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OPTIMIZATION AND COMPARATIVE EVALUATION OF NUCLEIC ACIDS EXTRACTION PROTOCOLS

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Abstract: Modern molecular applications have grown the need of biobanks, which contain DNA and RNA of high purety, quality, and quantity. Nucleic acid extraction methods have widely variate and evolve in time, from methods using toxic reagents to enzymatic protocols, and furthermore to DNA or RNA-binding polymers, separating membranes or highly eulogized commercially kits. In order to establish a constant, reproducible and ergonomic system in generating biobanks, we compared different available methods for the extraction of genomic DNA and total RNA, from peripheram blood or solid tumoral tissues. We evaluated the cost/effectiveness and time consumption of each method, tracking RNA/DNA quantity, quality and integrity. We imagined a “E-ratio” value to define these parameters, and a “NA-estimation” to integrate “E-ratio” with quality and integrity data.

INTRODUCTION

The use of nucleic acids in molecular biology has become increasingly important, covering applications as divers as genetic diagnosis, detection of human pathogens, gene expression, microarray applications, GWAS (genome-wide association studies) or NGS (Next-generation or exome-wide sequencing). Both DNA and RNA gain importance everyday in terms of usefulness for molecular advanced applications. More and more sources of nucleic acids become available, and the need of purification methods adapted to such sources becomes obvious. The purification of nucleic acids from difficult specimens like serum, urine, or tissues, however, has been laborious and time-consuming. Moreover, the many steps involved in the purification of nucleic acids from such specimens by classical procedures (involving detergent-mediated lysis, proteinase treatment, extractions with organic solvents, and ethanol precipitation) increase the risk of transmission of nucleic acids from sample to sample.

When the extremely sensitive polymerase chain reaction (PCR) or the transcription-based amplification system is used for the detection of a few nucleic acid molecules (which may be the case in quantifying low-expressed genes mRNA, or a pathogen DNA/RNA, the transmission of nucleic acids might easily lead to false-positive results. The binding of DNA to silica or glass particles is well known but has not, to our knowledge, resulted in methods for nucleic acids purification directly from clinical specimens like human serum or urine. GuSCN (guanidinium thiocyanate) has been shown to be a powerful agent in the purification and detection of both DNA and RNA because of its potential to lyse cells combined with its potential to inactivate nucleases.

In genetic linkage studies using the restriction fragment length polymorphism (RFLP) technique, it is essential to process effectively large numbers of blood samples. One of the problems faced when extracting DNA by standard methods is the requirement of deproteinizing cell digests with hazardous organic solvents like phenol, chloroform and isoamyl alcohol. Other methods avoid the use of any organic solvents. This is achieved by salting out the cellular proteins by dehydration and precipitation with a saturated sodium chloride solution. Most of the procedures also involve prolonged incubation with proteinase K. One of the obstacles encountered when extracting DNA or RNA from a large number of samples is the cumbersome method of deproteinizing cell digests with the hazardous organic solvents phenol and isochloroform. Several other non-toxic extraction procedures have been published, but require either extensive dialysis or the use of filters. Other methods involve salting out of the cellular proteins by dehydration and precipitation with a saturated NaCl solution.

Considering the growing diversity of applications performed by a modern molecular laboratory, we seriously considered the importance of evaluating different extraction procedures, with the aim of identifying the best way today to complete nucleic acid biobanks. A good biobank should contain enough pure gDNA, cDNA, mRNA and total RNA from as much samples as possible, patients, relatives or controls. A biobank must respect all requirements regarding quality/quantity of samples, tractability, anonymity and ergonomics. Therefore, the methods of nucleic acid extraction used in generation a biobank must be the most economical, safe and rapid, adapted to each source available.

In this paper, we describe a comparative evaluation of several commercially available methods for the extraction of gDNA and total RNA, considering the cases of peripheral blood and solid (tumoral) tissues. Three methods were compared for each source, quality and quantity of issued nucleic acids were compared, as well as the cost/effectiveness/time consuming aspects.

MATERIAL AND METHODS

DNA and RNA were extracted independently from peripheral blood and/or tumoral tissue. Samples were obtained from patients with breast/ovarian cancer, from relatives, and from healthy controls. All patients agreed by written informed consent. We gathered data from 200 patients/controls. For each source of nucleic acids, three different methods were compared in terms of cost/effectiveness and time consumption.

For peripheral blood, whenever possible, 12 ml were collected on anticoagulant tubes (heparin, EDTA or sodium citrate). DNA extraction was performed in parallel on 2 x 5 ml sample, while RNA extraction needed 2 x 1 ml. When such blood volumes were not available, lesser volumes were considered for extraction, with a minimum of 200 µl peripheral blood to extract DNA, and 100 µl for RNA. Nucleic acid extractions were performed the same day with blood collection, or blood samples were kept at 4°C for a maximum of 48 hours.

For extracting DNA from 1-10 ml peripheral blood, we compared the Wizard® Genomic DNA Purification Kit, Promega™, and the NucleoSpin® Blood XL, Macherey-Nagel™. For extracting DNA from lesser volumes of peripheral blood (< 1ml), we compared three different kits: Wizard® Genomic DNA Purification Kit, Promega™, QIAamp® DNA Blood Mini Kit, Qiagen™, and QuickGene® DNA whole blood kit, Fujifilm™. Depending on producer's instruction, extracted DNA was resuspended in nuclease-free water, or TE 1x buffer. DNA was stored at 4°C for immediate evaluation, or at -20°C in the biobank.

For extracting total RNA from less than 1 ml peripheral blood, we compared three different methods: SV® Total RNA Isolation System, Promega™, Genelute® Mammalian Total RNA Miniprep Kit, Sigma-Aldrich™, and TRIZOL® Reagent, Invitrogen™. RNA was resuspended in nuclease-free water, immediately evaluated for quality and quantity, and stored at -80°C in the biobank. Alternatively, RNA may be retrotranscribed immediately into cDNA, which can be stored for long term at -20°C.

For tumoral tissues, whenever possible, 300 mg were collected in RNAlater® solution, Sigma-Aldrich™. Solid tissues were frozen at -20°C before RNA or DNA extraction. Tissue harvesting was performed manually to avoid contaminations. 2 samples of 50 mg were used for DNA extraction, while 2 x 25 mg were sufficient for RNA.

In order to extract genomic DNA from fresh or frozen tumoral tissue, we compared the following kits: Wizard® Genomic DNA Purification Kit, Promega™, QIAamp® DNA Blood Mini Kit, Qiagen™ and QuickGene® DNA whole blood kit, Fujifilm™. Depending on producer's instruction, extracted DNA was resuspended in nuclease-free water, or TE 1x buffer. DNA was stored at 4°C for immediate evaluation, or at -20°C in the biobank.

RNA extraction for solid tissues was performed by comparing three different kits: SV® Total RNA Isolation System, Promega™, Genelute® Mammalian Total RNA Miniprep Kit, Sigma-Aldrich™, and TRIZOL® Reagent, Invitrogen™. RNA was resuspended in nuclease-free water, immediately evaluated for quality and quantity, and stored at -80°C in the biobank. Alternatively, RNA may be retrotranscribed immediately into cDNA, which can be stored for long term at -20°C.

All extractions were performed in duplicate, as mentioned above, and results were used for evaluation only if very similar results were obtained from both samples. DNA and RNA quantity was estimated by spectrophotometry, by measuring absorbance at 260 nm, using the DU®800 Spectrophotometer, *Beckman Coulter*™. Absorbance at 260 nm allowed the evaluation of nucleic acids purity, while a correction was performed against the 320 nm value. The quality of extracted nucleic acids was also tested by alternative methods. As the 260 nm absorbance value is only a quantitative indicating parameter, we evaluated the purity and integrity of RNA molecules by electrophoresis. 5 µl extracted RNA were mixed with 2 µl loading dye, and deposed in a 1% agarose gel stained with 0,5 µg/ml ethidium bromide. After a migration of 30 minutes at 5 V/cm, gels were visualized and interpreted using the G:BOX® Chemi system, Syngene™, and the GeneSnap® and GeneTools® software from the same producer. For DNA, an alternative method qPCR-based was used to verify integrity, but data are not shown here.

RESULTS AND DISCUSSION

Independently of the method performed, we considered only those samples whose extraction revealed a pure nucleic acid product, which means a $1{,}8 \leq DO_{260}/DO_{280} \leq 2{,}0$. We must admit that almost all samples obeyed this rule, and all compared protocols showed to be good ones in terms of the purity of extracted materials. We also normalized the results regarding the amount of starting material, calculated the mean time necessary to extract a sample (we considered the 1-sample procedure, as well as the modifications caused by the increasing number of samples), and evaluated the costs of kits, consumables and materials.

Although literature data generally do not recommend use of the heparin as anticoagulant for collecting blood samples, we did not observe any difference between heparin, sodium citrate or EDTA, neither for the effectiveness or DNA or RNA extraction, nor for an eventual inhibition of PCR-based downstream applications, therefore contradicting this hypothesis. Yet, a contribution of EDTA to 260 nm absorbance value seemed to be observed, either EDTA was used as anticoagulant, or component of the TE buffer.

Generally, the alternative methods to evaluate nucleic acids integrity corresponded to spectrophotometric evaluation, which seemed to be the case especially for RNA; however, an important disparity seems to interfere spectrophotometric values for gDNA compared to qPCR data, situation that will be discussed elsewhere.

We considered the extraction protocols indicated by the respective producers, but optimization steps were needed to obtain better results for each method. Although these modifications do not make the subject of the present paper, we strongly recommend optimization steps when implementing and using each of the methods presented below. Different data may be obtained when strictly using the protocols as they are given by the producers.

In all tables below we indicated the peripheral blood volume or solid tissue amount used, the final elution volume for extracted DNA/RNA, as well as the total quantity of DNA/RNA extracted. The efficiency of the method was calculated for 1 ml peripheral blood / 1 mg tissue, related to DNA/RNA yield obtained. By dividing the DNA/RNA yield by the initial volume/amount of sample, we identified each time a 100% efficiency method, the other methods being considered as % of this value. We obtained the effectiveness/cost ratio by dividing the whole effective quantity of DNA obtained to the cost of the extraction per sample, including reagents, consumable and materials. Finally, an "E-ratio" was generated by dividing the effectiveness/cost ratio by the time consumed for each extraction. We estimated by real-time measurement that time increment with the sample number is similar to all compared methods.

In table 1, we present the mean comparative results of gDNA extractions from peripheral blood (10 ml, 5 ml, 1 ml, 300 µl and 200 µl results are considered as well, data having been normalized), by all protocols used and described above. As 100% efficiency, we identified the kit Wizard® Genomic DNA Purification Kit, Promega™ (300 µl protocol). There is an important disparity between the big-volume and the little-volume kits regarding the effectiveness/cost ratio, which clearly indicates the importance of such choice in generating a biobank. Within big-volume kits, the Wizard® kit has less efficiency than the NucleoSpin® one, while effectiveness/cost ratio seems to favor the first mentioned above. This is confirmed by the E-ratio, Wizard® on 10 ml blood being the leader with a 0,11 value. Within little-volume kits, the difference between Wizard® and concurrence is even bigger, QIAmp® and QuickGene® being at a half value of E-ratio. The difference is even greater when considering only the effectiveness/cost values, although efficiency on itself didn't predict any important difference. Nevertheless, we could hardly ignore the purity level, much higher for QIAmp® and QuickGene®, as well as the important difference in time consumption, obviously in favour of the latter mentioned kits. We strongly recommend to carefully interpreting these data, taking always in account lab resources, time, and purity needs for further applications.

Table 1. Efficiency, effectiveness/cost and E-ratio for peripheral blood – extracted DNA

Method for DNA extraction	Producer	Blood volume (µl)	Elution volume (µl)	Extracted DNA (µg)	Efficiency (%)	DNA Purity (DO260/DO280 ratio)	Price/sample (EUR)	Time (hours)	Effectiveness/cost ratio	E - ratio
Wizard® Genomic DNA Purification Kit	Promega™	10000	2000	400	60	1,8 - 2,0	12	3	0,33	**0,11**
NucleoSpin® Blood XL	Macherey-Nagel™	10000	2000	450	68	1,8 - 2,0	15	3,5	0,30	**0,09**
Wizard® Genomic DNA Purification Kit	Promega™	300	100	20	100	1,8 - 2,0	2	2	0,10	**0,05**
QIAamp® DNA Blood Mini Kit	Qiagen™	200	200	8	60	1,95 - 2,0	3	1,5	0,03	**0,02**
QuickGene® DNA whole blood kit	Fujifilm™	200	200	10	75	1,9 - 2,0	2,5	1	0,04	**0,04**

In table 2, we present the mean comparative results of RNA extractions from peripheral blood (300 µl and 200 µl results are considered as well, data having been normalized), by all protocols used and described above. As 100% efficiency, we clearly identified the TRIZOL® Reagent, Invitrogen™, the two other protocols being of half of its value. Effectiveness/cost ratio, time consumption, and at a lesser extent E-ratio, are obviously confirming the fact, and everything seems to indicate "the perfect method". Still, one should always consider the toxicity of the phenol/chloroform compounds of the TRIZOL® system. Moreover, the purity of RNA obtained by this method is not "so perfect", the ratio 260/280 being rarely above 1,8. Therefore, considering TRIZOL® as the default method for RNA extraction from peripheral blood is always a difficult choice, linked to laboratory environment problems, as well as to downstream applications exigency with regard to RNA purity or integrity.

Table 2. Efficiency, effectiveness/cost and E-ratio for peripheral blood – extracted RNA

Method for RNA extraction	Producer	Blood volume (µl)	Elution volume (µl)	Extracted DNA (µg)	Efficiency (%)	DNA Purity (DO260/DO280 ratio)	Price/sample (EUR)	Time (hours)	Effectiveness/cost ratio	E - ratio
SV® Total RNA Isolation System	Promega™	1000	100	0,5	50	1,7 - 1,9	2	2	0,0025	**0,0013**
GenElute® Mammalian Total RNA Miniprep Kit	Sigma-Aldrich™	200	50	1	50	1,8 - 1,9	3	2	0,0033	**0,0017**
TRIzol® Reagent	Invitrogen™	1000	100	2	100	1,6 - 1,7	0,5	1,5	0,0400	**0,0267**

In table 3, we present the mean comparative results of gDNA extractions from solid tissues, by all protocols used and described above. As 100% efficiency, we identified the kit Wizard® Genomic DNA Purification Kit, Promega™ (tissue protocol). There is a confirming element on the effectiveness/cont ratio, while purity is "not so perfect" with Wizard® compared to QIAmp® and QuickGene®. Surprisingly, the QuickGene® automatic procedure clearly beats concurrence regarding our "E-value", time consumption, and has a great duration of protocol, which could indicate a serious candidate for DNA extraction from solid tissues, in view a good biobank.

Table 3. Efficiency, effectiveness/cost and E-ratio for solid tissue – extracted DNA

Method for DNA extraction	Producer	Blood volume (µl)	Elution volume (µl)	Extracted DNA (µg)	Efficiency (%)	DNA Purity (DO260/DO280 ratio)	Price/sample (EUR)	Time (hours)	Effectiveness/cost ratio	E - ratio
Wizard® Genomic DNA Purification Kit	Promega™	20	100	20	100	1,8 - 2,0	2	2	0,100	**0,0500**
QIAamp® DNA Blood Mini Kit	Qiagen™	25	200	20	80	1,95 - 2,0	3	1,5	0,067	**0,0444**
QuickGene® DNA whole blood kit	Fujifilm™	25	200	18	72	1,95 - 2,0	2,5	1	0,072	**0,0720**

In table 4, we present the mean comparative results of RNA extractions from solid tissues, by all protocols used and described above. Foreseeable, 100% efficiency was identified with the TRIZOL® Reagent, Invitrogen™, the two other protocols being of half of its value. Effectiveness/cost ratio, is hugely confirming the fact. Here again, one should always consider the toxicity of the phenol/chloroform compounds of the TRIZOL® system. Moreover, the purity of RNA obtained by this method is poor, the ratio 260/280 being largely above 1,8. There is another point concerning this comparison. The amount extracted from solid tissues seems to be always adequate or at least good enough for downstream applications, which wasn't the case for peripheral blood. Therefore, either QuickGene® or Wizard® can be reasonable non-toxic methods for RNA extraction from tissues. The E-value is, here again, immensely pending for TRIZOL®.

Table 4. Efficiency, effectiveness/cost and E-ratio for solid tissue – extracted RNA

Method for RNA extraction	Producer	Blood volume (µl)	Elution volume (µl)	Extracted DNA (µg)	Efficiency (%)	DNA Purity (DO260/DO280 ratio)	Price/sample (EUR)	Time (hours)	Effectiveness/cost ratio	E - ratio
SV® Total RNA Isolation System	Promega™	25	100	15	65	1,9 - 2,1	2	2	0,0750	0,04
Genelute® Mammalian Total RNA Miniprep Kit	Sigma-Aldrich™	40	50	20	57	1,9 - 2,0	3	2	0,0667	0,03
TRIzol® Reagent	Invitrogen™	75	200	70	100	1,6 - 1,7	0,5	1,5	1,4000	0,93

Quality, purity and quantity of DNA (either gDNA or cDNA) are essential for good efficiency and specificity of PCR or RT-PCT, therefore being parameters to be constantly verified. DO_{260} bring only information on nucleic acids quantity (either DNA or DNA), not on nucleic acids integrity, or about DNA/RNA contamination. Integrity is a very important parameter in PCR applications, especially if we consider fragile genomic regions or long mRNA molecules. Therefore, besides spectrophotometric measurement, one should always consider the evaluation of DNA/RNA quality or integrity. For gDNA, an alternative method qPCR-based was used to verify integrity, but data are not shown here. For RNA, we chose to perform the electrophoretic method for evaluating quality and integrity as well.

In figure 1, we can observe the migration profiles of extracted RNA by different methods, from solid (tumoral) tissues samples. Generally, RNA amount is insufficient to be observed in electrophoresis gel when extracted from peripheral blood. One can observe in figure 1 the identification of the 5 kb band (corresponding to the 28s ribosomal RNA), as well as the 2 kb (corresponding to the 18s ribosomal RNA), with an intensity band ratio 28s/18s of about 2, with the lack of gDNA contamination, and the lack of the “smear” which could indicate degrades RNA. We can conclude that highly quality RNA was obtained by all methods used, of enough quantity and good integrity. If comparing the methods, the TRIZOL® system (lanes 2, 4, 6 and 8) generates superior RNA yields, which also amplify the observed smear. The Wizard® kit (lanes 1 and 3) generates superior RNA quantities than the Genelute® kit, although no difference can be observed on the quality of extracted RNA between the two methods. The mRNA population is visible mostly with the TRIZOL® method.

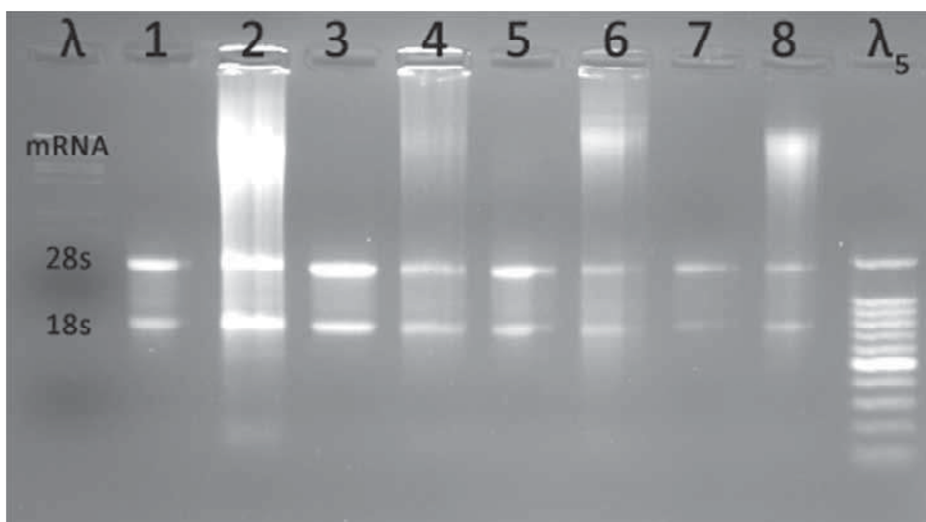

Figure 1. Electrophoretic profiles for RNA extracts from solid tissues, by different methods (λ= step ladder; 1: Wizard® kit, normal tissue; 2: TRIZOL® method, normal tissue; 3: Wizard® kit, tumoral tissue; 4: TRIZOL® method, tumoral tissue; 5: Genelute® kit, normal tissue; 6: TRIZOL® method, tumoral tissue; 7: Genelute® kit, tumoral tissue; 8: TRIZOL® method, tumoral tissue; $λ_5$ = 5 kb step ladder)

CONCLUSIONS

What method to choose? Which one is the best for each source? We did not intend to answer this kind of question. Our study highlights some particular aspects of nucleic acid extractions, regarding the amount of obtained DNA/RNA compared to the initial source, the price, the time consumption, the quality and purity of the nucleic acids. We imagined the E-ratio to define both cost-effectiveness and time consumption. An additional value, that we call NA-estimation, should bring further information on E-ratio with respect to nucleic acids integrity. We strongly recommend to carefully interpreting these data, taking always in account lab resources and environment, time, and purity needs for further applications.

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