APPLICATION NOTE 202102

Quantification of COX-1 & COX-2 Expression from iPSC Derived Neuroprogenitor Cells

Gabriella Ryan, M.S.; Zachary Morehouse, M.S.; Rodney Nash, Ph.D.
Omni International, a PerkinElmer company

Background

The applications of induced pluripotent stem cell (iPSC) research are reaching new depths as scientists make new discoveries about homeostasis and disease. Stem cell research has implications in many areas, from identification of disease mechanisms on a cellular level to development of treatments. A vital part of this research is focused on molecular identification of relevant genes. With that said, it is of utmost importance that researchers are able to obtain RNA from cultured iPSC's that are not only high yield, but also high-quality in order to conduct downstream studies like RT-qPCR, RNA-seq, and other RNA-based analyses.

More specifically, serving as a drug target for pain attenuation and topic of study for involvement in various inflammatory disease mechanisms, cyclooxygenase enzymes (COX) are known to convert arachidonic acid to prostaglandin in the tissues, and central and peripheral nervous systems (Rouzer, 2009). Acting in response to many inflammatory pathways, both isoforms of the COX enzymes serve as sites for selective and non-selective inhibition for non-steroidal anti-inflammatory drugs as well as targets for study in the case of pathophysiologic anomalies.

Herein, we evaluate the efficacy of the Bead Ruptor Elite™ in front end sample preparation of cultured iPSC derived neuroprogenitor (NP) cells for isolation and purification of RNA, and detection of COX-1 and COX-2 via RT-qPCR.

Materials and Methods

Materials

- Bead Ruptor Elite™ (Cat# 19-042E)
- Bead Ruptor Elite™ 2mL Tube Carriage (Cat# 19-373)
- 2 mL Soft Tissue Homogenizing Mix (Cat#19-627)
- Omni Tissue RNA Kit (Cat# 26-010)

Bead Ruptor Elite™ 2 mL Tube Carriage

Bead Ruptor Elite™ 2 mL Soft Tissue Homogenizing Mix

Bead Ruptor Elite™ Bead Mill

For research use only.
Not for use in diagnostic procedures.

OMNI International
a PerkinElmer company

Methods

Neuroprogenitor Cell Culture

NP Cells were obtained from consenting patients in accordance with CIRBI IRB protocol No. Pro00036306. NP Cells were cultured on tissue-culture treated plates (Corning, Cat No. 430167) coated with 0.1% vitronectin (Thermo, Cat No. A14700). Cells were supplemented with Stemflex Medium (Thermo, Cat No. A3349401) prepared with 5% supplement and 1% penicillin/streptomycin solution (Gemini Bio, Cat No. 400-109). NP cells were grown to 80% confluency, and detached from the plate with Accutase (Thermo, Cat No. A1110501). NP cell/Accutase solution was centrifuged, and the resulting supernatant was poured off and the pellet was re-suspended in PBS, pH 7.2 (Gibco, Cat No. 20012027).

Cell Counting

Resuspended cells were combined 1:1 with a 0.4% Trypan Blue solution (Millipore Sigma, Cat No. T8154) and counted using a hemocytometer to determine total number of intact cells.

RNA Extraction

No more than 1 x 10^7 cells/mL were added to each 2 mL Soft Tissue Homogenizing Mix tube (Omni International, Cat No. 19-627). Samples were homogenized on the Bead Ruptor Elite at 4.2 m/s for 30 seconds (Table 1). After homogenization, the remainder of the RNA extraction was carried out per the Omni Tissue RNA Kit manual (Omni International, Cat No. 26-010).

Samples were eluted with 100 µL of Nuclease-Free water supplied with the Omni Tissue RNA Kit. RNA concentration and integrity were determined by A_{260}/A_{280} spectrophotometry.

After spectrophotometric analysis, eluted RNA was further analyzed using the 2100 Bioanalyzer Instrument (Agilent, Cat. No. G2939B) with the Agilent RNA 600 Nano Kit (Agilent, Cat. No. 5067-1511) using the Eukaryote Total RNA Nano Assay. Standard kit protocol was followed when preparing samples for analysis on the Bioanalyzer. RNA Integrity Number (RIN) and gel electropherogram were obtained from the instrument readout upon protocol completion.

RT-qPCR

To prepare for RT-qPCR, eluted RNA was diluted to a final concentration of 1 ng/µl. One microliter of eluted NP cell RNA was added to RT-qPCR mix (New England Biolabs, Cat No. E3005S) along with either COX-1 primers, COX-2 primers or β-actin primers. All volumes of reaction mix, reverse transcriptase, primers, and nuclease-free water were prepared according to manufacturer's instructions as laid out for a 20 µl final reaction volume. COX-1 primers were selected from Pierzchalska et al. [2], COX-2 primers were selected from Fornai et al. [1], while β-actin primers were selected from Yasojima et al. [4]. COX-1 was targeted with forward primer 5'-CAGACGACCCGCCTCATCCTCATAG-3', reverse primer 5'-GCCTCAACCCCATAGTCCACCAACA-3' yielding a product size of 275 bp. COX-2 was targeted with forward primer 5'-TTC AAA TGA GAT TGT GGG AAA ATT GCT -3', reverse primer 5'- AGA TCA TCT CTG CCT GAG TAT CTT -3' yielding a product size of 305. β-actin was targeted with forward primer 5'-AGC GGG AAA TCG TGC GTG-3', reverse primer 5'-GGG TAC ATG GTG GTG CCG-3' yielding a product size of 307 bp. Reactions were loaded into the BioRad CFX Connect Real Time Instrument (BioRad Cat. No. 1855201) and amplified for 45 cycles. Amplicons were visualized on 2% agarose gel stained with EtBr for product visualization.

Sample Type	Speed (m/s)	Time (sec)	Cycles	Dwell Time (sec)
NP Cells	4.2 m/s	30 sec	1	N/A

Table 1. Bead Ruptor Elite Sample Homogenization Summary

OMNI International
a PerkinElmer company

Results

Sample Name	RNA Concentration (ng/µl)	A_{260}/A_{280}	A_{260}/A_{230}	RIN Value
NP Cells 1	92	2.06	1.8	9.6
NP Cells 2	92	2.08	1.8	9.8
NP Cells 3	72	2.08	1.9	9.0

Table 2. NP Cell RNA Concentration and Integrity Values

Sample Name	Average Cq Value	Standard Deviation
β-actin	19.9	0.49
COX-1	36.1	0.14
COX-2	34.4	0.47

Table 3. Average Cq Values and standard deviations obtained from RT-qPCR amplifying β-actin, COX-1 and COX-2 genes from NP RNA

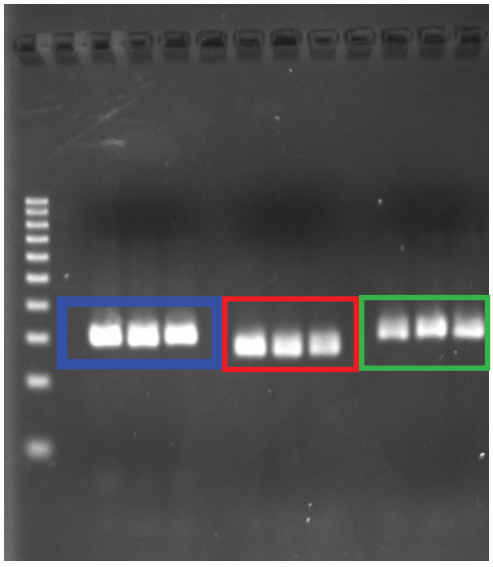

Figure 1. RT-qPCR Amplicon gel showing cDNA visualization on 2% Agarose gel. 100 bp molecular ruler is seen in lane 1. β-actin product is seen at 307 bp in lanes 3-5 (blue), COX-1 product is seen at 275 bp in lanes 7-9 (red), and COX-2 product is seen at 305 bp in lanes 11-13 (green).

Results

Ribonucleic acid isolated from NP cells resulted in high yields and purity after spectrophotometric analysis. A_{260}/A_{280} values were used to determine RNA purity with pure RNA ranging from values between 1.9-2.0 (Table 2). The data in Table 2 suggests high quantity and purity RNA can be obtained from NP cells using the Bead Ruptor Elite and Soft Tissue Homogenization Mix, along with the Omni Tissue RNA extraction kit.

The Agilent Bioanalyzer was used to visualize genomic RNA and determine RNA Integrity Number (RIN, out of 10) of each individual sample. RIN is determined by an algorithm based on a ratio of 28S to 18S ribosomal subunits along with electropherogram quality. All RIN were above 9 (Table 2) indicating the overall high integrity and intactness of the RNA sample. Showing distinctly separated bands, the electropherogram reinforces the high quality of isolated NP RNA and correlates with the high RIN values.

RT-qPCR was performed to confirm the presence of COX-1 and COX-2 gene sequences within RNA isolated from NP cells. β-actin was used as a structural positive control. Cq values for COX-1, COX-2 and β-actin were all within 1 cycle between replicates indicating reproducible results within triplicate samples (Table 3). A 2% agarose gel separation of the RT-qPCR products showed a hyperdense single band in each lane indicating that single products were formed at 275 bp (COX-1), 305 bp (COX-2) and 307 bp (β-actin) when compared to the 100 bp molecular ruler (Figure 1).

Conclusion

When used in conjunction with the Omni Tissue RNA kit and Soft Tissue Homogenization Mix, the Bead Ruptor Elite can be implemented in front-end sample preparation of cultured iPSC derived NP cells. The resulting homogenate is suitable for isolation of intact and high-quality RNA conducive to RT-qPCR analysis to identify genes of interest. The results also show that COX-1 and COX-2 gene sequences are present in genomic RNA from NP cells and acts as a stepping-stone for further investigation and in-depth research studies.

References (APA Style)

1. Fornai, M., Blandizzi, C., Colucci, R., Antonioli, L., Bernardini, N., Segnani, C., Baragatti, B., Barogi, S., Berti, P., Spisni, R., & Del Tacca, M. (2005). Role of cyclooxygenases 1 and 2 in the modulation of neuromuscular functions in the distal colon of humans and mice. Gut, 54(5), 608–616. https://doi.org/10.1136/gut.2004.053322

2. Pierzchalska, M., Soja, J., Woś, M., Szabó, Z., Nizankowska-Mogielnicka, E., Sanak, M., & Szczeklik, A. (2007). Deficiency of cyclooxygenases transcripts in cultured primary bronchial epithelial cells of aspirin-sensitive asthmatics. Journal of physiology and pharmacology: an official journal of the Polish Physiological Society, 58(2), 207–218.

3. Rouzer, C. A., & Marnett, L. J. (2009). Cyclooxygenases: structural and functional insights. Journal of lipid research, 50 Suppl(Suppl), S29–S34. https://doi.org/10.1194/jlr.R800042-JLR200

4. Yasojima, K., Schwab, C., McGeer, E. G., & McGeer, P. L. (1999). Distribution of cyclooxygenase-1 and cyclooxygenase-2 mRNAs and proteins in human brain and peripheral organs. Brain research, 830(2), 226–236. https://doi.org/10.1016/s0006-8993(99)01389-x

OMNI International
a PerkinElmer company

OMNI International, a PerkinElmer company
935 Cobb Place Blvd NW
Kennesaw, GA 30144
United States
770.421.0058 | 800.766.4431