qRT-PCR

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Method Article

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Abstract

This protocol describes how to perform qRT-PCR on microRNAs and mRNAs isolated from extracellular vesicles or other extracellular particles in order to quantify the amount of extracellular RNA present in the original sample.

Introduction

Extracellular RNAs \(exRNAs) have been identified in every biofluid that has been tested. They have been found in extracellular vesicles, ribonucleoprotein complexes and lipoprotein complexes. exRNAs are interesting because they may serve as signalling molecules between cells, they have the potential to serve as biomarkers for prediction and diagnosis of disease, and exRNAs or the extracellular particles that carry them might be used for therapeutic purposes. The Sample and Assay Standards Working Group of the Extracellular RNA Communication Consortium \(ERCC) is a group of laboratories funded by the U.S. National Institutes of Health to develop robust and standardized methods for collecting and processing of biofluids, separating different types of exRNA-containing particles and isolating and analyzing exRNAs. In our first joint endeavour, we held a series of conference calls and in-person meetings to survey the methods used among our members, placed them in the context of the current literature and used our findings to identify areas in which the identification of robust methodologies would promote rapid advancements in the exRNA field. A full list of the protocols developed during this effort is available at the exRNA Portal, the ERCC's website \("http://exrna.org/resources/protocols/":http://exrna.org/resources/protocols/). This protocol for performing qRT-PCR is one of the final steps in the process, allowing quantification of the amount of extracellular RNA found in a sample or biofluid.

Reagents

See figure in Figures section.

Procedure

For miRNAs 1.1 Normalize input RNA samples to a concentration of 1 ng/µL. 1.2 Thaw RT kit components on ice. Mix gently and centrifuge briefly. 1.3 Calculate number of RT reactions, including no-input control. 1.4 Make RT master mix on ice, using "attached spreadsheet":http://www.nature.com/protocolexchange/system/uploads/4051/original/Master_Mixes.xlsx?1447065271 to scale the following per reaction mixture: See figure in Figures section. 1.5 Mix and centrifuge briefly. 1.6 Combine in 0.2 ml qPCR well: 1 µL input RNA 11 µL RT master mix 3 µL 5X RT primer 1.7 Seal, mix, and centrifuge briefly. 1.8 Incubate on ice for 5 minutes. 1.9 Place in thermocycler and run the following program: 16°C 30 minutes 42°C 30 minutes 85°C 5 minutes 4°C Hold The reactions may be stored at -20°C at this point. 1.10 Thaw frozen PCR kit components on ice. Mix and centrifuge briefly. 1.11 Swirl mastermix bottle gently to mix. 1.12 Calculate number of samples, including no-input and no-template controls for each miRNA assay. 1.13 Set up triplicate PCR reactions for each template/assay combination, using the "attached

spreadsheet":http://www.nature.com/protocolexchange/system/uploads/4051/original/Master_Mixes.xlsx?1447065271 to scale the following per reaction mixture: See figure in Figures section. 1.14 Mix and centrifuge briefly. 1.15 Distribute into 0.2 ml qPCR wells, 10 µl per well. 1.16 Seal, mix, and centrifuge briefly. 1.17 Place in qPCR machine and run the following program: 1 cycle of: 95°C 10 minutes 40 cycles of: 95°C 15 seconds 60°C 60 seconds **For mRNAs** 2.1 Normalize input RNA samples to a concentration of 1 ng/µl. 2.2 Thaw RT kit components on ice. Mix gently and centrifuge briefly. 2.3 Calculate number of RT reactions, including no-input control. 2.4 Make RT master mix on ice, using "attached spreadsheet":http://www.nature.com/protocolexchange/system/uploads/4051/original/Master_Mixes.xlsx?1447065271 to scale the following per reaction mixture: See figure in Figures section. 2.5 Mix and centrifuge briefly. 2.6 Combine in 0.2 ml qPCR well: 1 µL input RNA 9 µL RT master mix. 2.7 Seal, mix, and centrifuge briefly. 2.8 Keep on ice until you are ready to put into thermocycler. 2.9 Place in thermocycler and run the following program: 25°C 10 minutes 37°C 120 minutes 85°C 5 minutes 4°C Hold The reactions may be stored at -20°C at this point. 2.10 Thaw frozen PCR kit components on ice. Mix and centrifuge briefly. 2.11 Swirl mastermix bottle gently to mix. 2.12 Calculate number of samples, including no-input and no-template controls for each miRNA assay. 2.13 Set up triplicate PCR reactions for each template/assay combination, using the "attached spreadsheet":http://www.nature.com/protocolexchange/system/uploads/4051/original/Master_Mixes.xlsx?1447065271 to scale the following per reaction mixture: • For the TaqMan Assays, set up this reaction mixture: See figure in Figures section. • For the Universal RNA Spike I Assay, set up this reaction mixture: See figure in Figures section. 2.14 Mix and centrifuge briefly. 2.15 Distribute into 0.2 ml qPCR wells, 10 µL per well. 2.16 Seal, mix, and centrifuge briefly. 2.17 Place in qPCR machine and run the following program: 1 cycle of: 50°C 2 minutes 95°C 10 minutes 40 cycles of: 95°C 15 seconds 60°C 60 seconds

Figures

Item	Vendor	Catalog #	Assay#	target
miR-16-5p TaqMan assay	Life Technologies	4427975	000391	miRNA
let-7a-5p TaqMan assay	Life Technologies	4427975	000377	miRNA
miR-223-3p TaqMan assay	Life Technologies	4427975	002295	miRNA
TaqMan MicroRNA Reverse Transcription Kit	Life Technologies	4366596		miRNA
18S rRNA TaqMan assay, FAM	Life Technologies	4331182	Hs99999901_s1	mRNA
GAPDH TaqMan assay, FAM	Life Technologies	4331182	Hs02758991_g1	mRNA
BRAF TaqMan assay, FAM	Life Technologies	4331182	Hs00269944_m1	mRNA
Universal RNA Spike I, FAM	TATAA Biocenter	RS25PFI		mRNA
High Capacity cDNA Reverse Transcription Kit, with RNase Inhibitor	Life Technologies	4374966		mRNA
TaqMan 2X Universal PCR Master Mix, no AmpErase UNG	Life Technologies	4324018		miRNA and mRNA
qPCR machine				miRNA and mRNA
96-well plates compatible with qPCR machine				miRNA and mRNA

Figure 1

Table 1 Reagents

Component	Volume per 15-µL reaction
100 mM dNTPs (with dTTP)	0.15 µL
MultiScribe™ Reverse Transcriptase, 50 U/µL	1 µL
10× Reverse Transcription Buffer	1.5 µL
RNase Inhibitor, 20 U/µL	0.19 µL
Nuclease-free water	8.16 µL
Total volume	11.00 µL

Figure 2

Table 2 RT Master Mix for miRNA

Component	Volume per 10-µL reaction
TaqMan® Small RNA Assay (20×)	0.5 µL
Product from RT reaction	0.66 µL
TaqMan® Universal PCR Master Mix II (2×), no UNG	5 µL
Nuclease-free water	3.84 µL
Total volume	10.00 µL

Figure 3

Table 3 PCR Master Mix for miRNA

Component	Volume per 10-µL reaction
10× RT Buffer	1 µL
25× dNTP Mix (100 mM)	0.4 µL
10× RT Random Primers	1 µL
MultiScribe™ Reverse Transcriptase	0.5 µL
RNase Inhibitor	0.5 µL
Universal RNA Spike I template	1 µL
Nuclease-free H2O	4.6 µL
Total per Reaction	9 µL

Figure 4

Table 4 RT Master Mix for mRNA

mRNA PCR Taqman

Component	Volume per 10-µL reaction
20X TaqMan® Gene Expression Assay	0.5 µL
Product from RT reaction	1 µL
TaqMan® Universal PCR Master Mix II (2×), no UNG	5 µL
Nuclease-free water	3.5 µL
Total volume	10.00 µL

Figure 5

Table 5 Taqman PCR Master Mix for mRNA

Component	Volume per 10-µL reaction
Spike I Assay primers	0.4 µL
Spike I Assay probe	0.2 µL
Product from RT reaction	1 µL
TaqMan® Universal PCR Master Mix II (2×), no UNG	5 µL
Nuclease-free water	3.4 µL
Total volume	10.00 µL

Figure 6

Table 6 Universal RNA Spike I PCR Master Mix for mRNA

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplement0.xlsx