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SPIDIA-RNA: First external quality assessment for the pre-analytical phase of blood samples used for RNA based analyses

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ABSTRACT

The diagnostic use of *in vitro* molecular assays can be limited by the lack of guidelines for collection, handling, stabilization and storage of patient specimens. One of the major goals of the EC funded project SPIDIA (www.spidia.eu) is to develop evidence-based quality guidelines for the pre-analytical phase of blood samples used for molecular testing which requires intracellular RNA analytes. To this end, a survey and a pan-European external quality assessment (EQA) were implemented. This report is the summary of the results of that trial.

With the European Federation of Laboratory Medicine (EFLM) support, 124 applications for participation in the trial were received from 27 different European countries, and 102 laboratories actually participated in the trial. Each participating laboratory described their respective laboratory policies and practices as well as blood collection tubes typically used in performing this type of testing. The participating laboratories received two identical blood specimens: in an EDTA tubes (unstabilized blood; $n = 67$) or in tubes designed specifically for the stabilization of intracellular RNA in blood (PAXgene® Blood RNA tubes; $n = 35$). Laboratories were requested to perform RNA extraction according to the laboratory's own procedure as soon as possible upon receipt of the tubes for one tube and 24 h after the first extraction for the second tube. Participants ($n = 93$) returned the two extracted RNAs to SPIDIA facility for analysis, and provided details about the reagents and protocols they used for the extraction.

At the SPIDIA facility responsible for coordinating the study, the survey data were classified, and the extracted RNA samples were evaluated for purity, yield, integrity, stability, and the presence of interfering substances affecting RT-qPCR assays. All participants received a report comparing the performance of the RNA they submitted to that of the other participants. All the results obtained by participants for each RNA quality parameter were classified as "in control", "warning", "out of control" and "missing" by consensus mean analysis.

From the survey data, the most variable parameters were the volume of blood collected and the time and storage temperature between blood collection and RNA extraction.

Analyzing the results of quality testing of submitted RNA samples we observed a data distribution of purity, yield, and presence of assay interference in agreement with expected values. The RNA Integrity Number (RIN) values distribution was, on the other hand, much wider than the optimal expected value, which led to an "in control" classification, even for partly degraded RNA samples. On the other hand, RIN values below 5 significantly correlated with a reduction of GAPDH expression levels. Furthermore, the distribution of the values of the four transcripts investigated (c-fos, IL-1β, IL-8, and GAPDH) was wide and the RNA instability between samples separated by 24 h were similar. Assuming the presence of at least two quality parameters "out of control" as an indication of a critical performance of the laboratory, 33% of the laboratories were included in this group.

The results of this study will be the basis for implementing a second pan-European EQA and the results of both EQAs will be pooled and will provide the basis for the implementation of evidence-based guidelines for the pre-analytical phase of RNA analysis of blood samples.

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1. Introduction

SPIDIA (www.spidia.eu) is a 4-year, large-scale, integrated project funded by the European Commission one aim of which is to standardize and improve pre-analytical procedures for *in vitro* diagnostics by developing quality assurance schemes (EQAs) and validated technologies for the collection, transport and processing of blood samples for RNA-based analyses. Ultimately, the results of these programs will be used as the basis for the development of specimen collection, transport, and handling guidelines for molecular diagnostic testing requiring RNA as the analyte.

Implementing an EQA program for the pre-analytical phase of blood samples collected for RNA based analyses, presents significant challenges. Whole blood is a complex mixture of various cell types in which the relative distribution of white blood cells may differ substantially between normal and diseased subjects. Furthermore gene expression in these cells may be affected by a number of factors that can either induce or repress gene expression changes or lead to degradation of RNA post-phlebotomy [1,2]. As a result of the pilot EQA designed to determine the deficiencies of sample handling for RNA based analyses, we defined the experimental protocol for a larger pan-European EQA [3]. We also proposed a panel of assays to test the quality of the purified RNA derived from blood which included the spectrophotometric measurement of total RNA yield and purity, and the RNA integrity (RIN) score as determined by algorithmic analysis of the RNA electropherogram [4]. Moreover, to test the performance of the resultant RNA in downstream assays, we measured transcript levels of selected genes (c-fos, IL-1β, IL-8, and GAPDH) known to be induced or repressed by *ex vivo* blood handling [3,5–8]. In order to recruit a representative number of participating laboratories, SPIDIA collaborated with the European Federation of Laboratory Medicine (EFLM; www.efcclm.org).

2. Materials and methods

2.1. Collection of applications

The announcement of the SPIDIA-RNA EQA was published on the EFLM web site (www.efcclm.org). A dedicated website was created which contained the description of the SPIDIA project (www.spidia.eu), the proposed protocols, the application form, and a questionnaire page (www.efcclm.org). Laboratories applying for participation were asked to describe the type of blood collection tube they usually use for RNA based analyses from blood samples: for instance tubes without an RNA stabilizer (e.g. EDTA Tube) or with an RNA stabilizer (e.g. the PAXgene® Blood RNA Tube).

Details on the content of these web pages are reported as Supplemental data: the Questionnaire (Supplement 1), the protocols describing the procedures (Supplements 2 and 3) to follow for blood storage/extraction, and the Results form (Supplements 4 and 5) to record the data and information used for blood samples storage/extraction/analysis. Two different protocols and results forms were developed depending on the type of blood collection tube used as specified by the applicant. All participants were informed in advance of the shipping date of the samples.

2.2. Blood collection and shipment conditions

Blood was collected from seven, consented healthy donors who were tested negative for HIV, HBV, and HCV. Venous whole blood (350 ml) was collected from each donor into blood collection bags containing citrate phosphate dextrose adenine (CPDA) as the anticoagulant/preservative. Blood from all seven donors was pooled in a sterilized flask, mixed under gently stirring conditions, and immediately aliquoted into K_2EDTA Tubes (Becton Dickinson) (5 ml per tube) and PAXgene Blood RNA Tubes (PreAnalytiX) (2.5 ml per tube). Participants received a box containing two blood tubes of the same type (labelled Tube A and Tube B), either K_2EDTA or PAXgene Blood RNA tubes and two empty vials to be used to send the extracted RNAs back to the SPIDIA facility for analysis. Boxes were shipped by an international courier the day after blood collection, and blood specimens were stored at 4 °C prior to packaging and shipment. The boxes contained a frozen soft-gel ice pack to maintain cool conditions during shipping.

2.3. Instructions for the participants

The participants received the box containing the blood samples and the protocols (Supplements 2 and 3) for performing the RNA extractions. To determine the stability of the RNA in each tube type, the laboratories were instructed, using their standard procedure, to extract RNA from Tube A preferably immediately after the arrival of the samples or at least within 24 h after arrival, and from Tube B 24 h after the extraction of Tube A Both Tube A and Tube B were to be stored at 4 °C prior to RNA extraction. The two extracted RNA samples (RNA A and RNA B) were analyzed spectrophotometrically by the participating laboratory for concentration and purity, and both purified RNA samples were returned to the SPIDIA facility.

2.4. Data reporting from participants

The participants recorded in the on-line Results form (Supplements 4 and 5) detailed information about the procedure used during the RNA extraction phase: day of sample arrival, temperature and time of blood sample storage, RNA extraction protocol, spectrophotometric evaluation, and temperature and time of storage of the extracted RNA prior to shipping.

2.5. Extracted RNA shipment and storage conditions

After RNA extraction the participants sent the two RNA samples on dry ice back to the SPIDIA facility where the extracted RNA samples were stored at −80 °C until analysis.

2.6. RNA quality parameters

The RNA quality parameters tested in this study included UV spectrophotometric analysis of RNA purity and yield as determined by the participants as well as the same measurements performed by the SPIDIA facility plus the RIN score (RNA Integrity Number, a software tool designed to estimate the integrity of total RNA sample by the evaluation of the entire electrophoretic trace by Agilent Bioanalyzer 2100, Agilent Technologies) for an overall evaluation of RNA integrity, measurement in RT-qPCR of the expression of c-fos, IL-1β, IL-8 and GAPDH transcripts, and kinetics analysis of the RT-qPCR data for the detection of the presence of interfering substances. Details on the reagents and methods used for these analyses are reported elsewhere [3].

2.7. Statistical analysis and results interpretation

In the absence of known reference values for each of the factors investigated, we measured the consistency of a given participant's results against all values excluding outliers (90%) of the results provided by the other participants as described in Orlando et al. [9]. Briefly, a two-step statistical procedure based on a distribution-free approach was adopted in order to process the data corresponding to each of the following variables: RNA purity, RNA yield, RIN, and concentration of transcripts of c-fos, IL-