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Effective Mixed-Type Tissue Crusher and Simultaneous Isolation of RNA, DNA, and Protein from Solid Tissues Using a TRIzol-Based Method

Kelly Karoline dos Santos [1], Isabelle Watanabe Daniel [1], Letícia Carani Delabio [1], Manoella Abrão da Costa [1], Júlia de Paula Dutra [1], Bruna Estelita Ruginsk [1], Jeanine Marie Nardin [2], Louryana Padilha Campos [3], Fabiane Gomes de Moraes Rego [3], Geraldo Picheth [3], Glaucio Valdameri [1] and Vivian Rotuno Moure [1,*]

[1] Graduate Program in Pharmaceutical Sciences, Laboratory of Cancer Drug Resistance, Federal University of Parana, Curitiba CEP 80210-170, PR, Brazil; gvaldameri@ufpr.br (G.V.)
[2] School of Medicine and Life Sciences, Pontifical Catholic University of Parana, Curitiba CEP 80215-901, PR, Brazil
[3] Graduate Program in Pharmaceutical Sciences, Federal University of Parana, Curitiba CEP 80210-170, PR, Brazil
* Correspondence: vivian.moure@ufpr.br

Abstract: One of the major challenges of studying biomarkers in tumor samples is the low quantity and quality of isolated RNA, DNA, and proteins. Additionally, the extraction methods ideally should obtain macromolecules from the same tumor biopsy, allowing better-integrated data interpretation. In this work, an in-house, low-cost, mixed-type tissue crusher combining blade and beating principles was made and the simultaneous isolation of macromolecules from human cells and tissues was achieved using TRIzol. RT-qPCR, genotyping, SDS-PAGE, and Western blot analysis were used to validate the approach. For tissue samples, RNA, DNA, and proteins resulted in an average yield of 677 ng/mg, 225 ng/mg, and 1.4 µg/mg, respectively. The same approach was validated using cell lines. The isolated macromolecule validation included the detection of mRNA levels of ATP-binding cassette (ABC) transporters through RT-qPCR, genotyping of *TNFR1* (rs767455), and protein visualization through SDS-PAGE following Coomassie blue staining and Western blot. This work contributed to filling a gap in knowledge about TRIzol efficiency for the simultaneous extraction of RNA, DNA, and proteins from a single human tissue sample. A low-cost, high yield, and quality method was validated using target biomarkers of multidrug resistance mechanisms. This approach might be advantageous for future biomarker studies using different tissue specimens.

Keywords: cancer; human tissues; TRIzol; total RNA; DNA; proteins; MDR; ABC transporters

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1. Introduction

Cancer is the main public health problem in the world and is a leading cause of death worldwide. Cancer is among the four main causes of premature death (before 70 years of age) in most countries, accounting for nearly 10 million deaths in 2020 [1]. Considering that most of the mortality in cancer is due to failure or lack of pharmacological intervention [2], it is mandatory to understand the biological alterations involved in disease development to identify new biomarkers, predict clinical outcomes, create personalized pharmacological therapies for patients, and investigate multidrug resistance (MDR) mechanisms [3–5]. Among the main biomarkers of MDR in cancer are the ATP-binding cassette (ABC) transporters, which are able to export chemotherapeutics drugs from cancer cells [6–8]. The ABC

family comprises 48 members in humans, which are divided into seven subfamilies, named ABCA to ABCG [9]. Among them, ABCB1/P-glycoprotein, ABCC1/MRP1 (multidrug resistance protein 1), and ABCG2/BCRP (breast cancer resistance protein) are the pivotal players in MDR [10,11]. Other relevant MDR causes involve anti-apoptotic mechanisms, since several chemotherapeutic drugs induce cell death through apoptosis [12–15]. Tumor necrosis factor (TNF) is involved in the extrinsic pathway of apoptosis through two membrane receptors, TNFR1 and TNFR2. TNFR1 (TNF receptor member 1, encoded by the *TNFR1* gene) has been involved in resistance to tyrosine kinase inhibitors (TKIs) in cancer cells [16–18]. Furthermore, recent findings demonstrated single-nucleotide polymorphisms (SNPs) in *TNFR1* as potential biomarkers in cancer [19]. Thus, investigation of ABC transporters and TNFR1 might uncover new insights into the coupling of these biomarkers and MDR in cancer [20–22].

Recent advances in molecular diagnostics, including gene and protein expression profiles, epigenetic profiling, DNA sequencing, and tumor genotyping, might facilitate the improvement of treatment efficacy and, consequently, an increase in patient survival [23]. These techniques require a good quality and quantity of RNA, DNA, and protein macromolecules. However, for the use of human tissues, other requirements must be considered, such as sample collection with full consent of the patient and in conformity with the ethical protocol, limited amount of sample, limited size of the cohort, and efficiency of tissue disrupting [20–22]. Furthermore, it remains difficult to establish a standardized method that allows the recovery of both nucleic acid and proteins to better correlate the clinical response with anticancer drugs [24,25]. There are several examples in the literature that isolate macromolecules following the order RNA, DNA, and protein from clinical samples using organic solvents or commercial kits [26]. Isolation of RNA and DNA mostly uses the commercial kits available, which offer the several advantages of speed and high yield and quality, but with the main disadvantage of high cost [27].

The organic extraction of RNA is mainly based on phenol–guanidine isothiocyanate. This reagent is commercialized by different companies, named TRIzol (Invitrogen, Carlsbad, CA, USA), TRI reagent (Sigma-Aldrich, St Louis, MO, USA), QIAzol (Qiagen, Hilden, Germany), TRIsure (Bioline, London, UK), RNA-Bee (Tel-Test, Friendswood, TX, USA), RNAzol (Molecular Research Center, Cincinnati, OH, USA), and PureZol (Bio-Rad, Hercules, CA, USA). To facilitate understanding, from here on, the solution based on phenol–guanidine isothiocyanate will be called TRIzol. The monophasic solution containing guanidine thiocyanate and phenol was initially used for RNA extraction [28], and later for RNA, DNA, and protein [29]. When chloroform is added to this mixture and the material is subjected to centrifugation, these solvents are separated into three liquid phases: an upper and aqueous phase (containing the RNA), a milky phase or interphase (containing the DNA), and an organic and lower phase (containing proteins dissolved in phenol and lipids dissolved in chloroform). Thus, RNA, DNA, and protein can be purified from a single sample, hence the prefix "tri" in the TRIzol name. Although TRIzol is commonly used for nucleic acid preparations, the literature is scarce regarding its use for protein isolation from human tissues [30–38].

Proteins are the most complex molecules to be isolated in a sample. Firstly, although the intracellular protein concentration is about 300 mg/mL [39], and human cultured cells supposedly express approximately 10,000 proteins [40], depending on the protein of interest its detection might be quite challenging. Secondly, proteins cannot be amplified, and, thirdly, they present diverse physic-chemical properties, contrary to the negatively charged nucleic acids that can be easily isolated and purified using silica columns. Thus, protein recovery remains the limiting step in both cellular and tissue samples to downstream expression analysis and it is based on manual procedures [41]. The main limitation of protein

recovery using TRIzol-based extraction is protein pellet solubilization after isopropanol precipitation [34,36,42–44]. Thus, simultaneous isolation of total RNA, DNA, and proteins from a single small biopsy specimen can be useful for integrated omics studies.

Cells in suspension, characterized by the absence of structurally complex extracellular matrices, such as cell lines and blood cells, can be efficiently lysed by vortexing samples with TRIzol [45,46]. However, the effective disruption of many tissues remains challenging due to the inherent structural and rheological complexities of multicellular matrices, which include heterogeneity in tissue stiffness, elasticity, and the composition of the extracellular matrix [47]. These matrices are constituted by a heterogeneous assemblage of biomolecules such as collagen, elastin, and proteoglycans, with their composition exhibiting significant variability across different tissue types [48].

Mechanical disruption methods are frequently utilized to overcome the challenges posed by tissue complexity [49]. Manual techniques, including needle–syringe systems that generate shear forces and grinding with a pestle and mortar, represent cost-effective strategies [50]. However, their efficacy is highly dependent on operator skill and technique, often resulting in considerable variability in the results [51]. In addition, these methods may not consistently provide uniform disruption, particularly in tissues with a heterogeneous composition or high mechanical resistance [51]. In contrast, automated systems, including bead-based homogenizers and blade-driven tissue grinders, offer more controlled and reproducible results [52]. These systems apply precise shear forces, pressure, or impact energy, ensuring consistent disruption and minimizing operator-dependent variability [50,53]. The main drawback of machine-based approaches is the high cost of the equipment [50]. Thus, the choice of method is contingent upon the tissue characteristics, intended downstream applications, cost, and the need to preserve the macromolecules' integrity [51].

Considering all these aspects, in this work we made an in-house, low-cost, mixed-type tissue crusher and revisited the extraction of macromolecules by TRIzol reagent in the low-cost and high-yield recovery of RNA, DNA, and proteins from a single sample of both human tissues and a cell line culture to further evaluate MDR biomarkers as a proof of concept. An integrative review was thus performed to obtain more information on the use of the TRIzol-based method in human cancer tissues. The analysis emphasizes the need for more validation studies in the field and argues for the integration of data of the examination of the genome, transcriptome, and proteome from a single biological sample.

2. Materials and Methods

2.1. Cell Culture

H460 cells and stably transfected HEK293-*ABCG2* were kindly provided by Dr. Attilio Di Pietro (IBCP, Lyon, France). The human fibroblast HEK293 cell line was transfected with expression vector (pcDNA3-*ABCG2*) coding for human ABCG2 (HEK293-*ABCG2*), as previously described [54]. The cells were maintained in high-glucose Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 2.50 µg/mL amphotericin B (Gibco, Grand Island, NY, USA), and 0.75 mg/mL G418 (Sigma-Aldrich, St Louis, MO, USA) at 37 °C and 5% CO_2 under controlled humidity in an incubator (ESCO, Singapore). Cells from culture flasks of 25 or 75 cm^2 were used, containing approximately 3×10^6 cells and 1×10^7 cells, respectively.

2.2. Tissue Sampling

All tissue samples were collected during surgery or needle core biopsy, and then immediately snap-frozen in liquid nitrogen in labeled cryovials. Frozen samples were stored at −80 °C. After the tissue disruption method standardization, 51 tissues samples were used for macromolecule extraction, including healthy breast (n = 16), breast cancer

(n = 22), stomach cancer (n = 2), healthy colon (n = 2), colon cancer (n = 4), and rectal cancer (n = 5), with an approximate volume of 0.5 cm^3 and 100 mg of weight [55].

2.3. Tissue Disruption

The tissues were disrupted using rotor/bead beating-based homogenization. The tissue samples were put into a 15 mL tube containing two sterilized inox steel beads (7 mm of diameter) and 1–2 mL of TRIzol. Homogenization was performed using a rotation of 4500 rpm up to 1 min.

2.4. Sample Processing

For samples obtained from cell lines, 1 mL of TRIzol was directly added to the cell pellet (2 mL tube), and the mixture was homogenized in a vortex (~30 s) and then incubated on ice for 5 min. For tissues samples, after disruption (Section 2.3), the supernatant was transferred to a 2 mL tube and incubated on ice for 5 min. For both samples, the tube was centrifuged for 5 min at 12,000 rpm at 4 °C to eliminate cellular debris. The supernatant was transferred to a new 2 mL tube, vortexed, and then incubated on ice for 5 min. After, 200 µL of chloroform was added, the tube was vortexed for 15 s, and then incubated for 3 min on ice. The samples were then centrifuged for 15 min at 12,000 rpm at 4 °C, allowing the separation into a lower red organic phase containing proteins, an interphase containing the DNA, and a colorless upper aqueous phase containing the total RNA. Each phase was aliquoted into different 2 mL tubes. Both the organic and interphase phases were stored in a freezer at −80 °C for 4–6 months until processing. RNA extraction immediately proceeded from the aqueous phase.

2.5. RNA Extraction

To the 2 mL tube containing the aqueous phase, 500 µL of isopropanol (for each 1 mL of TRIzol) was added and the solution was mixed by gentle inversion (10 times). The sample was centrifuged for 10 min at 12,000 rpm at 4 °C and then the supernatant was discarded. The pellet was washed with 1 mL of 75% ethanol, dried, resuspended in 40 µL of RNase-free water, and then kept at −80 °C until use. All plastics and reagents were RNase-free.

2.6. RNA Quantification and Integrity

Total RNA concentration was quantified by absorbance at 260 nm and the absorbance ratios 260/280 nm (A260/280) and 260/230 nm (A260/230) were obtained using a NanoDrop™ Spectophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was evaluated through 1% agarose gel electrophoresis. Gels were stained with sodium bromide ethidium (0.5 µg/mL) and visualized in a transilluminator under UV light (302 nm). Gel images were obtained using a L-PIX photodocumentation system (Loccus biotechnology, Sao Paulo, Brazil).

2.7. Reverse Transcription

cDNA was produced from 2 µg of total RNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instruction in a thermocycler (Applied Biosystems, Foster City, CA, USA), as follows: the volume reaction (20 µL) was incubated at 25 °C for 10 min, after at 37 °C for 120 min, and then successively warmed up to 85 °C for 5 min. The resulting cDNA was diluted 5-fold in RNAse- and DNAse-free ultrapure distilled water (Invitrogen, Carlsbad, CA, USA) and then stored at −20 °C until use.

2.8. Primer Design

Primers listed in Table 1 were purchased from Sigma-Aldrich (St Louis, MO, USA). The specificity of amplicons and primers pairs were analyzed by BLAST (National Center for Biotechnology Information) alignment tools. In this study, 3 different reference genes (*YWHAZ*, *RPS13*, and *HPRT1*) and 8 genes encoding ABC proteins were used. All primers were used at a final concentration of 1 µM.

Table 1. Sequence of gene-specific primers used in RT-qPCR analysis.

Gene	ID NCBI	Forward and Reverse Primer (5′–3′)	Length (pb)	Reference
ABCA1	NM_005502.4	F-GGGCATTTCTAGTTATGGCATCT R-GCAAGGTACCATCTGAGGTCT	104	This work
ABCB1	NM_000927.4	F-CCAGAAACAACGCATTGCCATA R-TTGGACAACCTTTTCACTTTCTGTA	109	This work
ABCC1	NM_004996.4	F-CTGGACTGATGACCCCATCG R-ACGGCGATCCCTTGTGAAAT	96	This work
ABCC2	NM_000392.5	F-ATGCACTCAATATCACACAAACCC R-CAGTTATTCGCTCAACAGCCA	90	This work
ABCC10	NM_033450.2	F-ACAGATGCCAAGATCCTGTGT R-CTGTCTTGTTGGCAAAGCGT	106	This work
ABCE1	NM_001040876.1	F-TGGTGATGCTGGGGGAAAAT R-AGAACTGGTACTTCTCCTCCTTC	97	This work
ABCF2	NM_007189.3	F-GGGCGTTACCATCAGCATTT R-TCTCCTTGATCTCTGGGTAGCA	91	This work
ABCG2	NM_001257386.2	F-ATGGTCTGTTGGTCAATCTCAC R-TTATGCTGCAAAGCCGTAAATCC	97	[56]
YWHAZ *	NM_001135702.1	F-GGGGGTGGTTGCGATACG R-GACTGGATGTTCTGACTTGAGAC	103	This work
RPS13 *	NM_001017.3	F-CGTCCCCACTTGGTTGAAGT R-TGAATCTCTCAGGATTACACCGA	109	[56]
HPRT1 *	NM_000194.3	F-CAGGGATTTGAATCATGTTTGTGT R-ACTCCAGATGTTTCCAAACTCAAC	98	[56]

* Genes used as reference in previous studies [56–58].

2.9. Quantitative Real-Time PCR

Quantitative real-time PCR (RT-qPCR) was performed with a 7500 Fast™ Real-Time PCR (Life Technologies/Applied Biosystems, Foster City, CA, USA) in 96 wells, using a final volume reaction of 6 µL, containing 2 µL of primer (forward and reverse primer), 3 µL of SYBR green master mix, and 1 µL of cDNA solution (1:5). The following protocol was applied: 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 1 cycle at 60 °C for 60 s. A melt curve was obtained after the amplification cycles. The temperature was increased by 0.5 °C per cycle, starting at 60 °C. A total of 8 tissues samples (4 breast cancer, 3 healthy breast, and 1 stomach cancer) were analyzed.

2.10. DNA Extraction and Quantification

Genomic DNA was extracted using the modified salting out method, with protein dehydration and precipitation in an environment saturated with sodium chloride [59]. An amount of 10 µL of 20% SDS and 120 µL of ultrapure water were added to the intermediary phase (interphase) containing DNA from cell or tissue samples. After homogenization with a vortex for 5 s, the samples were placed in a water bath at 65 °C for 40 min. After cooling the solution, 100 µL of 5 M NaCl was added following centrifugation for 10 min at 14,500 rpm at room temperature. The supernatant was transferred to a new tube, 700 µL of absolute ethanol was added, and then the solution was homogenized by inversion 10 times and centrifuged for 3 min at 14,500 rpm at room temperature. The pellet was washed with

700 µL of 70% ethanol, dried, and then reconstituted with 100 µL of sterile ultrapure water. The DNA-containing solution was heated for 1h at 65 °C and then stored at −20 °C for further analysis. The extracted DNA was quantified using a spectrophotometer at 260 nm (S60, Biochrom, Cambridge, UK). DNA was extracted from 51 tissue samples. As a control, DNA was also obtained from healthy human blood cells.

2.11. Genotyping

The genotyping of the tumor necrosis factor receptor 1 gene (TNFR1) rs767455 was performed using fluorescent probes TaqMan® (Taqman code: C_2298465_20) in the 7500 Fast™ Real-Time PCR (Life Technologies/Applied Biosystems, Foster City, CA, USA). Briefly, the reaction mixture (final volume of 8 µL) contained 3.0 µL TaqMan Genotyping Master Mix, 1.9 µL ultrapure water, 0.1 µL of fluorescent probe, and 3.0 µL DNA (20 ng/µL), which was added to each well of a 96-well plate. The reaction conditions were recommended by the manufacturer: 10 min at 95 °C (1 cycle), followed by 40 cycles of 15 s at 95 °C and 90 s at 60 °C. The genotyping quality was >99% for all samples measured by the 7500 Fast SDS system software v1.5.1. This analysis was performed for 3 breast cancer and 2 healthy breast tissues.

2.12. Protein Extraction

Protein was extracted using the phenolic phase after adding TRIzol to the tissue or cell pellet, according to Simões et al. (2013) [36]. Briefly, from the phenolic phase of sample processing, 300 µL of 100% ethanol was added and the solution was mixed by inversion. After centrifugation at 2000 rpm for 5 min at 4 °C, the supernatant was transferred to a new tube and 1.5 mL of ice-cold isopropanol was added. After mixing by inversion and incubation for 20 min at room temperature, the solution was centrifuged at 14,000 rpm for 15 min at 4 °C. The supernatant was discarded and 1 mL of solution containing 0.3 M guanidine hydrochloride in 95% ethanol was added to the pellet, following incubation for 20 min at room temperature and centrifugation at 7500 rpm for 5 min at 4 °C. The pellet was washed twice with 95% ethanol. The supernatant was discarded, and 2 mL of 100% ethanol was added to the pellet following incubation for 20 min at room temperature and centrifugation at 7500 rpm for 5 min at 4 °C. The supernatant was discarded, and the pellet was completely dried. Finally, the pellet was resuspended with 300 µL of 1% SDS and 300 µL of 8 M urea in Tris–HCl and further solubilized under sonication in a manual sonicator model FB120 (Fisher Scientific, Thermo Fisher Scientific, Waltham, MA, USA) using 5 cycles of 15 s, alternating with 30 s of rest on ice, with an amplitude of 20–40% and pulse of 10–20%. Proteins were extracted from 51 tissue samples.

2.13. Protein Analysis

Protein quantification using the Pierce bicinchoninic acid (BCA) Protein Assay Kit was performed according to the manufacturer's instructions, with incubation at 37 °C for 30 min and measurement of the absorbance at 595 nm. Protein electrophoresis was carried out by 12% SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) and gels were Coomassie Blue R250-stained.

2.14. Western Blot

Protein was obtained from HEK293-*ABCG2* cells from 25 cm^2 tissue flasks (at approximately 90% confluence). Proteins were separated by 8% SDS-PAGE and then transferred to a PVDF membrane using a semi-dry transference system (GE Healthcare). The membrane was incubated overnight at 4 °C with ABCG2 antibody BXP-21 (Abcam, Boston, MA, USA) diluted 1:500 and with GAPDH antibody (ABclonal, Woburn, MA, USA), diluted 1:5000 in Tris-buffered saline with 0.1% Tween 20 detergent (TBST) containing 1% powdered milk.

The membranes were washed in TBST, incubated with anti-mouse IgG HRP antibody, and then diluted 1:2000 in TBST containing 1% powdered milk at room temperature for 1 h. After, the membranes were washed with TBST and ECL Plus kit substrate (GE Healthcare, Chicago, IL, USA) was added. Protein bands were visualized through exposing the membranes in an Amersham Hyperfilm (GE Healthcare, Chicago, IL, USA) for 10 s.

2.15. Integrative Review

In this work, we asked the following questions: What are the human cancer tissues used for TRIzol extraction? What is the procedure to collect tumor samples? What are the macromolecules extracted from human tissues using TRIzol? What is the yield of RNA, DNA, and protein extracted using TRIzol? Based on these questions, the PubMed and Web of Science databases were searched until November of 2023 for abstracts matching the following keywords: ((“trizol” OR “guanidinium thiocyanate” OR “TRI Reagent” OR “TRI-Reagent” OR “QIAzol” OR “TRIsure”) AND (“cancer”)) AND (“RNA” OR “DNA” OR protein *) AND (human) AND (tissue) along with manual search.

3. Results

The flow chart in Figure 1 shows how RNA, DNA, and proteins were obtained from a single sample. To analyze the cellular macromolecules, standardization of four steps was needed, which can be adjusted depending on the sample nature and subsequent applications: (1) cell lysis or disruption; (2) isolation or purification; (3) resuspension; and (4) determination of yield and quality.

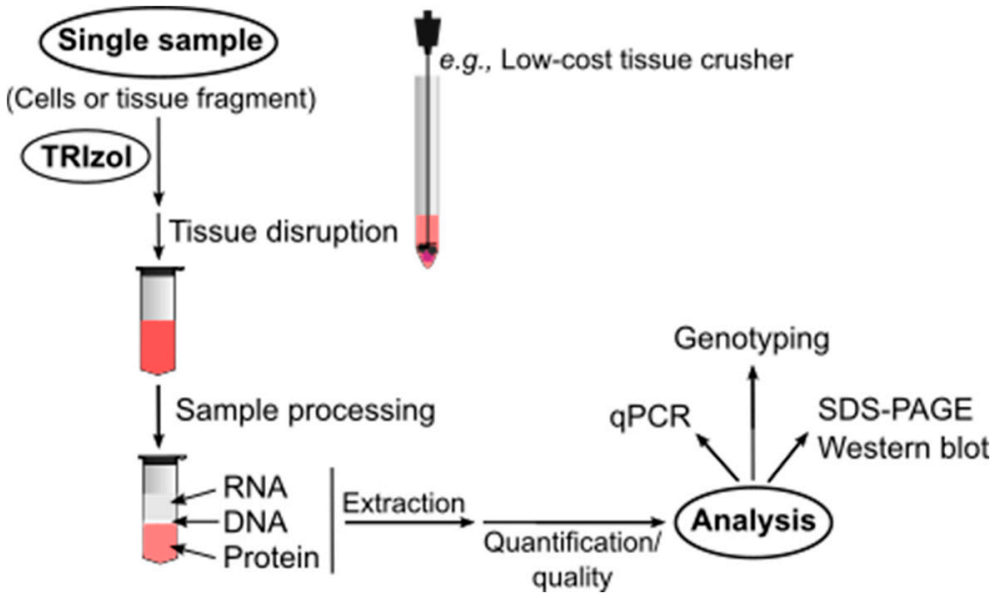


Figure 1. The flow chart illustrates the steps in analyzing RNA, DNA, and proteins obtained from a single sample using a low-cost tissue crusher (in-house prototype) and TRIzol reagent.

Cell or tissue disruption is the workflow’s first step. The disruption of cell lines can be easily achieved by vortexing the samples with TRIzol; however, efficient tissue disruption is challenging, and it is intrinsically associated with the tissue rheological properties [49]. Mechanical procedures are commonly used, including hands-on approaches (e.g., use of needle/syringes and pestle/mortars) or specific equipment, which allows lower variability in the process (e.g., beating-based homogenizer and tissue grinders based on blades).

In this study, different techniques were attempted, including (1) homogenization using a needle/syringe with a back-and-forth motion; (2) disruption through manual crushing

in liquid nitrogen using a mortar and pestle; (3) a rotating blade handheld homogenizer (BK-HG160, Biobase, Jinan, China); and (4) a beating-based homogenizer (PowerLyzer 24, Qiagen, Hilden, Germany). Among these approaches, only 3 and 4 were able to disrupt different tissues from surgical resection, such as the skin, kidney, liver, and breast. However, these pieces of equipment are expensive. To overcome this issue, we developed an in-house prototype combining the concept of the two most promising homogenizers: 3 and 4. After some optimizations, a stainless steel rod was coupled to a drill motor (4500 rpm). Two grooves were made in the bottom of the stainless steel rod to firmly receive two pieces of fishing nylon (0.9 mm) (Figure 2A).

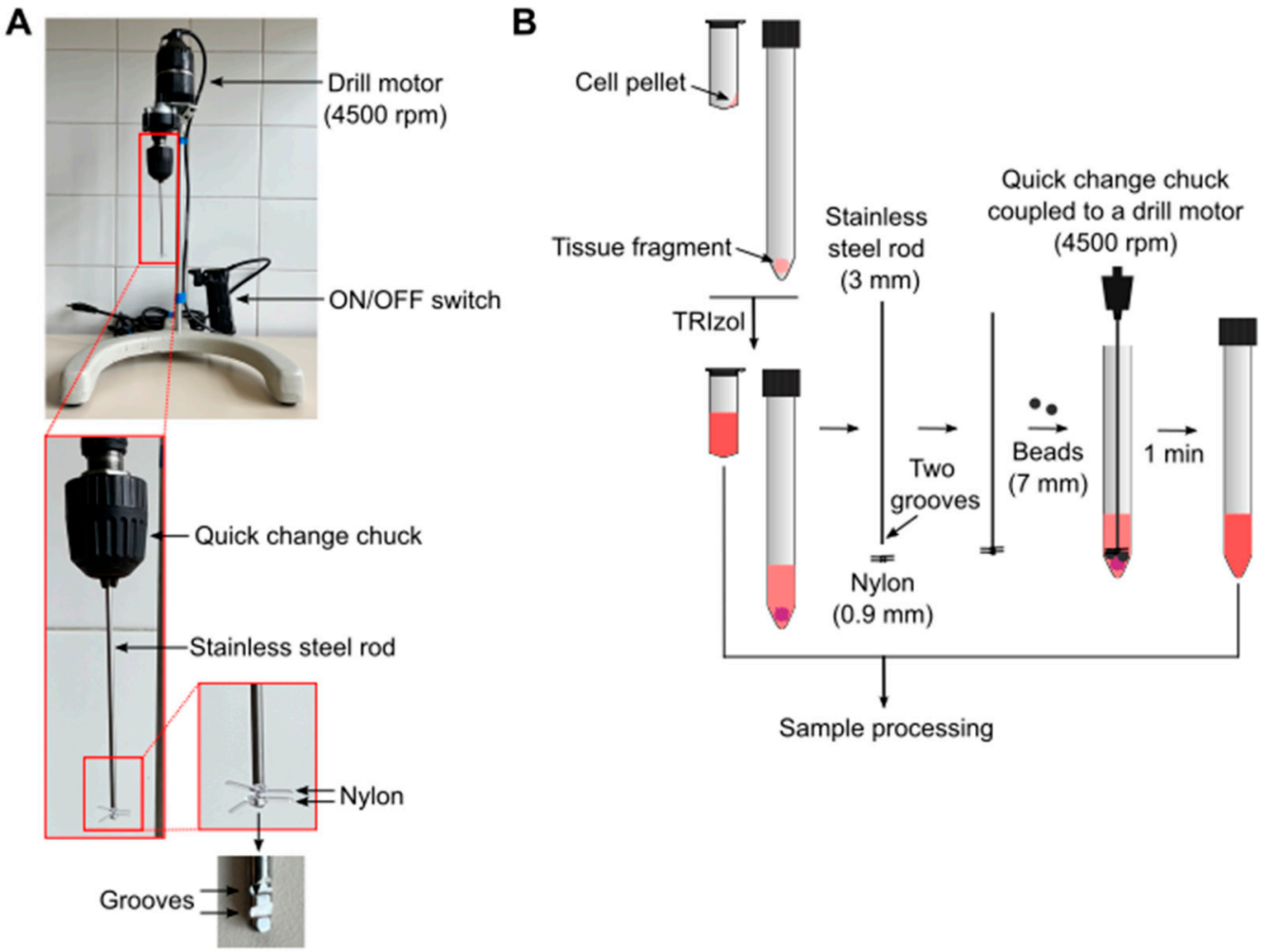


Figure 2. Prototype and overview of TRIzol sample processing. (**A**) Photos of the tissue crusher, denominated as the prototype. Some components and details appear in zoom images. (**B**) Overview of the tissue disruption using the mixed-type tissue crusher combining blade and beating principles and the TRIzol strategy. The illustration shows the coupling of nylon in the grooves of the stainless steel rod, the steel beads, and the coupling of the stainless steel rod in the quick-change chuck from a drill motor.

After cell lysis directly in TRIzol or tissue disruption using the rotor/bead beating system (Figure 2A), chloroform was added, and after centrifugation the solution was separated into three phases: an upper aqueous phase containing the RNA, an interphase containing the DNA, and a lower organic phase containing protein (Figure 2B). The RNA and DNA pellets were resuspended in water, while the protein pellet was resuspended in a 1:1 solution (1% SDS and 8 M urea in Tris-HCl pH 8.0). We validated TRIzol-based

extraction for recovering RNA, DNA, and protein from a unique sample using both human cell lines and tissues, following a schematic cascade (Figure 2B).

The total RNA and DNA yield were determined by absorbance at 260 nm. RNA and DNA quality were determined by absorbance ratios at 260/230 and 260/280 nm. RNA integrity was assessed through agarose gel electrophoresis followed by staining with ethidium bromide. The protein yield was assessed by determination of concentration using a BCA assay.

Table 2 summarizes all the results. The major target of this study was breast tissues; however, this study was extended for other samples, including different human tissues and stably transfected HEK293-*ABCG2* cells. The yield of total RNA depended on the source of tissue type and cell starting number, but it ranged between 0.5 and 4.3 μg/μL (on average for 677 ng/mg of tissue) or 1.3 and 7.7 μg/10^6 cells. The A260/280 ratios of the extracted RNA were generally 1.8–2.0; the A260/230 ratios were approximately 1.0. Although the average value of the A260/230 ratio was low, this might be due to contamination by guanidine thiocyanate, which absorbs at 220–230 nm and is present in the TRIzol reagent [60].

Table 2. Data of RNA, DNA, and proteins extracted from human cells and tissues using TRIzol.

Sample		RNA				DNA			Protein	
		n	**[μg/μL]**	**A260/280**	**A260/230**	***n***	**[ng/μL]**	**A260/280**	***n***	**[μg/μL]**
Cell Line	HEK293-*ABCG2*	4	0.92 ± 0.50	1.94 ± 0.06	1.31 ± 0.40	1	250	1.91	6	0.52 ± 0.24
Tissue	Breast cancer	22	1.08 ± 1.55	1.79 ± 0.41	0.84 ± 0.40	22	273 ± 261	1.55 ± 0.35	22	0.41 ± 0.31
	Healthy breast	16	0.46 ± 0.39	1.90 ± 0.49	0.78 ± 0.39	16	214 ± 136	1.50 ± 0.45	16	0.52 ± 0.38
	Stomach cancer	2	1.56 ± 0.48	1.87 ± 0.20	0.97 ± 0.55	2	251 ± 55	1.80 ± 0.02	2	0.30 ± 0.42
	Rectal cancer	5	1.18 ± 1.05	2.00 ± 0.10	1.33 ± 0.34	5	244 ± 176	1.29 ± 0.44	5	0.41 ± 0.30
	Colon cancer	4	1.54 ± 0.88	1.82 ± 0.14	0.76 ± 0.29	4	207 ± 151	1.71 ± 0.31	4	0.67 ± 0.31
	Healthy colon	2	4.34 ± 5.46	1.95 ± 0.00	1.61 ± 0.08	2	160 ± 71	1.59 ± 0.29	2	0.44 ± 0.54

Note: "*n*" corresponds to the number of samples used in this study. The values are presented as average ± standard deviation (SD).

Considering all samples of both cells and tissues, the DNA yields on average of 228 ng/μL (225 μg/mg of tissue) not carrying contamination with proteins. The protein yield from organic phase of TRIzol/chloroform extraction was 0.5 μg/μL for both cells and tissues (1.4 μg/mg of tissue).

To evaluate the quality of RNA extracted, besides UV spectrophotometry parameters, we used agarose gel electrophoresis analysis. Two distinct bands showing the 28S and 18S ribosomal RNA and no degradation were observed for all cell samples and for the vast majority of tissue samples. Representative results are shown in Figure 3A,D. Finally, the quality of the RNA was further confirmed by RT-qPCR. After reverse transcription, real-time PCR amplification was performed using specific primer pairs to the transcripts from reference genes and genes encoding for ABC transporters (Table 1).

Initially, the transcriptional analysis was carried out using RNA extracted from cells targeting ABCG2, since its exogenous overexpression was achieved by stable transfection of HEK293 cells (Figure 3B). Amplicons of RPS13, HPRT1, and ABCG2 revealed single peaks by melt curve analysis (Figure 3C). For tissue transcripts analysis, eight genes encoding ABC transporters were chosen, including ABCA1, ABCB1, ABCC1, ABCC2, ABCC10, ABCE1, ABCF2, and ABCG2. To facilitate the visualization of the results, an amplification plot and the melting temperature of one reference gene RPS13 and one target gene ABCG2 are represented for three healthy breast patients (Figure 3E,F). Additionally, representative tissue samples of breast and stomach cancer are shown to demonstrate the amplification

plots for the eight target genes encoding ABC transporters (Figure 3G). In accordance, amplicons from all transcripts revealed single peaks by melt curve analysis (Figure 3H).

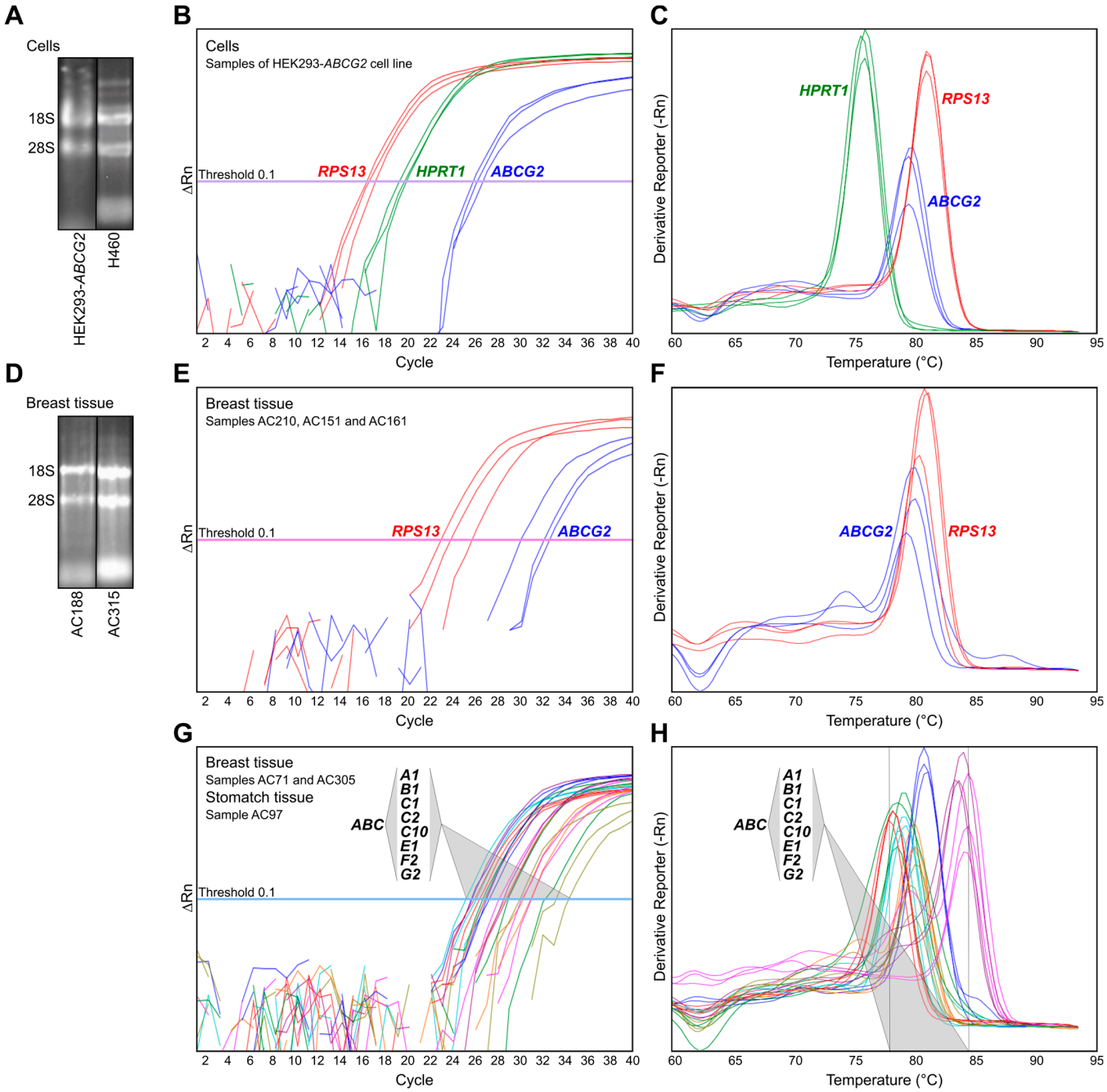


Figure 3. RNA bands, amplification plots, and melting curves. Agarose gel electrophoresis of 28S and 18S ribosomal RNA (rRNA) bands from RNA samples extracted using the TRIzol method for HEK293-ABCG2 and H460 cells (**A**) and fragment of breast tissues (**D**). Amplification plots (relative fluorescence of SYBR green versus cycle number) represent the accumulation of product over the duration of the real-time PCR experiment. The horizontal line indicates the threshold placement. The targets are indicated by color in the figures, in which the same color represents a single gene. Different samples were analyzed, including cells (**B**), breast tissues (**E**), and breast or stomach tissues (**G**). A melting curve (60–95 °C; in increments of 0.5 °C) was generated to verify the specificity of primer amplification. Melting curves from different samples were analyzed, including cells (**C**), breast tissue (**F**), and breast or stomach tissues (**H**).

To assess the use of the extracted DNA, a genotyping analysis was performed. Genotyping of TNFR1 gene variations was performed with specific fluorescent probes using the TaqMan system, consisting of fluorophores (VIC®, FAM™) covalently linked to the 5′ end of the oligonucleotide probe and a quencher at the 3′ end. The results of the genotyping of the breast cancer tissue samples (homozygous CC for the normal allele; heterozygous TC or homozygous TT for variant allele) are presented in the graphic form of allelic discrimination provided by the software analysis (Figure 4). As a control, DNA obtained from human blood cells was used for TNFR1 genotyping. One genomic DNA presented the rare genotype for this gene, while one was heterozygous and three presented the normal allele.

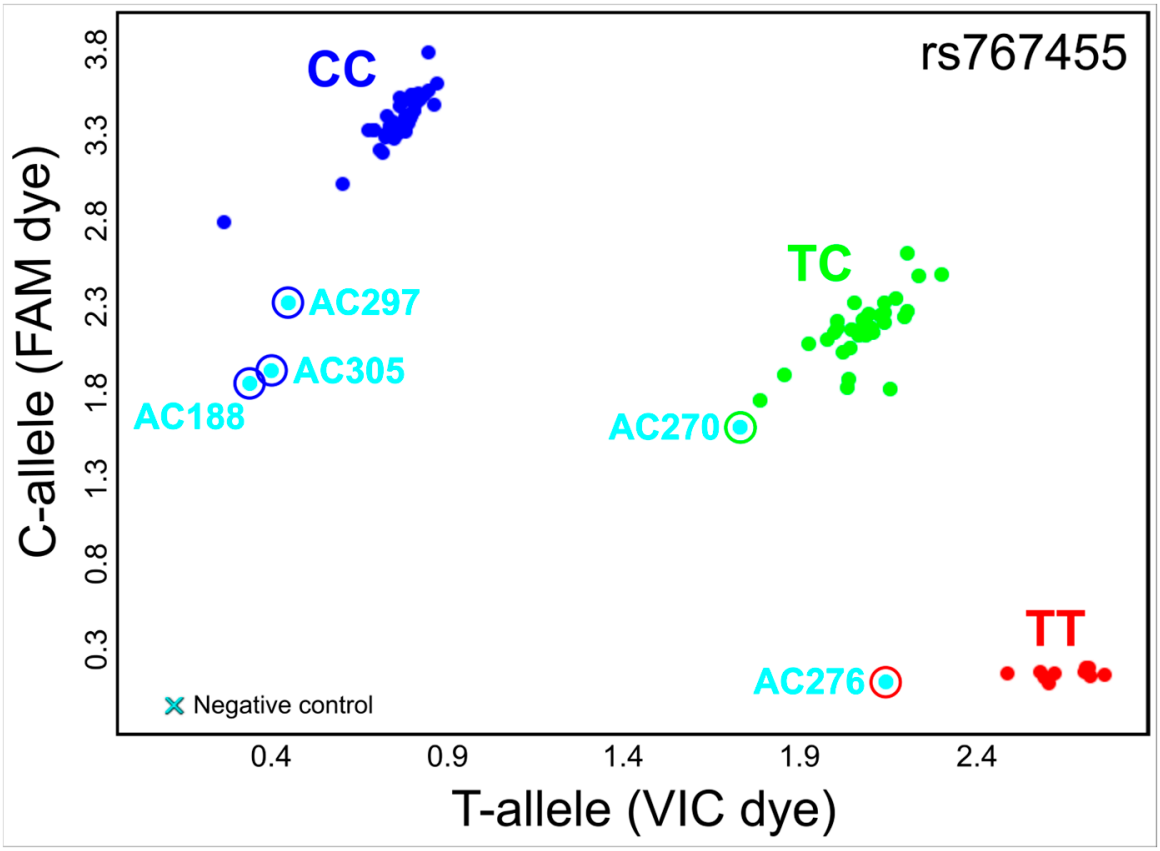


Figure 4. Allelic discrimination plot. Genotyping of rs767455 was performed using the TaqMan™ SNP genotyping method on a 7500 Fast Real-time PCR. The assay mix include unlabeled PCR primers and FAM™- and VIC® dye-labeled probes. Each dot corresponds to a different sample. Non-circled samples correspond to the DNA from human blood cells representing the genotypic controls to alleles CC (blue), TC (green), and TT (red). Circled samples correspond to five samples of human breast tissues (turquoise, AC297, AC305, AC188, AC270, and AC276). DNA was obtained using the TRIzol-based method.

Finally, to evaluate the protein patterns obtained from total protein extraction from cells and tissues, the samples were loaded on 8% SDS polyacrylamide gel and separated by electrophoresis, following Coomassie Blue staining. The results showed that the protein pattern was dependent on the sample nature. Nevertheless, a wide range of proteins with different molecular weights could be detected (Figure 5A). To further validate the performance of the extraction method, the proteins extracted from HEK293-*ABCG2* cells were subjected to Western blot to identify a housekeeping protein GAPDH and the ABCG2 transporter. A representative result is shown in Figure 5B to demonstrate that ABCG2 and GAPDH could be detected at an expected molecular weight of 72 (monomer) and 36 kDa, respectively.

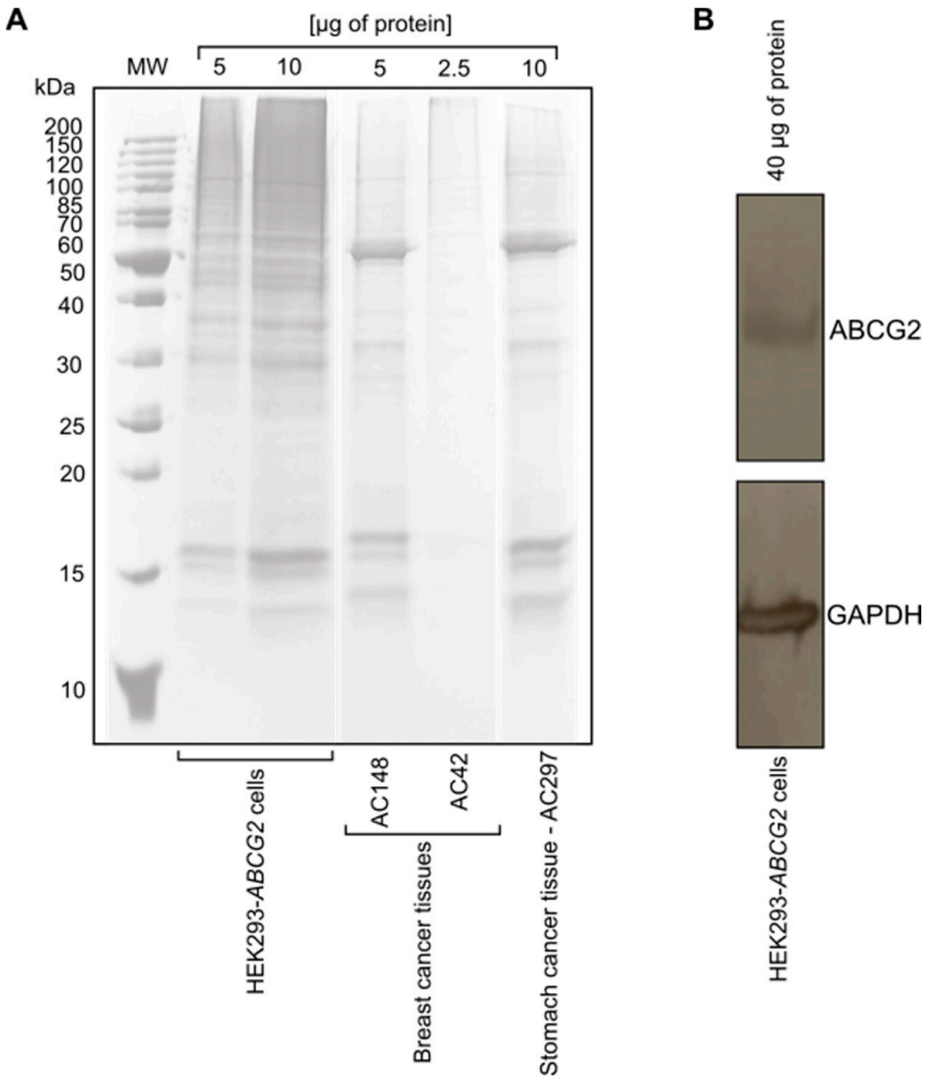


Figure 5. SDS-PAGE and Western blot. (**A**) Comparison of protein recovery from HEK293-ABCG2 cells and tissue cancer samples (AC148, AC42, and AC297). Proteins were extracted with TRIzol, resuspended in 1% SDS and 8 M urea in Tris–HCl, pH 8.0, and then solubilized using sonication. Protein concentration was determined by BCA and gels were Coomassie Blue-stained. (**B**) Representative Western blot analysis of total ABCG2 and GAPDH from HEK293-ABCG2 cells. The experiments were repeated at least three times, and the most representative image is shown. Total protein was loaded on 8% SDS-PAGE. PVDF membranes were incubated with an antibody against ABCG2 or GAPDH.

4. Discussion

Numerous variables that influence the oncogenic behavior of cancer cells have been recognized in the last decade [61]. The determination of the functional significance of either the presence of or changes in the biomarker expression signature among tissues, disease status, treatment therapies, and the detection of MDR is crucial to the management of patients with cancer [62]. Together, these facts stimulate research on methods for the efficient recovery of RNA, DNA, and protein from a single human tissue specimen. However, human samples, especially biopsies of tissue, are unique, mostly obtained by resected tumor samples from invasive surgeries, and a wasted recovery of nucleic acid or protein means that the opportunity to record data from this patient is lost, besides the wasting of financial resources [63].

Among the usual procedures of tissue disruption, mechanical hand disruption is widely used because of the low cost [64]. However, this approach presents some draw-

backs, such as incompatibility with small samples, low replicability, and being time-consuming [52]. The use of commercially available specific equipment improves experimental replicability and high throughput [49] but with the inconvenience of high cost. In addition, it is known that universal procedures are not recommended for all tissue types [47,49]. Considering that these types of equipment are not optimized for each sample and the low budget of the research laboratories in developing countries, low-cost devices are alternatively welcome.

TRIzol has been largely used, especially for the individual extraction of RNA [65], but the extraction of DNA and proteins still need to be optimized [32–34,66]. Despite TRIzol-based method limitations, which might involve the manual pipetting of the aqueous phase without contamination of the organic phase containing genomic DNA, the low quality of the extracted RNA due to chemical contaminants, which can affect downstream applications, and the time-consuming procedure, a major advantage of cost-effectiveness supports its use even in laboratories with limited resources for molecular biology experiments.

In order to gather knowledge of the TRIzol-based method for macromolecule extraction reported in the literature, we performed an integrative review, selecting experimental studies on human tumor tissue (Figure S1). The search resulted in 45 studies (Table S1); most of them included breast cancer samples (Figure S2) and carried out gene expression analysis from extracted RNA (Figure 6A). Only six studies isolated all the RNA, DNA, and proteins from a single sample [34,37,57–60]. Surprisingly, among them it was not possible to find the quantification or yield for all three macromolecules or even for a double combination (Figure 6B). Based on these data, we could assume that there is still a need for investigating whether TRIzol-based one-pot macromolecule extraction retains a high yield and quality for downstream integrated transcriptomic, genomic, and proteomic analysis targeting biomarkers in cancer, especially for clinical samples.

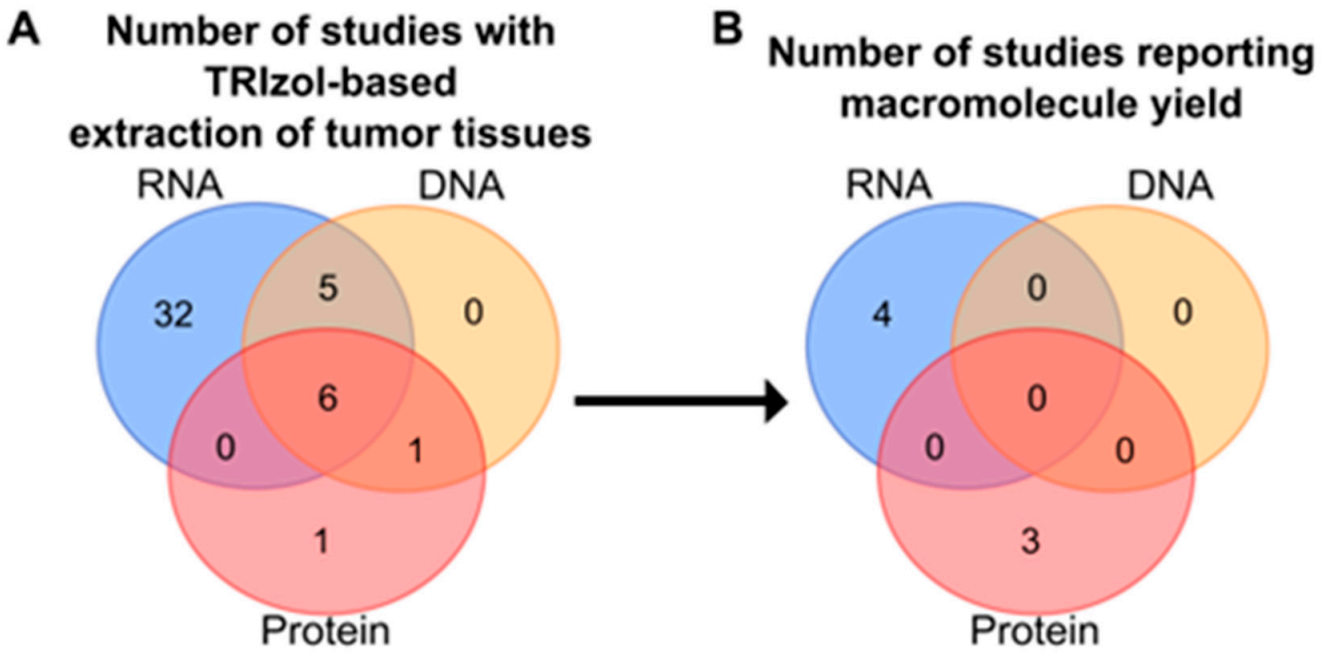


Figure 6. Integrative review of experimental studies on human tumor tissue extracted by a TRIzol-based method and reported yield of each macromolecule. (**A**) The studies were searched in the PubMed and Web of Science databases with a search strategy with the words TRIzol reagent (and analogs), cancer, RNA, DNA, protein, human, and tissue (n = 45). Based on the search findings (**A**), the arrow indicates (**B**) the number of studies reporting the number of macromolecules yield was assessed (n = 7).

In order to pursue the simultaneous extraction of RNA, DNA, and protein macromolecules from the same sample using TRIzol, we followed the steps of lysis or disruption, isolation, resuspension, and the determination of yield and quality. Although cell lysis or disruption could be achieved through physical or chemical methods [67], depending on the nature of the sample, the processing can be different. The lysis of cell lines was quite easy to achieve, requiring only an addition of TRIzol sufficient to solubilize the plasma membrane and disrupt the nuclear membrane, releasing RNA, genomic DNA, and cytoplasmatic and membrane proteins [68]. However, tissue disruption required further mechanical methods [22].

The tissue disruption method using rotor/bead beating through in-house-built equipment (Figure 2) was validated using a large cohort of 51 tissue samples. Considering that breast cancer is the most commonly diagnosed cancer and the leading cause of cancer death in women (1), 75% of the samples in this study comprise breast tissue. Table 2 summarizes the results of all tissue samples obtained from healthy and cancer patients. The same tissue samples were used for RNA, DNA, and protein extraction (Figure 1). For comparison, the results of these macromolecules extracted from human cell lines are also shown. RNA yielded on average of 677 ng/mg of tissue or 4.6 μg/10^6 cells, with an absorbance 260/A280 ratio of 1.8–2.0. DNA and proteins yielded on average of 225 ng/mg of tissue and 1.4 μg/mg of tissue, respectively. The calculated standard deviation (SD) among the results suggested the heterogeneous nature of the samples, indicating the need for large cohorts to allow correct subsequent data interpretation [33]. Herein, we did not focus on the comparison of different groups of clinical findings, but evaluated the performance of the proposed extraction of RNA, DNA, and proteins. Despite this heterogeneity, the results obtained for RNA are similar or even higher to those found in the literature [43,69,70], as well for DNA [66] and proteins [31,44], even using different pellet solubilizing procedures (Table S1). Nevertheless, we cannot assume that protein recovery is fully optimized. It is well known that RIPA (Radio-Immunoprecipitation Assay) buffer promotes cellular lysis and protein solubilization. The solubilization of TRIzol-precipitated protein from HEK293-*ABCG2* cells in urea/SDS buffer plus sonication resulted in 312 μg, while the yield was 948 μg when cell homogenization was performed through a syringe/needle in the presence of RIPA buffer [56].

To explore the performance of isolated RNA, DNA, and proteins for downstream analysis, we used RT-qPCR, genotyping, and SDS-PAGE followed by Western blot. Focusing on future analysis, we evaluated targets involved in MDR mechanisms. The overexpression of ABC transporters is a major factor associated with MDR in cancer [6,71,72]. Here, based on the expression profile involved with a poor treatment response in breast cancer patients [73] and transport of anticancer drugs [74], and to include all ABC subfamilies involved in MDR [75], the following eight genes, *ABCA1*, *ABCB1*, *ABCC1*, *ABCC2*, *ABCC10*, *ABCE1*, *ABCF2*, and *ABCG2*, were chosen to be evaluated.

Considering that the enrichment of specific mRNA targets might be considered for downstream analysis [76,77], as a proof of concept, the *ABCG2* gene as well housekeeping genes were studied in cells stably transfected to overexpress this ABC transporter (Figure 3B). All eight genes encoding ABC transporters, and three housekeeping genes, were efficiently detected in healthy and cancer tissues (Figure 3G). The reported design and validation of a RT-qPCR assay allowed reliable evaluation of gene expression modulations to determine the MDR profile mediated by ABC transporters using both biological models and clinical samples.

The TNFR1 genotyping of rs767455 [19] was performed using genomic DNA extracted using the TRIzol-based method. Usually, a whole-blood specimen is used for DNA extraction [78]. Considering that every cell contains the same DNA sequence, and, hence, the

same somatic mutations and polymorphisms, the tissue mast cell can be used to isolate this macromolecule, without other specimen collection from the patient. The genomic DNA obtained in this work was able to facilitate TaqMan allelic discrimination (Figure 4). Identifying the genetic associations between SNPs and gene expression in cells and tissues may be a promising approach to find functionally relevant pathways that contribute to MDR in cancer. Further studies might be performed with larger cohorts to obtain reliable associations with the genotyping of TNFR1 and ABC gene expression.

In addition, protein extracted from HEK293-*ABCG2* cells by the TRIzol-based method was subjected to SDS-PAGE followed by Western blot (Figure 5). Despite of the often-faced difficulties in detection by Western blot of transmembrane proteins [79], ABCG2 could be efficiently solubilized and detected through Western blot. Considering that the human genome encodes approximately 20% of membrane proteins [80], about 60% of the cellular membrane proteins are drug targets [81], and that the FDA now recommends the study of new drug candidates with membrane transporters, including ABCG2 [82], the results shown here clearly contribute to this emerging field.

5. Conclusions

This study introduces to the field some easy-to-perform and low-cost approaches to extract RNA, DNA, and proteins from cells and tissues with a good quality and high yield. We further demonstrated the use of each molecule using downstream analysis of RT-qPCR, genotyping, and Western blot. The additional advantage is that all analyses can be performed on the same tissue fragment, reducing the sample handling time and allowing direct comparisons of alterations in the genome, transcriptome, and proteome. The impact of these findings may be tremendous for the understanding of molecular mechanisms of cancer drug resistance, mainly for laboratories with a low budget in developing countries.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/j8010003/s1: Figure S1: Flow chart of the integrative review strategy. Studies were searched in PubMed and Web of Science database with the keywords TRIzol Reagent (and analogs), cancer, RNA, DNA, protein, human and tissue. Initially, the studies were filtered by language (english) and tittle. Then exclusion and inclusion criteria were applied after reading the full text of the articles. 45 studies were selected for analysis; Figure S2: Number of studies with TRIzol-based extraction for cancer tissue origin. The legend in colors represents the macromolecule extracted, being red for DNA, RNA and protein, dark blue for DNA and protein, light blue for DNA and RNA and white for only RNA. Note: Somes studies worked with more than one type of cancer, thus the number of studies it is not equal to the total of studies included in the integrative review (45); Table S1. Data analysis of 45 studies using a search strategy from the integrative review of TRIzol-based method to extraction of DNA, RNA and/or protein.

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Institutional Review Board Statement: This study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Ethics Committee of Federal University of Parana's Ethics Committee (CAAE: 17187219.5.0000.0102 and CAAE: 24676613.6.0000.0102) and Hospital e Maternidade Angelina Caron (CAAE: 17187219.5.3002.5226).

Informed Consent Statement: All subjects previously consented to inclusion. Written informed consent was obtained from the patients to publish this paper.

Data Availability Statement: The data presented in this study are available upon request from the corresponding author.

Conflicts of Interest: The authors declared that there are no conflicts of interest.

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