primers for qPCR (see Note 6).
2. Single Step quantitative real-time PCR (qRT-PCR) kit (Qiagen). The kit contains reverse transcriptase along with a master mix solution containing a hot-start Taq polymerase.
3. Absolutely RNA miniprep kit (Stratagene). The kit is used for RNA extraction and includes lysis buffer, beta mercaptoethanol, prefilter spin cups, RNA binding low and high salt wash buffers, 70% ethanol, and DNase I solutions.
4. Qiagen RNase-Free DNase in RNA Easy kit (Qiagen – see Note 7).
5. Spectrophotometer (e.g., Nano Drop, Wilmington, DE).
6. Heraeus Biofuge Fresco centrifuge (DJB Labcare Ltd, Buckinghamshire, England).
7. Vortex.
8. Air incubator.

⁶All gene-specific PCR primers discussed in this chapter and illustrated in Fig. 5, and with sequences shown in Table 1, were designed using GeneFisher software (26).

Nonetheless, alternative software is available for this purpose. A good, free program called Autoprime that specializes in qPCR primers for mRNA is now available. It has the additional benefit of having access to the genomes of over 50 common model organisms. The Autoprime Web site is: <http://www.autoprime.de/AutoPrimeWeb>. Another valuable software program for designing PCR primers is called Primer3, which can be found at <http://frodo.wi.mit.edu/primer3/>. The latter is a more general primer designing tool that is useful for all kinds of PCR, including qPCR.

The primers in Table 1 are shown for two intronic assays, (i.e., for measurements of intron 1 in VP hnRNA, of intron 2 in OT hnRNA) and for three exonic measurements (i.e., for c-fos, VP, OT, and GAPDH mRNAs, see Fig. 5 for primer locations). A typical PCR reaction mixture contains 0.6 μ M of primers and 10–20 ng of template RNA. Reaction volumes are 25 μ L for the Smart Cyclor system or 15 μ L for the Step One Plus. A very useful validation of the PCR primers is to electrophorese the resulting PCR reaction products on an agarose gel to confirm that the lengths of the amplicons are as expected (see Fig. 6).

⁷DNase treatment is necessary when qRT-PCR is done on heteronuclear RNA. We find that the DNase in the RNA Easy kit from Qiagen is superior to that supplied in the Stratagene Absolutely RNA miniprep kit.

9. Real-time PCR apparatus [e.g., Smart Cycler (Cepheid, Sunnyvale, CA), or Step One Plus PCR apparatus, 96 wells (Applied Biosystems)].

3. Methods

3.1. Quantitative In Situ Hybridization

In situ hybridization histochemistry (ISH) is an important method to detect and measure quantities of specific RNAs in tissue sections. Its principal value when compared to RT-PCR, which is a more sensitive assay (see Subheading 3.2), is the higher spatial resolution (even to the cellular level) of ISH. There are many ways to detect RNAs by ISH, e.g., by nonradioactive digoxigenin or biotin-labeled probe hybridization followed by immunodetection using either alkaline phosphatase or fluorescence-coupled antibodies or by qualitative/quantitative assays with ^{35}S labeled oligonucleotide or RNA probes (riboprobes). We focus here only on qISH assays using ^{35}S labeled riboprobes directed at exonic and intronic sequences. An example of such an ISH assay using ^{35}S -labeled probes of VP hnRNA and VP mRNA hybridized to sections at two different anatomic levels of hypothalamus is shown in Fig. 1. It is notable that the VP hnRNA levels appear to be similar between the supraoptic (SON) and suprachiasmatic nucleus (SCN), whereas the mRNA levels in the SON appear to be substantially greater than in the SCN. Quantitative in situ hybridization (qISH) (23) and quantitative PCR (20) confirm these impressions and show that the SON/SCN VPmRNA ratio was equal to about 15, whereas the SON/SCN VPhnRNA ratio of 2.8 is much lower (and this difference is not statistically significant). Thus, quantitative conclusions drawn from qISH and qPCR methods appear to be comparable.

3.1.1. Preparation of Intronic and Exonic OT and VP Riboprobes for qISH—

1. The rat VP intron 1 (735 bp), VP exonic (229 bp), and OT exonic (487 bp) DNA fragments are subcloned into pGEM-3 vectors (see Note 3). The OT intronic fragments were obtained and subcloned into pBlueScript II SK (+) as described from steps 2 to 5 below.
2. The PCR primers used to generate the OT intronic fragments are described in Note 4.
3. To obtain the the OT intronic fragments, the PCR is carried out in a 50- μL reaction volume, containing 0.8 μg rat genomic DNA, 1 $\times$ PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 200 μM each of dNTPs, 200 nM each of the primers, and 2.5 U Platinum Taq DNA polymerase (Invitrogen).
4. PCR is performed on a GeneAmp PCR System 9700 Thermal Cycler (Applied Biosystems) and consists of an initial 2 min, 94°C denaturation, followed by 30 cycles of denaturing (94°C, 30 s), annealing (60°C, 30 s), and extension (72°C, 45 s), followed by a final extension of 7 min at 72°C.
5. The PCR products are loaded on the 1.5% agarose gel and purified by the MinElute Gel Extraction Kit (Qiagen). Then, the purified PCR fragments were subcloned into pBlueScript II SK (+) (Stratagene, La Jolla, CA).

6. Modified T7 and SP6 primers are used to PCR-amplify all of the three fragments, VP intron1, VP and OT exonic fragments that are subcloned into pGEM-3 vectors and the OT intronic fragments that are subcloned into pBlueScript II SK (+). The PCR is carried out in a 50- μ L reaction volume, containing 20–50 ng plasmid, 1 $\times$ PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 200 μ M each of dNTPs, 0.2 pM each of the primers, and 2.5 U Platinum Taq DNA polymerase (Invitrogen). PCR is performed on the same conditions as described in step 4.
7. All the PCR products are loaded on the 1.5% agarose gel and purified by the MinElute Gel Extraction Kit, and the resulting PCR products are used as templates to synthesize the riboprobes.
8. The T7 RNA polymerase was used to synthesize the antisense of VP intronic and exonic probes, the SP6 polymerase is for the antisense of the OT exonic riboprobe and T3 polymerase is for the antisense of OT intronic riboprobes. SP6 RNA polymerase was used to synthesize the sense of VP intronic and exonic probes, T7 polymerase is for the sense OT exonic riboprobe and the sense OT intronic riboprobes.

3.1.2. ³⁵S Labeling and Purification of Riboprobes—

1. Label intronic and exonic riboprobes with ³⁵S by using 30–40 ng of PCR product, 50 μ Ci of ³⁵S UTP, and 10 mM DTT along with the reagents in the MAXIscript in vitro transcription kit (see Notes 8 and 9).
2. Adjust ³⁵S RNA sample to a volume of 100 μ L with 1 $\times$ TE pH 8.0. Add 350 μ L Buffer RLT and mix thoroughly.
3. Add 250 μ L ethanol (96–100%) to the diluted RNA and mix thoroughly by pipetting. Do not centrifuge. Continue immediately with step 4.
4. Apply the sample (700 μ L) to an RNeasy mini column placed in a 2-mL collection tube (supplied). Close the tube gently and centrifuge for 15 s at 8,000 $\times g$.
5. Take the flow-through with a pipette and Repeat step 4.
6. Discard the flow-through and collection tube.
7. Transfer the RNeasy column to a new 2-mL collection tube (supplied). Pipette 500 μ L Buffer RPE onto the RNeasy column. Close the tube gently and centrifuge for 15 s at 8,000 $\times g$ to wash the column. Discard the flow-through. Reuse the collection tube in step 7 (see Note 10).

⁸More specifically, labeling of the VP intronic, VP exonic and OT exonic riboprobes of optimal length used 40 ng, 60 ng, and 100 ng of the templates, respectively, and 50 μ Ci of ³⁵S UTP, 10 mM DTT, and a MAXI script in vitro transcription adequate kit.

⁹The shorter length riboprobe sequences such as for the OT intron 2 probe which contains only 13 uridines requires modification of the above labeling procedure for detection of heteronuclear RNA by ISH. Noting that the probe contained 31 cytidine nucleotides, versus the 13 uridine nucleotides, we found that use of both ³⁵S-UTP and ³⁵S-CTP together to label the OT intron 2 probe results in a very robust signal. Consequently, effective OT intron 2 riboprobe labeling was performed as described above but using 40 ng of the PCR product, 50 μ Ci of ³⁵S UTP, and 50 μ Ci of ³⁵S-CTP.

¹⁰Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use.

8. Add another 500 μ L Buffer RPE to the RNeasy column. Close the tube gently and centrifuge for 2 min at $8,000 \times g$ to dry the RNeasy silica-gel membrane. Continue directly with step 6, or, to eliminate any chance of possible Buffer RPE carryover (see Note 11).
9. To elute, transfer the RNeasy column to a new 1.5-mL collection tube (supplied). Pipette 50 μ L 1 $\times$ TE (pH 8.0) directly onto the RNeasy silica-gel membrane. Close the tube gently, incubate for 1 min, and centrifuge for 1 min at $8,000 \times g$ to elute.
10. Discard the column and measure the radioactivity using a scintillation counter. Take out 1 μ L of labeled probe and mix into 10 mL of scintillant in a scintillation vial. If the count is less than 300,000 cpm, it is suggesting that it is an inefficient labelling (see Note 12).

3.1.3. Tissue Preparation and Hybridization—

1. Cut serial 10- μ m brain sections in a cryostat and place them onto poly-L-lysine coated slides.
2. Dry sections on a slide warmer for 10–30 min at 37°C and store at –80°C until further use.
3. Before hybridization, fix the sections in 4% formaldehyde for 10 min at room temperature, rinse once in PBS, and wash twice for 5 min each in 1 $\times$ PBS.
4. Put into 0.1 M triethanolamine-HCl (pH 8.0) containing 0.25% acetic anhydride for 10 min at room temperature.
5. Rinse with 2 $\times$ SSC buffer, transfer through graded ethanol (EtOH:70% – 1 min, 80% – 1 min, 95% – 1 min, 100% – 1 min, 95% – 1 min.), and finally air-dry.
6. Mix probes and nucleotide mix, heat to 65°C, and cool on ice for 5 min. Then, add it to hybridization solution (20 mM Tris-Cl pH 7.4, 1 mM EDTA pH 8.0, 300 mM NaCl 50% formamide, 10% dextran sulfate, 1 $\times$ Denhardt's solution, 100 μ g/mL salmon sperm DNA, 250 μ g/mL yeast total RNA, 250 μ g/mL yeast tRNA, 0.0625% SDS, and 0.0625% sodium thiosulfate).
7. Carry out hybridization in 80 μ L hybridization solution containing approximately 10^6 cpm denatured S³⁵-labeled riboprobe for each section.

¹¹Optional: Place the RNeasy column in a new 2 mL collection tube (not supplied), and discard the old collection tube with the flow-through. Centrifuge in a microcentrifuge at full speed for 1 min. It is important to dry the RNeasy silica-gel membrane, since residual ethanol may interfere with downstream reactions. This centrifugation ensures that no ethanol is carried over during elution. Following the centrifugation, remove the RNeasy mini column from the collection tube carefully so that the column does not contact the flow-through as this will result in carryover of ethanol.

¹²The protocol for ethanol precipitation ends at 100 μ L, so the minimum count of radioactivity using the ethanol precipitation method will be 150,000 cpm.

8. Coverslip the slide and use a 4× SSC/50% formamide solution to saturate 3MM paper to humidify the hybridization chamber.
9. After an approximately 24 h (overnight) incubation at 55°C, remove the coverslips in 4× SSC/1 mM DTT at room temperature and wash with the same solution for 5 min.
10. Incubate the sections in TNE buffer [10 mM Tris–Cl pH 8.0, 0.5 M NaCl, and 0.25 mM EDTA pH 8.0] containing 20 µg/mL ribonuclease A for 30 min at 37°C.
11. Wash twice (5 min/wash) in 2× SSC/1 mM DTT, once in 1× SSC/1 mM DTT and then in 0.5× SSC/1 mM DTT (5 min each) at 65°C.
12. Let the sections cool to room temperature in 0.1× SSC/1 mM DTT.
13. Rinse the sections in graded ethanol solutions (1 min each in 70, 80, 90, 95, and 100% ethanol) and then air-dry.

3.1.4. Quantitation of Hybridization—

1. Before use, erase the screen to be employed with the Storm 860 Phosphor Imager System. Put the phosphor screen facing the surface on the eraser and switch on the eraser. Leave till the lights go off automatically.
2. Align the slides on the cassette. Use a liquid tape or tape to fix the slides so that the slides will not move during the exposure.
3. Lift the screen from the eraser and place it with the surface on the slides. Do not move the screen once you place it on the slides because the screen is sensitive enough to pick up signals at the time you start exposing. Also, do not place any material (wraps etc.) between the slides and the screen, since it will interfere with the exposure.
4. Place a spacer over the screen and shut the cassette at once. Be careful not to move the screen while closing the cassette.
5. Label the cassette with the exposure starting date and time. Store the cassette at room temperature. It is not needed to store in a dark place or –80°C.
6. Expose the screen for the required time. Usually, the phosphor screens are ten times more sensitive than regular autoradiography films, so you can estimate the days as 10% of the required time in autoradiography films. To get a better quality in the image (e.g., for making figures), longer exposure is better (see Note 13).
7. For scanning, open the “Storm Scanning Control” software (see Note 14).

¹³Half of the time required to expose an autoradiography film in the standard procedure is a good rule of thumb for selecting an exposure time using a phosphorimager. See Subheading 2.1.4 for materials.

¹⁴When scanning the large screens, it is mostly necessary to restart the computer; when scanning small (20 × 40) screens, it is mostly unnecessary.

8. Open the lid of the scanner and clean the glass surface with a Kimberly-Clark tissue wipe (or any kind of paper that can be used on microscopic glasses). Use distilled water if necessary. Do not use any detergent.
9. Mark the area that you are going to scan on the left panel of the “Storm Scanning Control” window. Then, go to the right panel and make sure that the acquisition mode is storage phosphor. Choose the pixel size of 50 μm .
10. Choose the image analysis to Image Quant if you want that the Image Quant software is opened automatically after the scanning is finished.
11. Click the scan button. This will prompt you to save your file before scanning. Name your file and save and then the scanning will start. It usually takes 40 min for a large screen and 20 min for a small screen to complete the scanning (see Note 15).
12. Start the “Image Quant 5.2” software and open the scanned files that you are going to analyze. The software enables you to evaluate the levels of mRNA or hnRNA in the brain nucleus/nuclei of interest (e.g., the SON) by measuring the average densities and unit areas per SON of phosphor screen in the sections from each experimental animal.
13. To optimize the display, first click the “Gray/color adjust” button on the top tool bar and check if the image is not saturated. The levels showing in the high should be less than 100,000, if it is exceeding 100,000 the image is saturating, and you should scan the image with a shorter exposure time. Then adjust the image contrast by dragging the slider above the histogram. Adjust the image till you see the first saturated signals (shown in red on the screen). If necessary, you can enlarge the image by using the “creating frame” option in the top tool panel, before adjusting the contrast. The quantitation results do not change by changing the contrast.
14. To select the region to quantitate, scroll to the section you would like to quantitate, enlarge it by using the “creating frame” option. Use the “region” tool in the left tool bar to select the area of interest that you need to quantitate. Each selected area will be numbered in the order you selected. For the background normalization, use the “ellipse” tool in the left tool bar to select an area that shows background hybridization.
15. After selecting the areas, go to analysis/background correction. Under the objects, you will see the numbered regions and ellipses that you selected. Select the regions that you would like to correct and select “object average” under the methods. After that, you will see the ellipses that you selected under the object average. Select the ellipse that you would like to use as the background and click the “set” button. Now you should see the regions marked as “OA” which means that it is corrected using certain

¹⁵If the scanning stops in the middle of the scanning for any reason (e.g., crashes), a border line will be created in your image and this will remain even though one starts a new scan. It is better to reexpose image if this happens.

background areas. After you finish the correction, close the inspector window.

16. Go to analysis/volume review. Your quantitation results will show up. Double-click the results and this will lead you to the results in an excel format. Save the data. The values shown in the volume columns are your corrected data (average intensity $\times$ area).
17. Determine statistical significance of differences between groups by one-way ANOVA followed by Fisher's protected least significant difference (PLSD) test or Student's test. Differences between groups are considered statistically significant when *P* values were less than 0.05. Experiments are routinely repeated on four animals and the results expressed as means $\pm$ SEMs.

3.2. Quantitative Real-Time PCR

The use of real-time reverse transcription PCR (aka real-time RT-PCR, or qRT-PCR) is a valuable alternative to qISH to do quantitative studies of pre-mRNAs. Because of its greater sensitivity, qRT-PCR can often detect intron-containing pre-mRNAs when qISH fails to do so. In a study of OT gene expression, we found that the short introns in the OT gene did not permit the generation of effective riboprobes to detect the intron 1 bearing hnRNAs by qISH (18). However, these pre-mRNAs could be readily studied by in qRT-PCR using primers directed against either intron1 or intron 2 (20). Real-time PCR can be done using either a one-step or two-step reaction. In the one-step method, the entire PCR procedure is performed in a single tube, whereas in the two-step procedure the reverse transcription and PCR amplification reactions occur in separate tubes. While a comprehensive comparison between these two PCR methods is beyond the scope of this article (but see (24, 25) for more discussion), we can state that we have studied both OT and VP hnRNAs and mRNAs by both of these approaches and have found both to be equally effective (19–22). However, because the single-step PCR method is obviously simpler to perform, we focus on this method here.

3.2.1. RNA Extraction—

1. Dissect a 6–10 mg tissue sample (approx. 10^6 cells) and place the sample into 250 μ L of Lysis Buffer containing an additional 1.8 μ L of beta mercaptoethanol (see Note 16).
2. Homogenize the sample by repeatedly pipetting (more than 20 times) and vortexing for about 15 s. Continue pipetting, if necessary, to completely homogenize the solution. A slightly opaque solution void of flocculent debris should result.
3. Transfer the homogenate to a Prefilter Spin Cup (blue), which is placed in a 2-mL receptacle tube, and spin the tube in a centrifuge at maximum speed ($17,900 \times g$ for 10 min).

¹⁶If storing for extended period of time, keep solution in the -80°C freezer.

4. Discard the blue spin cup but retain the filtrate. Add an equal volume (approx. 200 μ L, as some of the original solution is retained by the Prefilter Spin Cup) of 70% ethanol to the filtrate and vortex for 5 s.
5. Transfer the mixture to an RNA Binding Spin Cup (clear), which should be placed in a new 2-mL receptacle tube.
6. Centrifuge the spin cup for 1 min at $17,900 \times g$.
7. Retain the spin cup, aspirate/discard the filtrate, and replace the spin cup in the receptacle tube.
8. Add 550 μ L of 1 $\times$ Low-Salt Wash Buffer and centrifuge for 1 min at $17,900 \times g$.
9. Retain the spin cup, aspirate/discard the filtrate, and replace the spin cup in the receptacle tube.
10. Centrifuge for another 2 min at $17,900 \times g$ to completely dry the binding cup.
11. Prepare the DNase solution (see Appendix D in Qiagen RNeasy mini handbook, p. 70) by mixing 70 μ L of RDD (buffer) with 10 μ L of reconstituted RNase-Free DNase. Gently mix by pipetting three to four times.
12. Add DNase solution directly onto the matrix of the spin cup.
13. Incubate the solution at 37°C for 30 min, in an air incubator.
14. Add 550 μ L of 1 $\times$ High-Salt wash buffer to the spin cup and then centrifuge for 1 min at $17,900 \times g$.
15. Retain the spin cup, aspirate/discard the filtrate, and replace the spin cup in the receptacle tube.
16. Add 550 μ L of 1 $\times$ Low-Salt wash buffer to the spin cup and then centrifuge for 1 min at $17,900 \times g$.
17. Retain the spin cup, aspirate/discard the filtrate, and replace the spin cup in the receptacle tube.
18. Add 300 μ L of 1 $\times$ Low-Salt wash buffer to the spin cup and then centrifuge for 2 min at $17,900 \times g$.
19. Transfer the spin cup to a 1.5 mL microcentrifuge tube and discard the 2 mL receptacle tube with the filtrate.
20. Add 40 μ L of pure room-temperature water directly onto the center of the matrix inside the spin cup and wait for 2 min.
21. Centrifuge the tube for 1 min at $17,900 \times g$.
22. Immediately place the tube (with the eluted RNA) on ice.
23. Determine RNA concentration and purity using a spectrophotometer.

24. Acceptable purity: 260/280 ratio should be between 1.8 and 2.1.

3.2.2. qRT-PCR (see Note 17)—

1. To prepare the qRT-PCR reaction tubes, prepare 25 μ L master mix/tube containing the following: 0.6 μ M of total primers (1.5 μ L of a 100 μ M F + R primer mixture), 12.5 μ L of 2 $\times$ -Taq polymerase-SYBR Green buffer, 0.25 μ L Reverse Transcriptase, 10–20 ng of template RNA/reaction (see Note 18), and pure water to a final volume of 25 μ L (approximately an additional 9.75–10 μ L) (see Note 19).
2. Aliquot from the master mix to a reaction tube that has been placed in the cold (4°C) tube-holder rack that comes with the SmartCycler.
3. Annealing temperatures were 60°C for all primers shown in Table 1 for the qRT-PCR reactions.
4. Measure and calculate cycle threshold (Ct) values by the real-time PCR apparatus' software (see Note 20).
5. Run each individual qRT-PCR reaction in duplicate or triplicate, and run together samples from all animals in a given experiment (e.g., experimental and control samples).
6. At the end of every run, produce a melting curve (50–95°C with 0.1°C/s) to confirm the homogeneity of the PCR product.
7. Calculate an average Ct value for each PCR primer pair and each experimental condition.
8. If hnRNA are to be assayed, perform an additional reaction using the VP hnRNA primer pair in the absence of reverse transcriptases (–RT) to control for DNA contamination in the RNA template.
9. Include only templates with Ct differences >5 for the +RT versus –RT experiments, representing little if any DNA contamination, are included in the analysis (see Note 21).

3.2.3. Data Analyses and Calculations of Fold Changes—

1. Calculate average Ct values $\pm$ SEMs for each experiment. Perform control reactions with the PCR primers but without RT reactions for each PCR run to monitor the assays for DNA contamination and primer artifacts. Typical

¹⁷This protocol is based on Qiagen kit QuantiTect SYBR Green RT-PCR Handbook and a 25- μ L reaction volume in the Smart Cycler System.

¹⁸Volume of template solution is usually 2 μ L. The exact volume should be calculated based on concentration from spectrophotometer reading.

¹⁹This volume can be scaled up for performing duplicate or triplicate reactions.

²⁰Ct values indicate a PCR cycle number at which the measured fluorescence of the indicator dye (SYBR green) corresponding to the quantity of amplified products, is increasing in an exponential linear fashion above background.

²¹The –RT control is critical for PCR measurements using intronic primers, since in many cases the hnRNAs are of very low abundance.

fluorescent “growth” curves for high-abundance VP mRNA and low-abundance c-fos mRNA, and VP hnRNA, are illustrated in Fig. 2.

2. Calculate relative quantification of changes in the specific RNA's levels by the comparative Ct method using the formula, 2^{-Ct} (27–29) (see Note 22 for discussion about the appropriate method for the statistical analysis of Ct values).

The comparative Ct method assumes that the efficiency of the target gene of the PCR is close to one, and that the efficiencies of the PCRs of the gene of interest and the control gene are similar. In one study of the efficiencies of the PCRs of VP mRNA and VP hnRNA, this proved to be the case (21).

Hence, we use the simplified formula for the fold change in hnRNA or mRNA in any sample as the fold change or ratio = $2.0^{[(\text{the Ct of gene of interest minus the Ct of the control gene in the experimental RNA sample}) - (\text{the Ct of gene of interest minus the Ct of the control gene in the control RNA sample})]}$, and this is expressed as percent change from control.

Analyses of data obtained from in vivo in studies of the effects of acute and chronic salt loading on the changes in expression of OT and VP hnRNAs and OT and VP mRNAs in rat SONs are illustrated in Figs. 3 and 4, respectively. Note that in the acute salt loading (Fig. 3a) the Cts of the VPmRNAs did not change, and so the VP mRNA was used as the control gene to calculate the percent change in the c-fos mRNA and VP hnRNA (Fig. 3b). By contrast, in the chronic salt loading study in which the VPmRNA which, as expected, did undergo significant change (Fig. 4a), we used the GAPDH mRNA, which did not change, as the control gene to calculate the percent changes in the other genes' expression after salt loading (Fig. 4b).

Acknowledgments

We wish to thank various past and present members in our LNC, NINDS, NIH laboratory, Noriko Mutsuga, Ray Fields, Shirley House, Daniel Lubelski, and Madison Stevens, and colleagues in other NIH institutes, Eva Mezey, Sharon Key and Zuzanna Toth in the NICDR, and W. Scott Young in NIMH for their help in establishing the qISH and qPCR methods in our laboratory. This work was supported by the intramural program of the NIH, NINDS.

References

1. Audibert A, Weil D, Dautry F. In vivo kinetics of mRNA splicing and transport in mammalian cells. *Mol. Cell. Biol.* 2002; 22:6706–6718. [PubMed: 12215528]
2. Clement JQ, Qian L, Kaplinsky N, Wilkinson MF. The stability and fate of a spliced intron from vertebrate cells. *RNA.* 1999; 5:206–220. [PubMed: 10024173]
3. Hargrove JL. Microcomputer-assisted kinetic modeling of mammalian gene expression. *FASEB J.* 1993; 7:1163–1170. [PubMed: 8375615]

²²An important issue in qPCR is how to evaluate variation in the data and how to determine whether there are statistically significant differences between the control and experimental data. The exponential nature of PCR argues against using the raw Ct values in standard statistical tests to determine statistical significance between the control and experimental samples. According to several experts in the qPCR field variation in the data should not be calculated using raw Ct values since these are logarithmic units, and the data used for calculation of coefficients of variations must be linear values (25, 30). Therefore, it is recommended that first a log transformation of normalized relative values should be done, followed by mean centering and autoscaling to remove outliers before statistical analysis (for details about these methods, see (30)).

4. Perry RP, Bard E, Hames BD, Kelly DE, Schibler U. The relationship between hnRNA and mRNA. *Prog. Nucleic Acid Res. Mol. Biol.* 1976; 19:275–292. [PubMed: 1019347]
5. Fremeau RT Jr, Lundblad JR, Pritchett DB, Wilcox JN, Roberts JL. Regulation of pro-opiomelanocortin gene transcription in individual cell nuclei. *Science.* 1986; 234:1265–1269. [PubMed: 3775385]
6. Fremeau RT Jr, Autelitano DJ, Blum M, Wilcox J, Roberts JL. Intervening sequence-specific in situ hybridization: detection of the pro-opiomelanocortin gene primary transcript in individual neurons. *Brain Res. Mol. Brain Res.* 1989; 6:197–201. [PubMed: 2615595]
7. Chang MS, Hahn MK, Sved AF, Zigmond MJ, Austin MC, Sherman TG. Analysis of tyrosine hydroxylase gene transcription using an intron specific probe. *J. Neurosci. Methods.* 2000; 94:177–185. [PubMed: 10661837]
8. Fox CA, Mansour A, Thompson RC, Bunzow JR, Civelli O, Watson SJ Jr. The distribution of dopamine D2 receptor heteronuclear RNA (hnRNA) in the rat brain. *J. Chem. Neuroanat.* 1993; 6:363–373. [PubMed: 8142073]
9. Herman JP, Schafer MK, Watson SJ, Sherman TG. In situ hybridization analysis of arginine vasopressin gene transcription using intron-specific probes. *Mol. Endocrinol.* 1991; 5:1447–1456. [PubMed: 1775130]
10. Herman JP, Schafer MK, Thompson RC, Watson SJ. Rapid regulation of corticotropin-releasing hormone gene transcription in vivo. *Mol. Endocrinol.* 1992; 6:1061–1069. [PubMed: 1324419]
11. Herman JP, Spencer R. Regulation of hippocampal glucocorticoid receptor gene transcription and protein expression in vivo. *J. Neurosci.* 1998; 18:7462–7473. [PubMed: 9736665]
12. Kovacs KJ, Sawchenko PE. Regulation of stress-induced transcriptional changes in the hypothalamic neurosecretory neurons. *J. Mol. Neurosci.* 1996; 7:125–133. [PubMed: 8873896]
13. Ma XM, Levy A, Lightman SL. Rapid changes in heteronuclear RNA for corticotrophin- releasing hormone and arginine vasopressin in response to acute stress. *J. Endocrinol.* 1997; 152:81–89. [PubMed: 9014842]
14. Paskitti ME, McCreary BJ, Herman JP. Stress regulation of adrenocorticosteroid receptor gene transcription and mRNA expression in rat hippocampus: time-course analysis. *Brain Res. Mol. Brain Res.* 2000; 80:142–152. [PubMed: 11038247]
15. Rusnak M, Gainer H. Differential effects of forskolin on tyrosine hydroxylase gene transcription in identified brainstem cate-cholaminergic neuronal subtypes in organotypic culture. *Eur. J. Neurosci.* 2005; 21:889–898. [PubMed: 15787695]
16. Brooks PJ, Kaplitt MG, Kleopoulos SP, Funabashi T, Mobbs CV, Pfaff DW. Detection of messenger RNA and low-abundance heteronuclear RNA with single-stranded DNA probes produced by amplified primer extension labeling. *J. Histochem. Cytochem.* 1993; 41:1761–1766. [PubMed: 8245424]
17. Douglas AJ, Meeren HK, Johnstone LE, Pfaff DW, Russell JA, Brooks PJ. Stimulation of expression of the oxytocin gene in rat supraoptic neurons at parturition. *Brain Res.* 1998; 782:167–174. [PubMed: 9519260]
18. Yue C, Mutsuga N, Scordalakes EM, Gainer H. Studies of oxytocin and vasopressin gene expression in the rat hypothalamus using exon- and intron-specific probes. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2006; 290:R1233–R1241. [PubMed: 16357095]
19. Yue C, Mutsuga N, Sugimura Y, Verbalis J, Gainer H. Differential kinetics of oxytocin and vasopressin heteronuclear RNA expression in the rat supraoptic nucleus in response to chronic salt loading in vivo. *J. Neuroendocrinol.* 2008; 20:227–232. [PubMed: 18088359]
20. Yue C, Ponzio TA, Fields RL, Gainer H. Oxytocin and vasopressin gene expression and RNA splicing patterns in the rat supraoptic nucleus. *Physiol. Genomics.* 2008; 35:231–242. [PubMed: 18765859]
21. Ponzio TA, Yue C, Gainer H. An intron-based real-time PCR method for measuring vasopressin gene transcription. *J. Neurosci. Methods.* 2007; 164:149–154. [PubMed: 17540451]
22. Kawasaki M, Ponzio T, Yue C, Fields RL, Gainer H. Neurotransmitter regulation of c-fos and vasopressin gene expression in the rat supraoptic nucleus. *Experimental Neurology.* 2009; 219:212–222. [PubMed: 19463813]

23. Young WS 3rd, Mezey E, Siegel RE. Vasopressin and oxytocin mRNAs in adrenalectomized and Brattleboro rats: analysis by quantitative in situ hybridization histochemistry. *Brain Res.* 1986; 387:231–241. [PubMed: 3828759]
24. Wacker MJ, Goddard MP. Analysis of one-step and two-step Real time PCR using superscript III. *J. Biomolecular Techniques.* 2005; 16:266–271.
25. Wong ML, Medrano JF. Real time PCR for mRNA quantitation. *Biotechniques.* 2005; 39:75–85. [PubMed: 16060372]
26. Giegerich R, Meyer F, Schleiermacher C. GeneFisher – software support for the detection of postulated genes. *Proc. Int. Conf. Intell. Syst. Mol. Biol.* 1996; 4:68–77. [PubMed: 8877506]
27. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(–Delta Delta C(T)) Method. *Methods.* 2001; 25:402–408. [PubMed: 11846609]
28. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 2001; 29:e45. [PubMed: 11328886]
29. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat. Protoc.* 2008; 3:1101–1108. [PubMed: 18546601]
30. Willems E, Leyns L, Vandesompele J. Standardization of real-time PCR expression data from independent biological replicates. *Anal. Biochem.* 2008; 379:127–129. [PubMed: 18485881]

Fig. 1.

Photomicrograph of ISHH of VP mRNAs and hnRNAs in rat brain sections at two different anatomic levels of hypothalamus. *Two top panels* illustrate VP mRNAs by hybridization with VP exon-specific probe. The VP mRNA levels in the SON appear to be similar as in the PVN, but significantly greater than in the SCN. *Two bottom panels* illustrate VP hnRNAs by hybridization with VP intron 1-specific probe. The VP hnRNA levels appear to be quantitatively similar among all these three hypothalamic nuclei. Note that the signal to background ratio is much better for the abundant mRNAs than that for the much less abundant mRNAs (from ref. 19).

Fig. 2.

Representative fluorescence “growth curves” produced by quantitative real-time (qRT) PCR of *c-fos* mRNA, VP mRNA, and VP hnRNA in the SON in control rats. Locations of the qRT-PCR primers in the RNAs are shown in Fig. 2 (from ref. 22).

Fig. 3.

Changes in Ct values for c-fos mRNA, VP mRNA and VP hnRNA in rat SONs after acute osmotic stimulation as measured by the qRT-PCR assay. **(a)** Average $\pm$ SEM Cycle threshold (Ct) values from control SONs in rats injected with 0.15 M NaCl (*black bars*) versus SONs in rats injected with 2 M NaCl (*stripped bars*) are shown. **(b)** Calculated percent changes in c-fos mRNA and VP hnRNA in the SON 30 min after the injection of hyperosmotic 2 M compared to rats injected with isotonic 0.15 M NaCl injection. Statistically significant differences between Ct values in the 0.15 M and 2 M injected rats are shown by *asterisks* where $P < 0.05$ (unpublished data).

Fig. 4.

Changes in Ct values for GAPDH mRNA, VP mRNA, and VP hnRNA in rat SONs after chronic osmotic stimulation as measured by the qRT-PCR assay. **(a)** Average $\pm$ SEM Cycle threshold (Ct) values from control SONs in rats given normal drinking water (*black bars*) versus SONs in rats given 2.1% NaCl solution ad lib as their only drinking fluid for 5 days (*stripped bars*) are shown. **(b)** Changes in Ct values for GAPDH mRNA, OT mRNA, and OT hnRNA in rat SONs after chronic osmotic stimulation as measured by the qRT-PCR assay. **(a)** Average $\pm$ SEM Cycle threshold (Ct) values from control SONs in rats given normal drinking water (*black bars*) versus SONs in rats given 2.1% NaCl solution ad lib as their only drinking fluid for 5 days (*stripped bars*) are shown. In **(c)** are the calculated percent changes in VP mRNA and VP hnRNA in the SON in rats given 2.1% NaCl solution ad lib as their only drinking fluid for 5 days compared to rats given normal drinking water. In **(d)** the calculated percent changes in OT mRNA and OT hnRNA in the SON in rats given 2.1% NaCl solution ad lib as their only drinking fluid for 5 days compared to rats given normal drinking water. Statistically significant differences between Ct values in the control and 2.1% NaCl given rats are shown by *asterisks* where $P < 0.01$ (unpublished data).

mRNA

c-fos mRNA

VP mRNA

OT mRNA

GAPDH mRNA

hnRNA

OT hnRNA

VP hnRNA

Fig. 5.

Locations of qRT-PCR primers in the c-fos mRNA, VP mRNA, VP heteronuclear (hn)RNA, OT mRNA, OT hnRNA and GAPDH mRNA species used for quantitative real-time PCR (qRT-PCR). The *arrows* depict the positions of forward and reverse PCR primer binding sites (See Table 1 for specific sequences). In single step qPCR, the reverse PCR primer also serves as the primer for reverse transcription.

Fig. 6.

(a) Diagram of VP RNA species and PCR primer binding sites. Top, Pre-mRNA containing both introns and exons. Only the intronic primer pair can bind to this species. Bottom, following the removal of both introns, the exonic regions are joined, thereby allowing the binding of the exonic primer pair. (b) Gel analysis of exonic and intronic PCR products generated from VP mRNA and VP hnRNA from rat supraoptic nucleus. PCR triplicates were subjected to gel electrophoresis on a 2.5% agarose gel containing ethidium bromide.

The products are the predicted sizes for the amplicons and are reproducible (modified from ref. 21).

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Table 1

PCR primers used to assay *c-fos*, vasopressin (VP), oxytocin (OT), and GAPDH RNAs by single-step, real-time, quantitative PCR

Primers	Sequence	Amplicon length	T _M (°C) ^a
<i>c-fos</i> mRNA primer pairs	5'-AGCATGGGCTCCCTGTCA (forward) 5'-GAGACCAGAGTGGGCTGCA (reverse)	134 bp	83.4
VP mRNA primer pairs	5'-TGCCTGCTACTTCCAGAACTGC (forward) 5'-AGGGGAGACACTGTCTCAGCTC (reverse)	77 bp	80.7
VP hnRNA primer pairs	5'-GCCCTCACCTCTGCCTGCTA (forward) 5'-CCTGAACGGACCACAGTGGT (reverse)	100 bp	81.5
OT mRNA primer pairs	5'-GGCATCTGCTGTAGCCCG (forward) 5'-AAGGCAGACTCAGGGTCG (reverse)	62 bp	84.5
OT hnRNA primer pairs	5'-CCGTCGGGTTTGCTGCTCA (forward) 5'-AAGGCAGACTCAGGGTCG (reverse)	87 bp	84.3
GAPDH mRNA primer pairs	5'-CAGAGCTGAACGGGAAGCT (forward) 5'-CTTACCACCTTCTTGATGTCA (reverse)	125 bp	81.2

T_A, annealing temperature and time for each primer pair is 60°C, and 30 s

All the sequences shown in this Table were designed using GeneFisher software (26)

^aT_M, PCR product melting temperature is shown for each primer