Chapter 2

Quantification of NMDAR Subunit Genes Expression by qRT-PCR

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Abstract

Transcription is the initial and generally the most sensitive step to cellular needs and environmental cues. Thus, it serves as a major mechanism controlling gene expression. Using reverse-transcription quantitative polymerase chain reaction technology (RT-qPCR), we will present how to quantify the transcriptional expression of NMDARs subunits during brain development and in both healthy and pathological conditions.

Key words RNA, RT-qPCR, Reverse transcription, cDNA, Relative quantification

1 Introduction

N-Methyl-D-aspartate receptors (NMDARs) are glutamate-gated cation channels that are expressed throughout the brain and play an important role in proper brain functioning [1]. To date, seven different subunits of NMDARs have been characterized [2–4]:

- the GluN1 subunit required for the normal function of the NMDA ionophore [5–8];
- the four distinct GluN2 subunits (GluN2A, GluN2B, GluN2C, and GluN2D), which are encoded by four separate genes and appear to serve a modulatory role in NMDA channel function [7, 8, 9, 10];
- and two GluN3 subunits (GluN3A and GluN3B) encoded by two distinct genes [11–13].

NMDARs are generated by the heteromeric assembly of the GluN1 subunit with specific members of the GluN2 and/or GluN3 subunits. These different NMDARs subtypes have distinct physiological properties, which may produce synaptic plasticity by expression in specific neurons or other cells. When present, GluN3 subunits may function as dominant-negative subunits in a NMDA

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receptor complex [12]. Therefore, transcription of each NMDAR subunit gene in a given neuron must be coordinately controlled but also differentially responsive to cell type, developmental stage, and environmental signals to maintain healthy cellular function. In this section, we present how to quantify the transcriptional expression of NMDARs subunits during brain development using RT-qPCR. RT-qPCR utilizing primer–probe combinations directed to exons flanking the breakpoint junctional region, offers very high levels of both specificity and sensitivity, in a scalable, robust, and cost-effective assay.

2 Materials

2.1 RNA Extraction and Quantification

2.1.1 Columns Extraction Method

1. RNeasy Mini Kit.
2. 14.3 M β-mercaptoethanol.
3. 70% Ethanol.
4. 96–100% Ethanol.
5. 1 mL Syringes.
6. 18 G and 26 G needles.
7. Sterile, RNAse-free pipet tips.
8. 1.5 mL microcentrifuge capped tubes.
9. Microcentrifuge (with rotor for 1.5 and 2 mL tubes).

2.1.2 Trizol Extraction Method

1. TRIzol® RNA Isolation Reagent.
2. Chloroform.
3. Cooled Isopropanol.
4. Cooled 75% Ethanol.
5. Sterile RNAse-DNAse water.
6. 1 mL Syringes.
7. 18 G and 26 G needles.
8. Sterile, RNAse free pipet tips.
9. 1.5 mL microcentrifuge capped tubes.
10. Microcentrifuge (with rotor for 1.5 and 2 mL tubes).

2.1.3 RNA Quantification

1. Sterile water.
2. Sterile, RNAse free pipet tips.
3. Kimtech paper.
4. Nanophotometer.

2.2 cDNA Synthesis

1. QuantiTect® Reverse Transcription kit.
2. Thermocycler (SimpliAmp Thermal Cycler).

3. Micropipets.
4. Disposable plasticware (tubes, filters tips).

2.3 qPCR

1. Forward and reverse stock primers at the concentration of 100 μM.
2. Ready-to-use hot start reaction mix for SYBR Green I-based real-time PCR using the LightCycler®480 Instrument.
3. Sterile RNAse-DNAse water.
4. cDNA (Reverse transcripts and control reactions).
5. Optical 96 wells PCR plates.
6. Microseal film.
7. Plastics tubes.
8. Single repeater pipettes.
9. qPCR thermalcycler (LightCycler® 480 Instrument II) (*see* **Note 1**).

3 Methods

3.1 Extraction and Quantification of RNA

Whole dissected mouse brain or parts of the cerebral cortex are frozen in dry ice and directly stored at −80 °C. Immediate freezing of fresh tissue samples preserves good quality RNA for gene expression studies. Two alternative protocols are described depending on the weight of the available frozen tissue.

3.1.1 Extraction of RNA from Frozen Brain Tissue <30 mg (See Note 2)

The RNeasy Mini Kit provides a fast purification of high-quality RNA from brain tissues

1. Add 600 μL RLT containing 6 μL of β-mercaptoethanol.
2. Homogenize by passing the lysate at least ten times through a blunt 18 G needle fitted to an RNAse-free syringe.
3. Homogenize carefully by passing the lysate at least 20 times through a blunt 26 G needle fitted to the same RNAse-free syringe.
4. Centrifuge the lysate for 3 min at full speed.
5. Transfer the supernatant by pipetting to a new microcentrifuge tube and add 600 μL of 70% ethanol.
6. Transfer the mix to an RNeasy spin column placed in a 2 mL collection tube (*see* **Note 3**).
7. Centrifuge for 15 s at 15,000 × *g* and discard the flow-through.
8. Add 700 μL Buffer RW1 to the spin column and centrifuge for 15 s at 9000 × *g*, discard the flow-through, and replace the 2 mL collection tube (*see* **Note 4**).

9. Add 500 µL RPE wash buffer to the spin column and centrifuge for 15 s at 9000 × *g*, discarding the flow-through.
10. Add 500 µL RPE washing buffer to the spin column and centrifuge for 2 min at 9000 × *g*, then transfer the spin column to a new 2 mL collection tube and discard the old collection tube with the flow-through.
11. Centrifuge for 1 min at 15,000 × *g*.
12. Place the RNeasy Spin column in a new 1.5 mL collection tube.
13. Add 30 µL of RNAse-free water to the spin column and incubate for 1 min before elution of RNA by centrifuging for 1 min at 15,000 × *g*.
14. Repeat the previous step with 25 µL of RNAse-free water. The spin column can then be discarded.
15. Freeze RNA at −20 °C for one night before quantification.

3.1.2 Extraction of RNA from Frozen Brain Tissue >30 mg

This technique is a common method to extract total RNA from tissues. It takes slightly longer than column-based methods, but it has higher capacity and can yield more RNA.

1. Add 1 mL TRIzol Reagent per 50–100 mg of tissue sample.
2. Homogenize sample by passing the lysate at least ten times through a blunt 18 G needle fitted to an RNase-free syringe and at least 20 times through a blunt 26 G needle fitted to the same RNase-free syringe.
3. Incubate the homogenized sample for 5 min at room temperature.
4. Add 200 µL of chloroform per 1 mL of TRIzol Reagent and shake vigorously by hand for 15 s.
5. Incubate for 3 min at room temperature (20–22 °C).
6. Centrifuge at 10,000 × *g* for 15 min at 4 °C. The mixture will separate into three phases. The upper aqueous phase containing RNA represents approximatively half the total volume.
7. Transfer the aqueous phase to another new tube by pipetting carefully.
8. Add 1 volume of cooled isopropanol (−20 °C).
9. Incubate for 10 min at room temperature.
10. Centrifuge at 10,000 × *g* for 15 min at 4 °C.
11. Remove the supernatant and wash the pellet with 1 volume of cooled 75% ethanol.
12. Briefly vortex the sample and centrifuge at 7500 × *g* for 5 min at 4 °C. Discard the wash. Do not allow RNA to dry completely as the pellet might lose solubility. Partially dissolved RNA samples have an A260/280 ratio <1.6.

13. Resuspend the RNA pellet in RNAse-free water.
14. Store at −80 °C.

3.1.3 Quantification of RNA

The usual method for assessing RNA concentration and purity is UV spectroscopy. The absorbance of a diluted RNA sample is measured at 260 and 280 nm. The nucleic acid concentration is calculated using the Beer-Lambert law, which predicts a linear change in absorbance with concentration.

1. Defroze RNA on ice.
2. Quantify RNA using a nanodrop (nanophotometer) according to the manufacturer's instructions. An A260/A280 ratio of 1.8–2.1 indicates highly purified RNA.

3.2 cDNA Synthesis

1. Thaw template RNA, genomic DNA (gDNA) wipeout buffer and Quantiscript Reverse Transcriptase on ice. Thaw Quantiscript RT buffer, RT Primer Mix, and RNase-free water at room temperature.
2. Prepare the gDNA wipeout reaction on ice to a final total volume of 14 µL. The components are: 2 µL of gDNA Wipeout Buffer (7×), and 500 ng to 1 µg of RNA (*see* **Note 5**). One reaction tube, called minus-RT control, has to be prepared for reverse transcription but no reverse transcriptase enzyme will be added (*see* **Note 6**).
3. Incubate for 2 min at 42 °C in a thermal cycler. Then place immediately on ice.
4. Prepare the reverse-transcription master mix on ice. The mix contains 1 µL of Quantiscript reverse transcriptase, 4 µL de Quantiscript RT buffer, and 1 µL RT primer Mix per reaction.
5. Add reverse transcription master mix (6 µL) to each tube containing template RNA from **step 4** (14 µL). In the minus-RT control tube add 1 µL of DNAse-RNAse-free water, 4 µL of Quantiscript RT buffer, and 1 µL RT primer Mix per reaction.
6. Incubate for 20 min at 42 °C in a thermal cycler.
7. Incubate for 3 min at 95 °C to inactivate Quantiscript Reverse Transcriptase.
8. Store cDNA at −20 °C.

3.3 qPCR Primers

3.3.1 qPCR Primers Design [14]

1. Log on to www.universalprobelibrary.com/ (*see* **Note 7**).
2. On the assay design center screen, select your organism of interest from the drop down menu.
3. Specify the name or the sequence ID of the gene of interest.
4. Verify that the intron-spanning assay option is active and Click "design."

5. Select the right RefSeq sequence in the transcript table and click again on "Design."
6. Copy-paste the complete exon sequence in the text box. For real-time PCR purposes, we set "product size ranges" to "100–200," and "number to return" to "50." Short PCR products are typically amplified with higher efficiency than longer ones, but the PCR product should be at least 100 bp long to easily distinguish it from any primer-dimers that could potentially form.
7. Try to select two to three different primer pairs, preferably targeting different regions in the cDNA, except the untranslated regions. Annealing temperature must be between 58 °C and 61 °C and the percentage of Guanine cytosine (GC) contents between 45 and 55%. Primer length should be around 20 bp. Forward and reverse primers must span at least one intron within the gene of interest. Indeed, primers were designed to anneal to exons on both the sides of a given intron; this helps in discriminating between amplification of cDNA and amplification of potentially contaminating genomic DNA.
8. Check primer pairs for cross-hybridization with other genes using NCBI/Primer-BLAST at http://www.ncbi.nlm.nih.gov/tools/primer-blast. If there is cross-hybridization, go back to **step 7**. No mismatch in the primer set is acceptable.
9. To validate qPCR assay with the correct primers pair, at least two different pairs are ordered.
10. Primers are shipped in a dry form. Briefly spin the tube before you open the cap to avoid loss of DNA pellet. Dissolve the oligonucleotide in DNAse/RNAse-free water to make a primer stock at concentration 100 μM. Dilute from this stock 1:20 (in water) to make a working solution of mixing solution (forward and reverse) at 5 μM for use in setting up qPCR reactions.

3.3.2 Specificity (Melting Curve) and Validation (Standard Curve/Efficiency) of Primers

Each qPCR reaction must efficiently amplify a single product corresponding to a unique peak in post-amplification melting curve analysis, indicating that the design of the corresponding primers pair is correct. This analysis is frequently used as a diagnostic tool for assessing qPCR amplicon sequence and primer-dimers (*see* **Note 8**). The melting temperature of the peak correlates with the length and the GC content of the amplicon but not with the concentration template.

Amplification efficiency of these primers should be independent of cDNA template dilutions and should exhibit a twofold increase in copy numbers during each cycle of exponential amplification.

1. Make five serial dilutions of cDNA (could be any cDNA sample, but it is preferable to use a mixture of target samples to reduce heterogeneity). cDNA amount units of templates could be dilution values at 0.5, 0.1, 0.05, 0.01, and 0.002. Minus-RT and water will be used as control conditions.
2. Set up PCR reactions (*see* Subheading 3.4 for protocols). Do duplicated reactions for each diluted template. Add the appropriate controls (minus RT and water samples).
3. RT-qPCR conditions and instrument setup are described above (*see* Subheading 3.4 for protocols).
4. Data will be analyzed using LightCycler 480 SW 1.5.1 software and following the manufacturer's instructions.

3.4 qPCR (*See* Note 1)

We routinely use Sybrgreen-based application. SYBR® Green I is a fluorescent dye that binds to the minor groove of double-stranded DNA and fluoresces when bound to DNA and excited by a light source. It is useful as a screening tool to quickly assess the relative expression levels of a variety of genes in different sample types. All the procedures should be done on ice. It is advisable to run at least a triplicate for each sample.

1. Prepare one mix per reaction: 1 μL of primers solution (mixture of forward and reverse, each 5 μM) (*see* **Note 9**), 7.5 μL ready-to-use hot start reaction mix for SYBR Green I-based real-time PCR, and 2.75 μL water.
2. Pipet reaction mixture on a PCR plate, using a single channel repeater pipette.
3. Add 3.75 μL of 1/10 or 1/20 diluted cDNA per reaction (*see* **Note 10**).
4. Cover the PCR plate with optical seal.
5. Incubate the plate in the thermal cycler at the following temperatures: 10 min at 95 °C, then 50 cycles of 10 s at 95 °C, 20 s at 60 °C, and 10 s at 72 °C. At the end, a melting curve analysis needs to be performed. This is done after a denaturation step of 5 s at 95 °C followed by 1 min at 65 °C and increasing the temperature from 65 to 95 °C continuously. Five acquisitions are obtained per °C. The ramp rate is of 0.11 °C/s.
6. As standardization of the expression levels, reference housekeeping genes need to be taken along for qPCR (*see* **Note 11**).
7. Data will be analyzed using LightCycler 480 SW 1.5.1 software and following the manufacturer's instructions (*see* **Note 12**).

4 Notes

1. If using different instrument or fluorescent dye, the protocol needs to be adapted.
2. It is essential to use the correct amount of starting material to obtain optimal RNA yield and purity. A maximum amount of 30 mg fresh or frozen tissue can generally be processed. For most tissues, the RNA binding capacity of the RNeasy spin column and the lysing capacity of Buffer RLT will not be exceeded by these amounts.
3. As the volume exceeds 700 μL, centrifuge the mix two times in the same RNeasy spin column and discard the flow-through after each centrifugation.
4. After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.
5. If using >1 μg RNA, scale up the reaction linearly to the appropriate volume.
6. The amount of amplification in the samples that is attributable to genomic DNA contamination can be evaluated by running this kind of control (Minus-RT controls) in a run of qPCR.
7. Specific primers for qPCR should be designed with the aid of primer design software to avoid primer-dimers and secondary structures. ProbeFinder is a web-based software tool based on Primer3 software using optimized settings as default.
8. If a dissociation curve gives multiple peaks (even though some are very weak), we recommend not using the corresponding primers pair for further experiments.
9. If the initial set of 300 nM primer does not work, optimization of primer concentration might be required. Lower primer concentrations decrease the accumulation of primer-dimer formation and nonspecific product formation, which is critical in using SYBR Green I dye in quantitative PCR.
10. Generally, the volume of reverse-transcription reaction added should not exceed 10% of the final PCR volume. Having a look at the Ct value gives a rough idea about how much RNA/cDNA is needed for further experiments. If Ct value is high (>30), it is better to increase the amount of template for further experiments.
11. Before selecting the most appropriate reference gene, test several housekeeping genes with preliminary experiments.
12. In some studies standards curves are not determined and target quantities are reported as a fraction of the reference, a technique

termed comparative quantitation. In other studies, it is assumed that amplification efficiencies of target and reference are optimal. However in the most accurate relative quantification techniques, amplification efficiencies of both the reference and target are measured and a correction factor is determined. This process, termed normalization, requires a sample containing known concentrations of both target and reference and the generation of two standard curves. We perform relative quantification of our samples. Indeed, the ratio between the amount of NMDA-R subunit transcripts and of housekeeping transcripts (such as *Ppia1*, *Hprt*, *Rpl13a* in rodents and *HPRT*, *β-Actin* in humans) can be calculated at different stages of development and in different regions of the brain in rodents and humans.

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References

1. Burnashev N, Szepetowski P (2015) NMDA receptor subunit mutations in neurodevelopmental disorders. Curr Opin Pharmacol 20:73–82. doi:10.1016/j.coph.2014.11.008
2. Paoletti P (2011) Molecular basis of NMDA receptor functional diversity. Eur J Neurosci 33 (8):1351–1365. doi:10.1111/j.1460-9568.2011.07628.x
3. Paoletti P, Bellone C, Zhou Q (2013) NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease. Nat Rev Neurosci 14(6):383–400. doi:10.1038/nrn3504
4. Traynelis SF, Wollmuth LP, McBain CJ, Menniti FS, Vance KM, Ogden KK, Hansen KB, Yuan H, Myers SJ, Dingledine R (2010) Glutamate receptor ion channels: structure, regulation, and function. Pharmacol Rev 62 (3):405–496. doi:10.1124/pr.109.002451
5. Hollmann M, Heinemann S (1994) Cloned glutamate receptors. Annu Rev Neurosci 17:31–108. doi:10.1146/annurev.ne.17.030194.000335
6. McBain CJ, Mayer ML (1994) N-methyl-D-aspartic acid receptor structure and function. Physiol Rev 74(3):723–760
7. Moriyoshi K, Masu M, Ishii T, Shigemoto R, Mizuno N, Nakanishi S (1991) Molecular cloning and characterization of the rat NMDA receptor. Nature 354(6348):31–37. doi:10.1038/354031a0
8. Petralia RS, Yokotani N, Wenthold RJ (1994) Light and electron microscope distribution of the NMDA receptor subunit NMDAR1 in the rat nervous system using a selective anti-peptide antibody. J Neurosci 14(2):667–696
9. Anson LC, Chen PE, Wyllie DJ, Colquhoun D, Schoepfer R (1998) Identification of amino acid residues of the NR2A subunit that control glutamate potency in recombinant NR1/NR2A NMDA receptors. J Neurosci 18 (2):581–589
10. Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, Lomeli H, Burnashev N, Sakmann B, Seeburg PH (1992) Heteromeric NMDA receptors: molecular and functional distinction of subtypes. Science 256(5060):1217–1221
11. Das S, Sasaki YF, Rothe T, Premkumar LS, Takasu M, Crandall JE, Dikkes P, Conner DA, Rayudu PV, Cheung W, Chen HS, Lipton SA, Nakanishi N (1998) Increased NMDA current and spine density in mice lacking the NMDA receptor subunit NR3A. Nature 393 (6683):377–381. doi:10.1038/30748

12. Nishi M, Hinds H, Lu HP, Kawata M, Hayashi Y (2001) Motoneuron-specific expression of NR3B, a novel NMDA-type glutamate receptor subunit that works in a dominant-negative manner. J Neurosci 21(23):RC185
13. Pachernegg S, Strutz-Seebohm N, Hollmann M (2012) GluN3 subunit-containing NMDA receptors: not just one-trick ponies. Trends Neurosci 35(4):240–249. doi:10.1016/j.tins.2011.11.010
14. Lozovaya N, Gataullina S, Tsintsadze T, Tsintsadze V, Pallesi-Pocachard E, Minlebaev M, Goriounova NA, Buhler E, Watrin F, Shityakov S, Becker AJ, Bordey A, Milh M, Scavarda D, Bulteau C, Dorfmuller G, Delalande O, Represa A, Cardoso C, Dulac O, Ben-Ari Y, Burnashev N (2014) Selective suppression of excessive GluN2C expression rescues early epilepsy in a tuberous sclerosis murine model. Nat Commun 5:4563. doi:10.1038/ncomms5563

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