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Preanalytical aspects of quantitative TaqMan real-time RT-PCR: Applications for TF and VEGF mRNA quantification

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Abstract

Objectives: The present paper focuses on preanalytical aspects of tissue factor (TF) and vascular endothelial growth factor (VEGF) mRNA quantification: the choice of blood collection tubes and defining the time frame allowed before processing the sample.

Design and methods: Blood was collected from healthy volunteers in K_3 EDTA tubes, CPT™, endotoxin-free EndoTube™ tubes and in PAXgene™ tubes. Total RNA concentration was determined by absorbance readings at 260 nm with a GeneQuantII UV spectrophotometer. RNA quantity and quality were also determined by the Lab on a Chip technique (Agilent 2100 Bioanalyzer). Real-time RT-PCR assays were performed by the TaqMan technology.

Results: The more expensive PAXgene and CPT tubes and the Endo tubes did not give superior results from those obtained in inexpensive routine K_3 EDTA tubes. The PAXgene tubes preserved high molecular mass rRNA better than the other tubes.

Conclusion: Both the PAXgene™ system and routine EDTA tubes are suitable for clinical purposes aimed at quantitation of mRNA for TF and VEGF. PAXgene™ yielded rRNA that was less degraded but had lower mRNA per μg extracted RNA. A time frame up to 24 h until sample processing is acceptable for TF and VEGF mRNA.

Keywords: Tissue factor; Vascular endothelial growth factor; mRNA; RNA isolation; Real-time PCR; Human blood; Agilent bioanalyzer; RNA quantity

Introduction

Quantitative methods for detection of mRNA levels in blood, utilising quantitative real-time reverse transcriptase PCR (real time QRT-PCR) by various techniques, have been devised [1–3]. Topics currently being investigated include avoidance of DNA and other contaminants [4,5], construction of standard curves [6–9], selecting a suitable housekeeping gene as the mRNA reference [10,11] and comparisons of different types of specimen collection tubes for blood sampling [12]. For the latter purpose, a commercially available system, PAXgene™ [13], was recently presented on the market. A bioanalyzer has been launched which, in addition to a fluorescent dye-dependent RNA quantification, offers a qualitative image of the extracted RNA by mini-electrophoresis through automated scanning of the electrophoresed samples.

We present here our data on the evaluation of some preanalytical variables of importance when designing the specimen collection phase for clinical studies of tissue factor (TF) and vascular endothelial growth factor (VEGF) in cancer patients. The special focus of the present paper has been on the choice of blood collection tubes and on defining the time frame allowed before processing the sample.

Materials and methods

Blood sampling and total RNA isolation

Blood was collected from healthy volunteers in K_3 EDTA tubes, CPT™ (both from BD Vacutainer™, Preanalytical Solutions, Plymouth UK) and in endotoxin-free EndoTube™ tubes (CoaChrom Diagnostica GmbH, Vienna, Austria). The CPT™ tubes are especially designed to separate white blood cells and plasma from the erythrocytes by centrifugation. CPT™ tubes were centrifuged before extraction according to

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the manufacturer's instructions, and total RNA was extracted from 1 mL of the cell suspension (plasma and mononuclear cells) by the QIAamp RNA Blood Mini Kit® (Qiagen Inc., Valencia, CA, USA) spin column procedure in accordance with the instructions of the manufacturer. Total RNA was extracted from 1 mL of whole blood from K_3 EDTA tubes and EndoTube, also by the QIAamp RNA Blood Mini Kit®. Total RNA was extracted from PAXgene™ tubes with the PAXgene™ Blood RNA Kit (QIAGEN Inc., Valencia, CA, USA). Two tubes from each brand were drawn from each subject. One of the tubes was extracted within an hour (PAXgene after 2 h according the manufacturer's instructions) of phlebotomy which simulates transportation time to laboratory. The other tubes were left at an ambient temperature for 24 h and were then extracted as above. This 24-h period was chosen to simulate transport by mail from another laboratory.

RNA quality and quantity

Total RNA concentration was determined by absorbance readings at 260 nm with a GeneQuantII spectrophotometer (Pharmacia Biotech, England) [14]. We typically obtained values of 1.9–2.1 (mean 1.96 ± 0.0764, $n = 40$).

RNA quantity and quality were also determined by capillary electrophoresis using the Lab on a Chip technique (Agilent 2100 Bioanalyzer, Agilent Technologies, GmbH, Waldbronn, Germany) in accordance to the manufacturer's instructions on the RNA 6000 Nano Labchip®. The chip also generates a quantitative measure of the amount of RNA loaded into the wells (3 μL).

cDNA synthesis

One microgram of total RNA was transcribed into cDNA (single stranded complementary DNA) in a 20 μL reaction volume using Omniscript reverse transcriptase for first-stand cDNA synthesis, two tube RT-PCR (Omniscript® RT Kit for reverse transcription, GmbH, Hilden, Germany) using random hexamer primers and RNase OUT Ribonuclease Inhibitor (Invitrogen, Karlsruhe, Germany). The master mix was prepared according the manufacturer's instructions and incubated for 60 min at 37°C in a Gene Amp® PCR System 2700 PCR instrument (Applied Biosystems, Foster City, CA, USA).

Real-time QRT-PCR

Real-time RT-PCR assays were performed by the TaqMan technology on an ABI PRISM 7000 SDS (Applied Biosystems, Weiterstadt, Germany). Each PCR reaction contained 3 μL cDNA template (corresponding to approximately 150 ng of the total extracted RNA), 12.5 μL TaqMan Universal PCR master mix 2× (Applied Biosystem), 0.25 μL of each sense and antisense primer and 1.25 μL TaqMan probe, supplemented with water to a final volume of 25 μL, according to the manufacturer's instructions. Both samples and standards (see below) were performed in duplicate. A non-template control was included in every assay. Samples were heated for 2 min at 50°C and 10 min at 95°C and then subjected to 40 cycles of denaturation at 95°C for 15 s and annealing and elongation at 60°C for 1 min. Primer and probe sequences (purchased from SGS DNA, Köping, Sweden) for β-actin and VEGF are available on request. For the tissue factor, we used Assays on Demand™ (Applied Biosystem, Gene Expression Products, No. 4331182).

Preparation of standards

Blood was collected from healthy volunteers in K_3 EDTA tubes (BD Vacutainer™). Total RNA was extracted from 1 mL of whole blood by the QIAamp RNA Blood Mini Kit® (Qiagen) spin column procedure in accordance with the instructions of the manufacturer, and RT-PCR was performed as described above. The amplicons generated by the primer pairs specified above were used as standards in the QRT-PCR reaction. The same PCR programs as above were used. The concentrations of the amplicons were measured by the GeneQuantII. Appropriate dilutions of the amplicon stock solution were used to generate a standard curve in the ABI PRISM 7000 SDS instrument. All standards were stored undiluted at −20°C.

Results

Qualitative assessment of extracted total RNA

The quality of extracted total RNA was assessed by the Agilent Bioanalyzer. The RNA extracted by the PAXgene system yielded fairly sharp peaks of the expected 18S and 28S sizes corresponding to human rRNA. The baseline was usually almost flat (data not shown).

RNA extracted from EDTA tubes also displayed 18S and 28S rRNA peaks, but the peaks were always broad-based, indicative of substantial degradation of rRNA: some samples in addition showed signs of the presence of DNA in the RNA extracts (data not shown). CPT and Endo tubes were not subjected to this test for reasons stated in Quantitation of extracted mRNA from different types of specimen collection tubes.

Quantitative assessment of extracted total RNA

The total amount of extracted RNA was quantified by two different methods. The values obtained by the Agilent Bioanalyzer were about 38% higher (slope of the regression = 1.378) than the GeneQuantII values over the assay range studied. However, when analyzed with a Bland–Altman plot, the difference between the methods actually varied from approximately −20% in the lowest range to +80% in the high range of mRNA levels (Fig. 1, lower panels). When the regressions were studied separately for RNA samples extracted by the PAXgene system and from EDTA blood, respectively, the Bioanalyzer and GeneQuantII values were seen to agree