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An optimized QIAzol-based protocol for simultaneous miRNA, RNA, and protein isolation from precision-cut lung slices (PCLS)

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Abstract

Background Precision-cut lung slices (PCLS) are ex vivo models with preserved lung cell populations and maintained tissue architecture. PCLS are, therefore, a powerful tool in respiratory research to study molecular mechanisms that closely reflect whole tissue biology. High-quality RNA and protein extraction from PCLS is, however, challenging as agarose significantly interferes with the yield and purity of extracted material. The present study aimed to optimize QIAzol-based isolation protocol for high-yield and quality RNA, miRNA, and protein extraction from PCLS.

Materials and methods PCLS were prepared from 10 to 15-week-old Wistar rats and cultured for 7 days in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) supplemented with 0.1% FBS, penicillin, and streptomycin. LDH release to PCLS culture media was measured to determine cellular cytotoxicity. To select the optimal miRNA/RNA isolation protocol, we tested two different times (10 min, 2 h) and temperatures (room temperature, 4 °C, and −20 °C) of precipitation with isopropanol. Finally, we also assessed isolation with GHCL (guanidinium hydrochloride) extraction buffer. To select the optimal protein isolation protocol, we tested protein precipitation for 10 min at room temperatures (21 ± 1 °C) with 1.5 volumes of isopropanol and 3 volumes of acetone per 1 volume of phenol-ethanol supernatant. Additionally, we also tested protein precipitation for 3 h at −20 °C with 3, 5, and 7 acetone volumes per 1 volume of phenol-ethanol supernatant. We also validated protein precipitation with back extraction buffer instead of 100% ethanol. To measure the general efficiency of the optimized QIAZ-4 protocol, we used native rat lungs. PCLS for the ex vivo model of allergic inflammation were treated with IL-13 at a concentration of 80 ng/ml.

Results Standard QIAzol isolation protocol provided RNA, miRNA, and protein with low yield and poor quality. We found that 2-h isopropanol precipitation at 4 °C with a high concentration of salts significantly increased the yield and quality of extracted RNA and miRNA and provided acceptable qPCR efficiency (between 90 and 110%). Surprisingly, 2-h isopropanol precipitation at −20 °C significantly increased qPCR efficiency above the acceptable range (average efficiency: 120.4%). As for protein extraction, we found that 3-h acetone precipitation at −20 °C provided the highest yield with linear protein detection on Westen Blot. Optimized QIAZ-4 provided significantly higher miRNA and RNA yield compared to standard QIAzol protocols. We also found a significantly increased expression of Eotaxin-1 in PCLS treated with IL-13 as compared to the untreated controls.

Conclusions In our study, we described a simple QIAzol-based method for the simultaneous isolation of RNA, miRNA, and protein from PCLS.

Keywords Extraction yield, QIAzol, miRNA, RNA, Protein

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Background

Precision-cut lung slices (PCLS) are ex vivo models generated by trimming agarose-inflated lungs to uniform-thickness slices [1]. As PCLS preserve all lung cell populations with maintained tissue architecture, they are a powerful tool in respiratory research to study molecular mechanisms that closely reflect whole tissue biology [2]. High-quality RNA extraction from PCLS is, however, challenging as agarose significantly interferes with the yield and purity of extracted material. Agarose contamination of RNA significantly hampers standard molecular biology techniques (e.g. reverse transcription, real-time PCR) and high-throughput methods (e.g. next-generation sequencing, microarrays) that limit the use of PCLS in respiratory studies [3, 4]. Column-based protocols are predominantly used for extracting RNA from PCLS. These methods are, however, expensive and provide limited RNA yield that entails pooling several PCLS for single isolation, therefore increasing costs and the number of animals required for the experiment. Additionally, column-based protocols usually provide only full-length RNA (mRNA) with a cut-off for small RNA fractions (e.g. miRNA, piRNA) that have a crucial role in the pathogenesis of respiratory diseases [5–7]. Moreover, as agarose shows similar physicochemical properties to RNA, it often binds to silica columns and co-elutes with RNA, making complete removal of agarose impossible with unmodified isolation protocols [8]. Finally, to our knowledge, none of the commercially available column-based RNA isolation protocols allow for simultaneous protein extraction from PCLS that limits the correlation of these two fractions in subsequent multi-omic analyses.

QIAzol Lysis Reagent is a monophasic solution of phenol and guanidine thiocyanate originally described by Chomczynski et al. [9] for simultaneous RNA, DNA, and protein isolation from a single sample. Isolation with QIAzol (or alternative reagents such as TRIzol and TRI Reagent) is superior to other methods as it provides a high yield of RNA, preserves small RNA fractions (e.g. miRNA and piRNA), and is susceptible to modifications for challenging samples [10]. However, simultaneous isolation of RNA, miRNA, and protein from PCLS using QIAzol has not been previously described, as the standard protocol is not designed for agarose-rich samples.

Therefore, to overcome these challenges, in this study, we developed a modified, cost-effective QIAzol-based protocol providing high-yield, pure miRNA, RNA, and protein from a single PCLS.

Materials and methods

Generation of rat PCLS

Wistar rats, 10–15 weeks old, were sacrificed by an intraperitoneal overdose of ketamine and xylazine, and the inferior vena cava was immediately cut. Pulmonary perfusion was performed through the right ventricle with warm 1X phosphate-buffered saline (PBS) to remove blood from the lungs. The trachea was then intubated, and low gelling 1.5% agarose (Sigma-Aldrich) in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12, Thermo Fisher Scientific) was slowly injected into the lungs and left to fully solidify. The whole lungs were then cut out, divided into individual lobes, and cut into 500 µm slices with VT1000s Vibratome (Leica) equipped with the cooling system. Lung tissues were sliced at a frequency of 100 Hz and amplitude of 1.0 mm. PCLS were cut from slices with 4-mm diameter biopsy punchers and transferred to DMEM/F-12 medium supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), and 0.1% fetal bovine serum (FBS) and cultured at 37 °C, 5% CO_2 in a humidified atmosphere. PCLS were cultured in a 96-well plate (one PLCS per well submerged in 200 µl of media) for 7 days and the culture medium was changed every 24 h.

To induce allergic inflammation, PCLS (n = 3) were treated with 80 ng/ml of rat IL-13 (PeproTech) for 7 days with a fresh medium replacement (supplemented with the same concentration of IL-13) every other day. PCLS (n = 3) treated with 0.9% saline in the same way was used as a control in this experiment. PCLS (n = 3), treated with 0.9% saline in the same manner, were used as a control in this experiment.

After that, PCLS were snap-frozen in liquid nitrogen and stored at −80 °C (single PCLS per Eppendorf tube without any cryoprotectant) for RNA/protein isolation. Culture medium from each well was transferred to Eppendorf tube and stored at −80 °C for lactate dehydrogenase (LDH).

LDH viability test

LDH release to PCLS culture media was measured with Cytotoxicity Detection Kit (Sigma-Aldrich) according to the manufacturer instructions. Briefly, 100 µl of PCLS culture medium or cell-free medium (background control) was gently transferred to a 96-well flat-bottom microplate and mixed with 100 µl of freshly prepared reaction mixture, followed by incubation for 30 min at room temperature (21 ± 1 °C). The optical density was measured at 490 nm with Asys UVM 340 microplate reader (Biochrom). The background control (mean OD ~ 0.082) was subtracted from all optical density data obtained from the PCLS media.

MiRNA and RNA isolation

PCLS (1 PCLS per isolation) were lysed in an appropriate lysis buffer using an ultrasonic tissue sonicator (Sonoplus

HD 3200, Bandelin). To homogenize PCLS, we used 4 cycles of 5 pulses at 10% of sonicator efficiency. Each miRNA/RNA isolation was performed in 3 technical replicates. We used 200 µl of QIAzol Lysis Reagent (Qiagen) for the following protocols: QIAZ-1 (standard QIAzol protocol), QIAZ-2, and QIAZ-3, and 100 µl for QIAZ-4. Samples were incubated for 5 min at room temperature (21 ± 1 °C) to fully dissociate the nucleoprotein complexes, followed by mixing with chloroform (0.2 ml of chloroform per 1 ml of QIAzol Lysis Reagent). Samples were then incubated for 3 min at room temperature and centrifuged for 15 min at 12 000 × g at 4 °C. The aqueous phase was carefully transferred to a new tube and supplemented with 20 µg (in a volume of 1 µl) of UltraPure RNA-free glycogen (Thermo Fisher Scientific). The interphase and the phenol–chloroform phase were saved for subsequent protein isolation. The aqueous phases from QIAZ-2, QIAZ-3, and QIAZ-4 protocols were additionally supplemented with 5 M sodium chloride (NaCl) to a final concentration of 1.2 M. The aqueous phase from the QIAZ-1 protocol was not supplemented with NaCl. RNA was precipitated with isopropanol (0.5 ml of isopropanol per 1 ml of QIAzol Lysis Reagent) for 10 min at room temperature (QIAZ-1), 2 h at -20 °C (QIAZ-2) or 4 °C (QIAZ-3 and QIAZ-4). Samples were then centrifuged for 10 min at 12000 × g at 4 °C to precipitate RNA, followed by washing the pellet three times with 75% ethanol. RNA pellet was air dried for 5–10 min, dissolved in 15 µl of RNase-free water, and incubated in a heat block set at 55 °C for 10 min.

For GHCL-1 and GHCL-2 protocols, PCLS were lysed in 200 µl of guanidinium hydrochloride (GHCL) extraction buffer (0.1 M β-mercaptoethanol, 6.5 M guanidinium hydrochloride, 100 mM Tris–HCl pH 7.4, 0.1 M sodium acetate pH 5.5). To precipitate agarose, GHCL buffer was supplemented with potassium acetate to a final concentration of 0.2 M, incubated for 10 min at room temperature, and centrifuged for 15 min at 12 000 × g at 4 °C. The supernatant was transferred to a new tube, mixed with 100 µl of QIAzol Lysis Reagent, and incubated for 5 min at room temperature. Each tube was then shaken with 40 µl of chloroform and incubated for 3 min at room temperature. Samples were then centrifuged for 15 min at 12 000 × g at 4 °C, and the aqueous phase was transferred to a new tube, followed by supplementation with 20 µg of UltraPure RNA-free glycogen. Samples isolated with GHCL-2 were additionally supplemented with 5 M NaCl to a final concentration of 1.2 M. RNA was precipitated with isopropanol (0.5 ml of isopropanol per 1 ml of GHCL buffer) for 2 h at 4 °C and centrifuged (10 min at 12,000 × g at 4 °C). RNA pellets were washed three times with 75% ethanol and dissolved in RNase-free water, similar to QIAZ protocols. The overview of RNA isolation protocols is shown in Fig. 1. PCLS treated with IL-13 and 0.9% saline (control) were extracted with QIAZ-4 protocol. Lung tissues (~ 5 mg) were isolated with QIAZ-1 (100 µl of QIAzol Lysis Reagent) and QIAZ-4 protocol.

Protein isolation

Each protein isolation was performed in 3 technical replicates using phenol–chloroform phase collected during miRNA/RNA isolation. All samples were supplemented with 100% ethanol (0.3 ml of 100% ethanol per 1 ml of QIAzol Lysis Reagent), thoroughly mixed, and incubated at room temperature for 2–3 min. Samples were centrifuged for 5 min at 2,000 × g at 4 °C to precipitate DNA, and supernatants were transferred to new tubes. For QIAZ-P1 and QIAZ-P2 protein was precipitated with, respectively, isopropanol (1.5 ml of isopropanol per 1 ml of QIAzol Lysis Reagent) or acetone (3 volumes of acetone per 1 volume of phenol-ethanol supernatant) and incubated for 10 min at room temperature. For QIAZ-P3, QIAZ-P4, and QIAZ-P5 samples were supplemented with, respectively, 3, 5, and 7 volumes of acetone per 1 volume of phenol-ethanol supernatant and incubated for 3 h at −20 °C. All abovementioned samples were centrifuged for 10 min at 12 000 × g at 4 °C and washed three times with 0.3 M guanidine hydrochloride in 95% ethanol (2.0 ml of wash buffer per 1 ml of QIAzol Lysis Reagent) followed by a single wash with 2 ml of 100% ethanol. During each wash cycle, the protein pellets were stored in a washing solution for 20 min at room temperature and then centrifuged for 5 min at 7 500 × g at 4 °C. Protein pellets were air dried for 5–10 min, dissolved in 50 µl of 4 M Urea/1% SDS solution, and incubated in a heat block set at 50 °C for 10 min at the agitation rate of 500 rpm. Samples were centrifuged for 10 min at 10 000 × g at 8 °C, supernatants were transferred to new tubes and stored at -80 °C for further analysis.

For RIPA protocol, we used one PCLS in 50 µl of RIPA Lysis Buffer (Sigma-Aldrich) supplemented with cOmplete ULTRA Tablets (Sigma-Aldrich) protease inhibitor cocktail according to manufacturer instructions. PCLS were homogenized as in the miRNA/RNA isolation protocols with an ultrasonic sonicator. Lysates were centrifuged at 8 000 × g for 10 min at 4 °C to pellet the cell debris, and the remaining supernatants were stored at −80 °C.

For QIAZ-P6, back extraction buffer (4 M guanidine thiocyanate, 50 mM sodium citrate, and 1 M Tris pH 7.4) was vigorously mixed with the interphase-organic phase (0.5 ml of back extraction buffer per 1 ml of QIAzol Lysis Reagent) and incubated for 10 min at room temperature. Samples were then centrifuged at 12 000 × g for 15 min at 4 °C, and the upper phase (containing DNA) was discarded. The organic phase was supplemented with 5

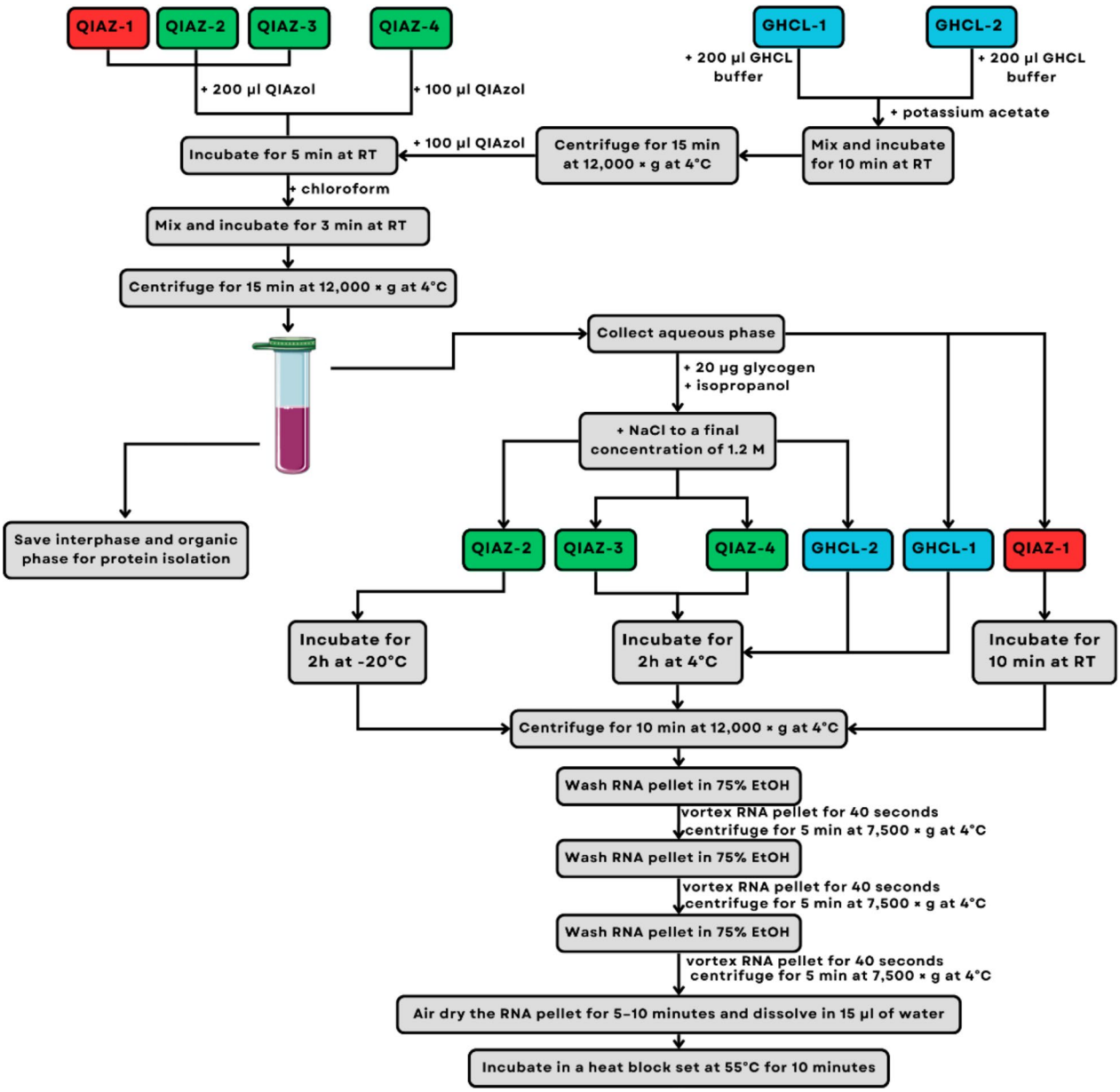

Fig. 1 Schematic presentation of miRNA and RNA isolation protocols

volumes of acetone, mixed vigorously, and incubated at room temperature for 10 min. Protein pellet washing and solubilization were performed similarly to the QIAzol-based protocols. The overview of protein isolation protocols is shown in Fig. 2. PCLS treated with IL-13 and 0.9% saline (control) were extracted with QIAZ-P3 protocol. Lung tissues (~5 mg) were isolated with RIPA Lysis Reagent and QIAZ-P3 protocol.

RNA quantification and quality control

The RNA concentration and purity were determined by measuring absorbance with NanoDrop2000 (Thermo Fisher Scientific) spectrophotometer. We used Quantus Fluorometer (Promega) for fluorescent RNA, and miRNA quantification with, respectively, QuantiFluor RNA System (Promega) and Qubit microRNA Assay Kit (Thermo Fisher Scientific). RNA integrity number (RIN) was assessed using RNA ScreenTape System (Agilent) with Agilent 2200 TapeStation System (Agilent).

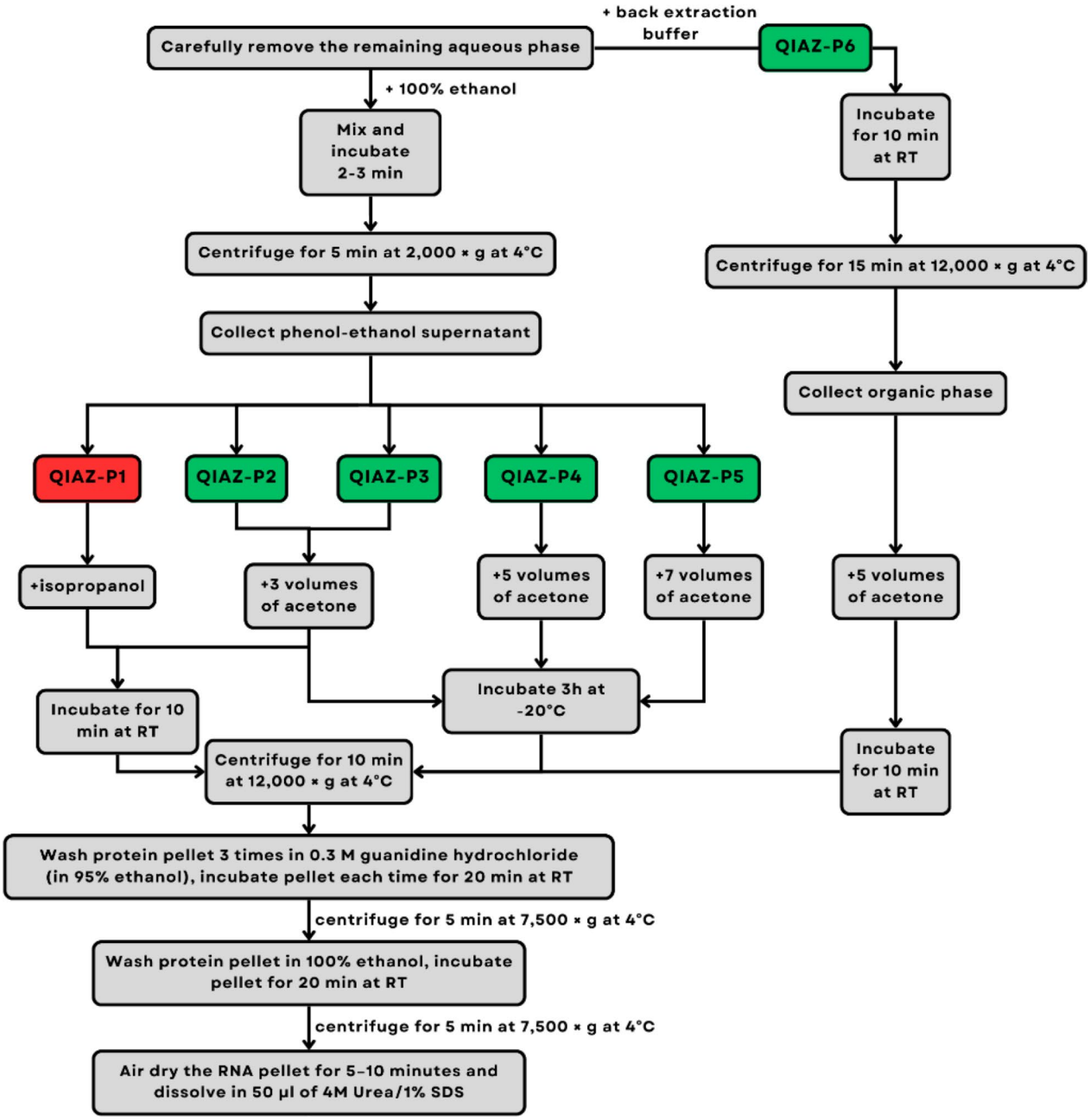

Fig. 2 Schematic presentation of protein isolation protocols

Real-time PCR

RNA and miRNA were converted to cDNA using, respectively, Reverse Transcription Kit (Promega) and TaqMan Advanced miRNA cDNA Synthesis Kit (Thermo Fisher Scientific). *GAPDH* and Exotoxin-1 expression was measured with SYBR Green MasterMix (Promega) using the comparative method (ΔΔCt) and the following primers: *GAPDH* forward 5'-AACTCC CTCAAGATTGTCAGCAA-3' and *GAPDH* reverse 5'-GGCATGGACTGTGGTCATGA-3', Eotaxin-1 forward 5'- CTATTCCTGCTGCTCACGGC-3' and Eotaxin-1 reverse 5'- CAGGAAGTTGGGATGGAA CCTGG -3'. Rno-miR-223-3p was quantified with TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific) and TaqMan Advanced miRNA Assay (Thermo Fisher Scientific) as a hybridization probe (assay ID: rno481007_mir). We chose rno-miR-223-3p

as in our previous study, this miRNA was expressed in rat lungs and its crucial role in the pathogenesis of human respiratory diseases [11]. Data acquisition was performed in ABIPrism 7900HT (Applied Biosystems) using SDS 2.4 software. Data analysis was performed using DataAssist v3.01.

Western blot analysis

Protein concentration was measured using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). For Western blot, we used 1.25 µg, 2.5 µg, 5 µg and 10 µg of total protein. Samples were mixed with Tris–Glycine SDS Sample Buffer (2X) (Thermo Fisher Scientific) and NuPAGE Sample Reducing Agent (10X) (Thermo Fisher Scientific) and heated at 85 °C for 2 min, followed by SDS-PAGE gel electrophoresis. For total protein normalization, the gel was stained with No-Stain Protein Labeling Reagent (Thermo Fisher Scientific) and visualized on a UV transilluminator (ChemiDoc Imaging System, Bio-Rad). Proteins were transferred to a nitrocellulose membrane with iBlot 2 Dry Blotting (Thermo Fisher Scientific) system using pre-programmed P0 method: 20 V for 1 min, 23 V for 4 min, 25 V for 2 min. The membrane was then blocked for 1 h in 5% BSA dissolved in phosphate-buffered saline containing 0.05% Tween-20 (PBST), followed by overnight incubation with rabbit monoclonal anti-FKBP51 (ab126715) primary antibody (1:1000) at 4 °C. We quantified FKBP51 as in our previous experiments, this protein was highly expressed in rat lungs (data not published). The membrane was washed three times for 10 min with PBST, followed by incubation with anti-rabbit IgG HRP-conjugated (HAF008) secondary antibody (1:1000) for 1 h at room temperature. The membrane was washed three times for 10 min with PBST, followed by protein band detection with ECL SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific) in ChemiDoc Imaging System (Bio-Rad). The band and total protein intensities were measured in ImageJ version 1.54.

Statistical analysis

One-way ANOVA with Tukey HSD (Honestly Significant Difference) post-hoc test was used to calculate differences between groups. The normality of data distribution and variance homogeneity were assessed using Kolmogorov–Smirnov and Cochran's C test, respectively. Statistical analyses were performed in Statistca version 13, and graphs were plotted in GraphPad Prism version 9.0.

Results

LDH viability test

We first compared the release of lactate dehydrogenase (LDH) to the culture medium from PCLS used for miRNA, RNA, and protein isolations. The increased release of LDH indicates damage to the plasma membrane and thus is commonly used to determine cell viability [12]. As shown in Fig. 3, no significant differences in cell viability were found among groups.

MiRNA and RNA yield

The absorbance at 260 nm was measured using a NanoDrop spectrophotometer to compare the RNA yield between different isolation protocols. Each sample was measured in triplicate. As shown in Fig. 4A, RNA concentration was significantly increased in QIAZ-2, QIAZ-3, QIAZ-4, and GHCL-2 compared to the standard QIAzol extraction protocol (QIAZ-1). Interestingly, modified QIAzol-based protocols provided significantly higher RNA yield compared to GHCL-1 and GHCL-2, but no differences were found between QIAZ-2, QIAZ-3, and QIAZ-4.

As UV spectrophotometry measurements might be interfered with protein, DNA, and salt contaminations [13], we validated the RNA and miRNA concentrations with fluorimetric-based detection methods. We found that both RNA and miRNA concentrations in all modified protocols were significantly increased compared to QIAZ-1 (Fig. 4B, C). Overall, modified QIAzol-based protocols provided significantly higher RNA concentrations than GHCL-based protocols. Surprisingly, no

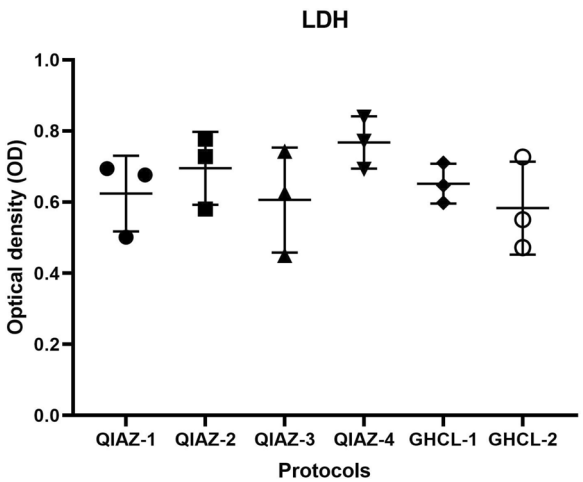

Fig. 3 LDH release to PCLS culture medium showed as optical density (OD). Values are presented as grouped scatter plot with mean ± standard deviation (whiskers). One-way ANOVA with Tukey HSD post-hoc test was used to calculate differences between groups, and $p < 0.05$ was considered statistically significant

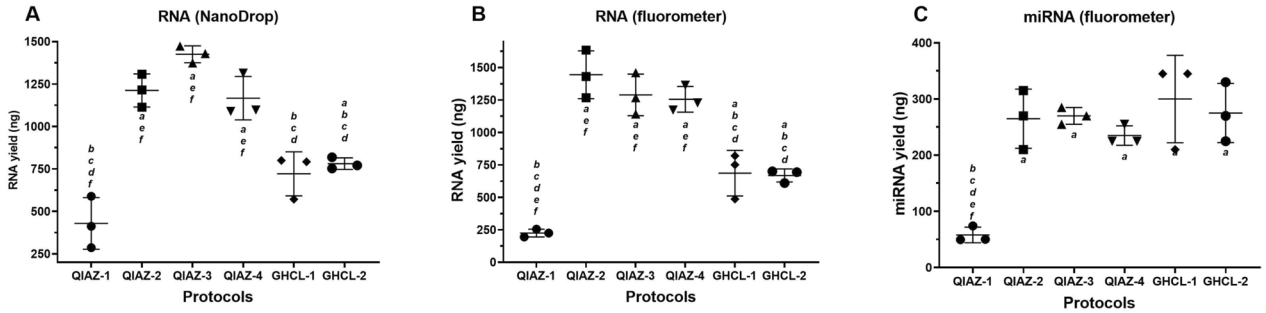

Fig. 4 Comparison of miRNA and RNA yield. **A** RNA yield determined with NanoDrop spectrophotometer (absorbance at 260 nm), **B** RNA yield determined with Quantus Fluorometer (fluorescent-based method), **C** miRNA yield determined with Quantus Fluorometer (fluorescent-based method). Values are presented as grouped scatter plots with mean ± standard deviation (whiskers). One-way ANOVA with Tukey HSD post-hoc test was used to calculate differences between groups, and $p < 0.05$ was considered statistically significant. Statistical significance is indicated as follows: *a*-significant difference from QIAZ-1, *b*-significant difference from QIAZ-2, *c*-significant difference from QIAZ-3, *d*-significant difference from QIAZ-4, *e*-significant difference from GHCL-1, *f*-significant difference from GHCL-2

significant differences in miRNA concentrations were found between modified QIAzol and GHCL-based protocols.

RNA quality and integrity

To evaluate the purity of RNA, we compared the ratios of absorbance at 260 nm vs. 280 nm (A_{260}/A_{280}) and 260 nm vs. 230 nm (A_{260}/A_{230}) that indicate, respectively, protein and salt contaminations [14]. Ratios of ~ 1.8 for A_{260}/A_{280} and ~ 2.0 for A_{260}/A_{230} indicate pure RNA, suitable for downstream applications [15]. We found that A_{260}/A_{280} ratio for QIAZ-1 was significantly decreased compared to other protocols. No other significant differences in A_{260}/A_{280} ratios were found (Fig. 5A). The A_{260}/A_{230} ratio for standard QIAzol protocol (QIAZ-1) was under the acceptable threshold (average A_{260}/A_{230}: 0.39 ± 0.19) that indicates salt contamination, whereas, for all modified protocols, the mean value equaled approximately 2.0 (QIAZ-2: 2.04 ± 0.03; QIAZ-3: 1.97 ± 0.02; QIAZ-4: 2.09 ± 0.05; GHCL-1: 1.99 ± 0.14; GHCL-2: 1.93 ± 0.06) that corresponds to pure RNA (Fig. 5B). To compare RNA integrity between the protocols, we measured the RNA integrity number (RIN) that indicates the ratio intensity of 28S to 18S ribosomal RNA. RIN values above 8 indicate that the RNA is intact and acceptable for standard molecular techniques (e.g. real-time PCR) and high-throughput methods (e.g. next-generation sequencing, microarrays), whereas values below 5 indicate sample degradation [16]. For all QIAzol-based protocols, the mean RIN values ranged between 9 and 10, indicating intact RNA in analyzed samples. In contrast, GHCL-based protocols showed significantly decreased RIN (mean value ~ 6) that indicates RNA degradation (Fig. 5C).

Amplification efficiency of real-time PCR

To further explore the applicability of reverse-transcribed RNA (cDNA), we measured the amplification efficiency

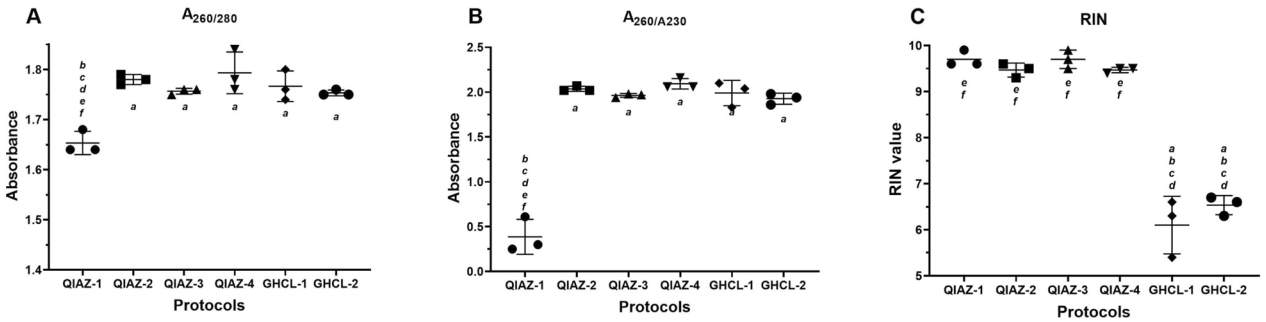

Fig. 5 Assessment of RNA purity with A_{260}/A_{280} (**A**), A_{260}/A_{230} (**B**) ratios, and RNA integrity with RIN values (**C**). Values are presented as grouped scatter plot with mean ± standard deviation (whiskers). One-way ANOVA with Tukey HSD post-hoc test was used to calculate differences between groups, and $p < 0.05$ was considered statistically significant. Statistical significance is indicated as follows: *a*-significant difference from QIAZ-1, *b*-significant difference from QIAZ-2, *c*-significant difference from QIAZ-3, *d*-significant difference from QIAZ-4, *e*-significant difference from GHCL-1, *f*-significant difference from GHCL-2

in real-time quantitative PCR. The range between 90 to 110% indicates acceptable real-time PCR efficiency. We used cDNA from QIAZ-2, QIAZ-3 and QIAZ-4 as only these protocols provided acceptable RNA purity ($A_{260}/A_{280} > 1.8$ and $A_{260}/A_{230} > 2.0$) and integrity (RIN > 8.0). We prepared twofold serial dilutions of cDNA from QIAZ-2, QIAZ-3, and QIAZ-4 and quantified the expression of *GAPDH*. Each reaction was performed in triplicate. As shown in Fig. 6A–C, the amplification efficiency only for QIAZ-3 and QIAZ-4 was within the acceptable range. We finally aimed to verify the amplification efficiency of miRNA extracted with QIAZ-3 and QIAZ-4 that showed acceptable *GAPDH* amplification efficiency. For this, we quantified the expression of rno-miR-223-3p, that is highly expressed in rat lungs [11]. As shown in Fig. 7A, B, the amplification efficiency of rno-miR-223-3p for QIAZ-3 and QIAZ-4 was within an acceptable range and equaled approximately 105.4% and 100.7%, respectively.

Protein yield

For protein isolation, we used the phenol-ethanol phase collected according to the manufacturer protocol. We first verified if the precipitation of protein with acetone instead of isopropanol improves protein yield. Although precipitation with acetone (QIAZ-P2) provided higher protein yield than precipitation with isopropanol (QIAZ-P1), that difference was not significant. To further increase protein yield, we precipitated protein for 3 h at −20 °C with 3 (QIAZ-P3), 5 (QIAZ-P4), and 7 (QIAZ-P5) volumes of acetone per 1 volume of phenol-ethanol supernatant. We found that overnight protein precipitation significantly increased protein yield, but no significant differences were found between different volumes of acetone as well as protein yield obtained with RIPA. Finally, as DNA precipitation with 100% ethanol might potentially coprecipitate protein, we verified alternative isolation with back extraction buffer (QIAZ-P6), but this did not significantly increase protein yield compared to standard QIAzol protocol (QIAZ-P1). The comparison of protein yield between different isolation protocols is summarized in Fig. 8.

Protein linear detection

We verified if protein extracted with QIAzol-based modified protocols is suitable for specific and linear immunodetection. For this, we used two-fold dilutions of total

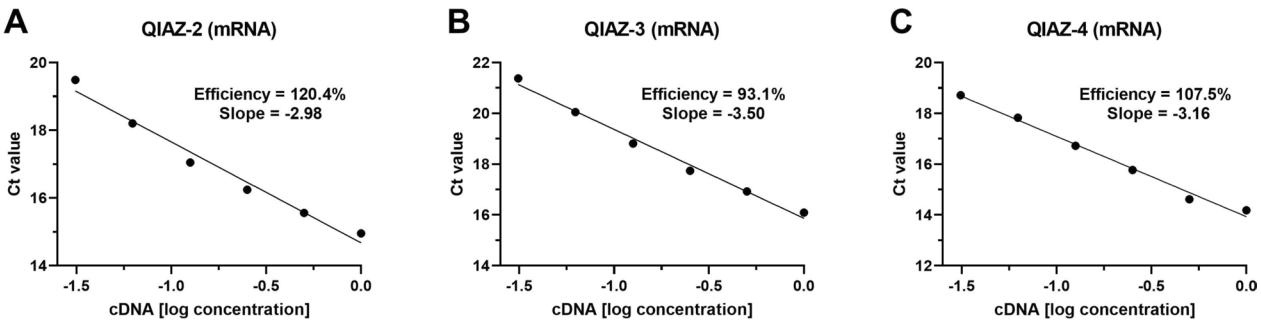

Fig. 6 Comparison of *GAPDH* amplification efficiency of cDNA from QIAZ-2 (**A**), QIAZ-3 (**B**) and QIAZ-4 (**C**)

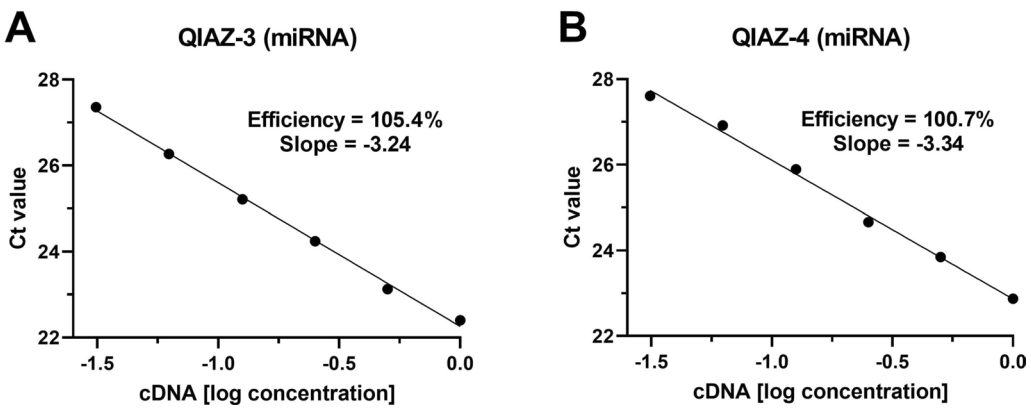

Fig. 7 Comparison of rno-miR-223-3p amplification efficiency of cDNA from QIAZ-3 (**A**), QIAZ-4 (**B**)

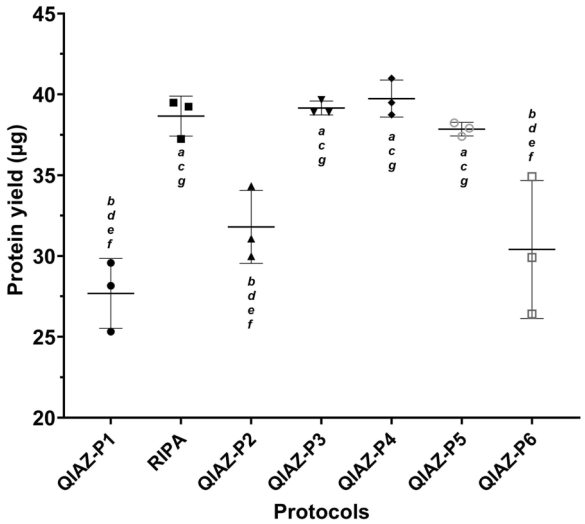

Fig. 8 Assessment of protein yield from different isolation protocols. Values are presented as grouped scatter plots with mean ± standard deviation (whiskers). One-way ANOVA with Tukey HSD post-hoc test was used to calculate differences between groups, and $p < 0.05$ was considered statistically significant. Statistical significance was indicated as follows: *a*-significant difference from QIAZ-P1, *b*-significant difference from RIPA, *c*-significant difference from QIAZ-P2, *d*-significant difference from QIAZ-P3, *e*-significant difference from QIAZ-P4, *f*-significant difference from QIAZ-P5, *g*-significant difference from QIAZ-P6

protein lysates (1.25, 2.5, 5, and 10 ug) extracted with QIAZ-P4, that provided the highest yield. We verified the linear detection of total protein and level of FKBP51 with No-Stain Protein Labeling Reagent and Western blot, respectively. As shown in Fig. 9A, B, either FKBP51 level or total protein intensity showed a linear detection range from 1.25 to 10 µg with a coefficient of determination (R-Pearson Squared) equaled to 0.961 (Fig. 9C).

General efficiency of the optimized protocol

To measure the general efficiency of optimized QIAZ-4 and QIAZ-P4 protocols compared to standard QIAzol protocol and RIPA buffer, we used rat lungs without the agarose. As shown in Fig. 10A–C, RNA and miRNA concentrations were significantly higher in the QIAZ-4 protocol compared to QIAZ-1. Moreover, we found that A260/A230 ratios for QIAZ-1 were significantly below 1.8 that indicates salt contamination (Fig. 10E). No significant differences were found in RIN values (Fig. 10F) and A260/A280 ratios (Fig. 10D) between QIAZ-1 and QIAZ-4. Additionally, there were no significant differences in protein yield between QIAZ-P4 and RIPA buffer (Fig. 10G).

The efficiency of *GAPDH* and rno-miR-223-3p amplification for QIAZ-1 was slightly below the acceptable range and equaled, respectively, 115.1% and 88.3% (Fig. 11A, C). As for QIAZ-4, the amplification efficiency was within the acceptable range (Fig. 11B, D).

FKBP51 level and total protein intensity showed a linear detection range from 1.25 to 10 µg with a coefficient of determination (R-Pearson Squared) for RIPA and QIAZ-P4 equaled, respectively, 0.997 (Fig. 12A) and 0.956 (Fig. 12B)

Ex vivo model of allergic inflammation

To assess the clinical relevance of our optimized QIAZ-4 and QIAZ-P4 protocols, we tested them on PCLS treated with interleukin-13 as an ex vivo model of allergic inflammation. No significant differences in miRNA/RNA concentration, purity, or integrity were observed between PCLS treated with IL-13 and untreated controls (Fig. 13A–F). Additionally, protein concentration (Fig. 13G) or qPCR amplification efficiency for *GAPDH* and rno-miR-223-3p (Fig. 14A–D) did not differ significantly between the analyzed groups.

FKBP51 level and total protein intensity showed a linear detection range from 1.25 to 10 µg with a coefficient of determination (R-Pearson Squared) for PCLS treated with IL-13 and untreated equaled, respectively, 0.941 (Fig. 15A) and 0.937 (Fig. 15B).

Finally, we utilized our optimized QIAZ-4 protocol to measure the gene expression of Eotaxin-1 in PCLS treated with IL-13 as compared to untreated controls. As shown in Fig. 16, the expression of Eotaxin-1 was significantly increased following treatment with IL-13.

Discussion

Despite intensive research, the number of chronic respiratory cases (e.g. asthma, idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease, and lung cancer) significantly increased over the last decades. One of the factors that strongly limits the development of novel therapeutics is the poor correlation of results between in vitro and in vivo models with human tissues [17].

Over the last decade, precision-cut lung slices (PCLS) have emerged as powerful tools in respiratory studies as they preserve all lung cell subpopulations and tissue architecture. PCLS generated from human non-transplantable lungs or surgical resections provides, therefore, a unique opportunity to study the pathophysiology of human respiratory diseases [18]. PCLS are, therefore, an excellent translational lung model to fill the gap between basic and clinical research [19].

RNA extraction from PCLS is, however, extremely challenging as agarose precipitates with RNA and significantly hampers downstream analyses. So far, optimization of RNA and miRNA isolation from PCLS was described in two studies by Niehow et al. [20, 21] with

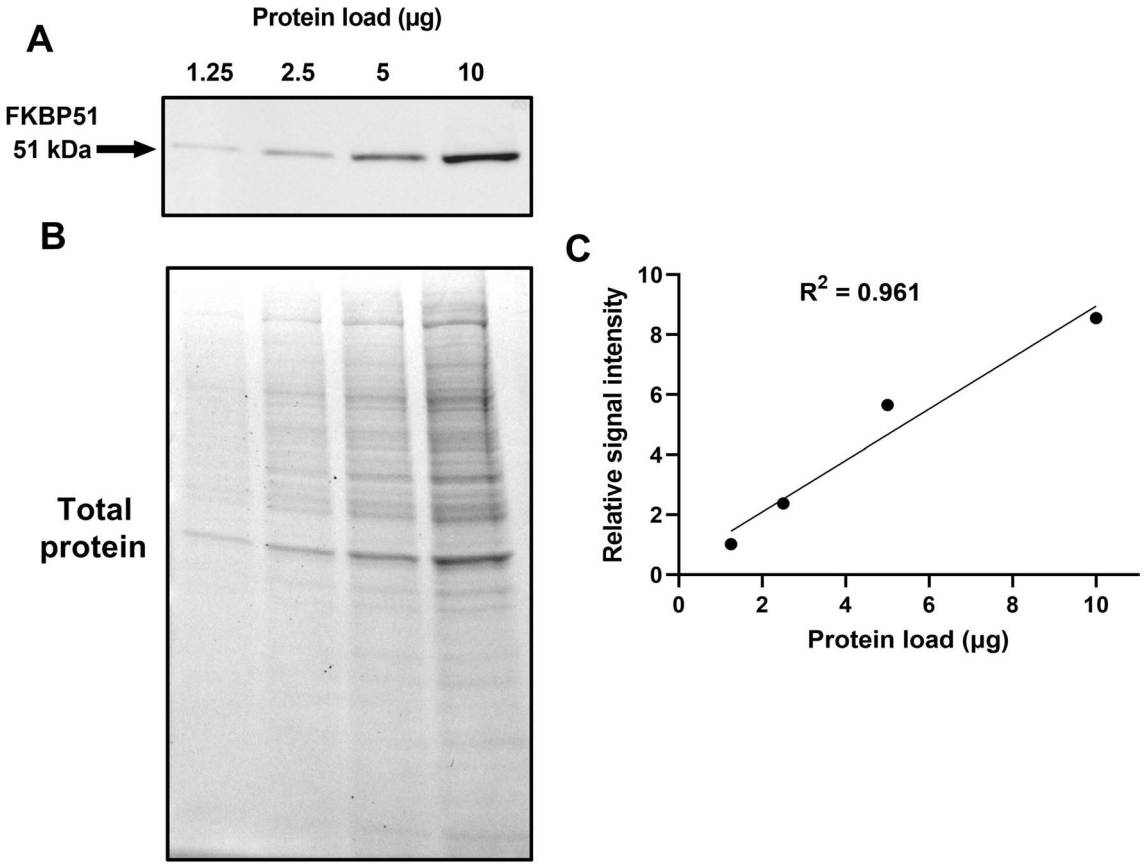

Fig. 9 Comparison of linear detection of FKBP51 (**A**), total protein (**B**), and normalized FKBP51 band intensity (**C**) with Western Blot

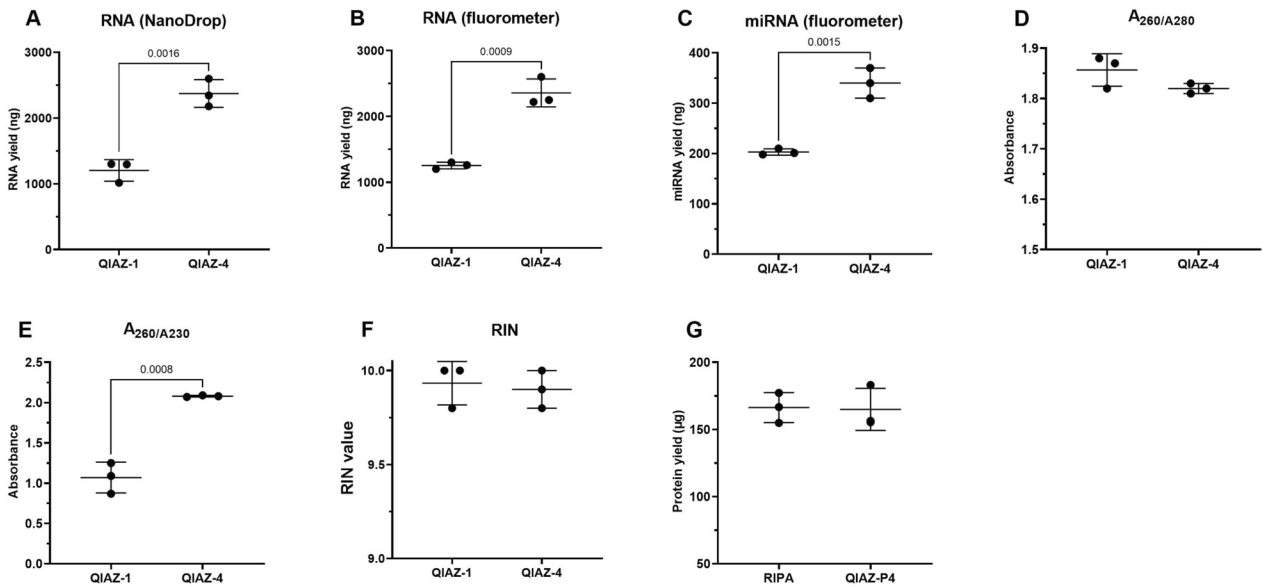

Fig. 10 Assessment of miRNA, RNA, and protein concentration, purity, and integrity between QIAZ-1 and QIAZ-4 (**A**–**F** and RIPA and QIAZ-P4 (**G**)

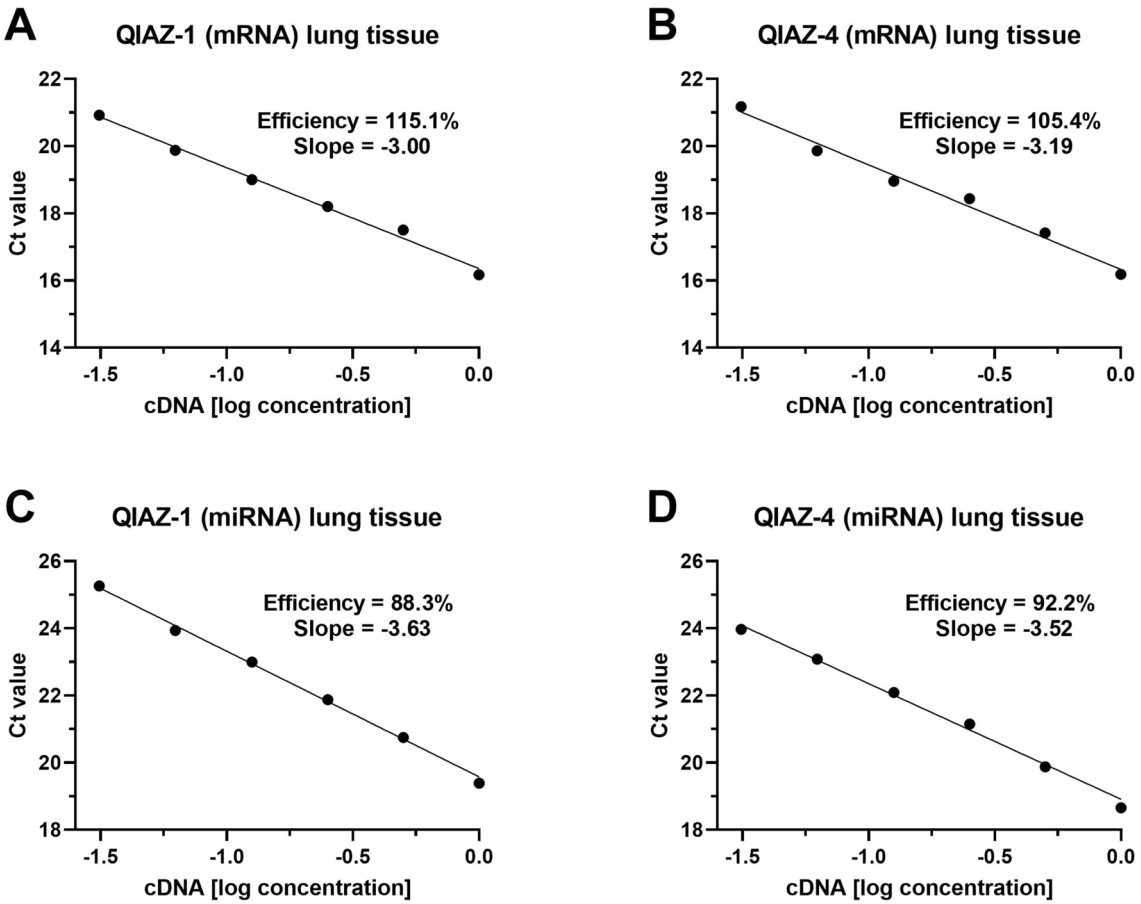

Fig. 11 Comparison of *GAPDH* and rno-miR-223-3p amplification efficiency of cDNA from QIAZ-1 (**A**, **C**) and QIAZ-4 (**B**, **D**)

magnetic bead-based MagMAX Total Nucleic Acid Isolation kit from Thermo Fisher Scientific. These protocols are, however, time-consuming, require expensive reagents, and do not allow for simultaneous protein isolation from the same sample. Additionally, they require 4 PCLS to be pooled in one isolation to obtain RNA yield of around 1500 ng (~ 375 ng of RNA per 1 PCLS). Pooling several PCLS for a single isolation, however, significantly increases the number of required reagents and animals for the experiment.

Previously, we have shown that modified column-based extraction with RNeasy (Qiagen) provides high-quality RNA suitable for low- (real-time PCR) and high-throughput (RNA sequencing) analyses [4]. However, similar to MagMAX-based protocol, we used 4 PCLS per 1 isolation, but without the possibility of simultaneous protein extraction.

To overcome these challenges, we developed a novel, QIAzol-based, column-free protocol for simultaneous miRNA, RNA, and protein isolation from a single PCLS. As agarose is a polysaccharide derived from seaweed, we adopted methods dedicated to isolating RNA from plant tissues. Therefore, to remove agarose contamination, we used the modification of TRI Reagent protocol by Chomczyński et al. [22] for polysaccharide-rich samples that include the supplementation of the aqueous phase with a high concentration of salts (e.g. sodium chloride). Increased salt concentration in the aqueous phase keeps polysaccharides dissolved while RNA is precipitated with isopropanol. Thus, according to the modification, we supplemented the aqueous phase with NaCl to a final concentration of 1.2 M. Then, to completely remove NaCl contamination after precipitation with isopropanol, we washed the RNA pellet three times with 75% ethanol. Furthermore, to increase RNA yield, we tested different precipitation temperatures and times of the aqueous phase with isopropanol and NaCl. To increase yield, RNA is usually precipitated with isopropanol for 1 h to overnight at −20 °C [22]. In our modification, however, precipitation of RNA at −20 °C (QIAZ-2) significantly increased real-time PCR efficiency above the acceptable range that indicates agarose contamination.

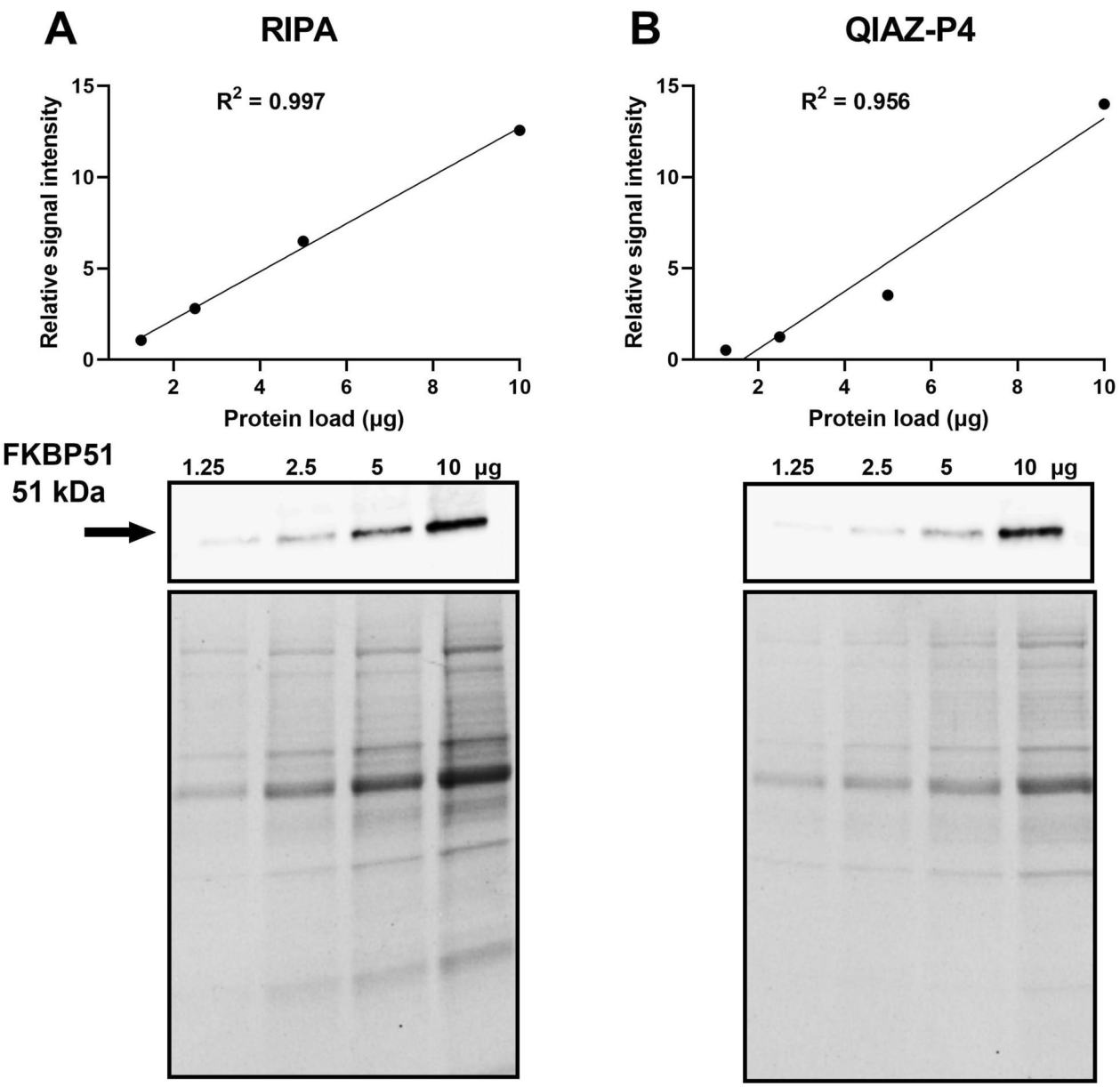

Fig. 12 Comparison of linear detection of FKBP51, total protein and normalized signal intensity between RIPA buffer (**A**) and QIAZ-P4 (**B**) in native rat lungs

As temperatures below 0 °C precipitate salts (including NaCl), we hypothesized that agarose does not remain dissolved in the aqueous phase and coprecipitates with RNA, affecting *GAPDH* amplification efficiency in real-time qPCR. To verify this hypothesis, we tested RNA precipitation at 4 °C for QIAZ-3 and QIAZ-4 and found acceptable real-time PCR efficiency for these two protocols with preserved RNA yield and integrity compared to QIAZ-2. We then selected QIAZ-3 and QIAZ-4 to verify their suitability for analyzing miRNA, and, similarly to RNA, we found that the amplification efficiency of rno-miR-223-3p was within the acceptable range.

To verify alternative RNA isolation approaches that efficiently remove polysaccharides within the lysis buffer, we adapted the protocol by Dos Reis Falcao et al. [23] that includes lysis with TRIZOL and GHCL buffer followed by supplementation with 0.2 M potassium acetate. The latter precipitates polysaccharides within the lysis buffer, which are then discarded during centrifugation. Although RNA and miRNA yield was increased in GHCL-1 and GHCL-2, the RIN numbers

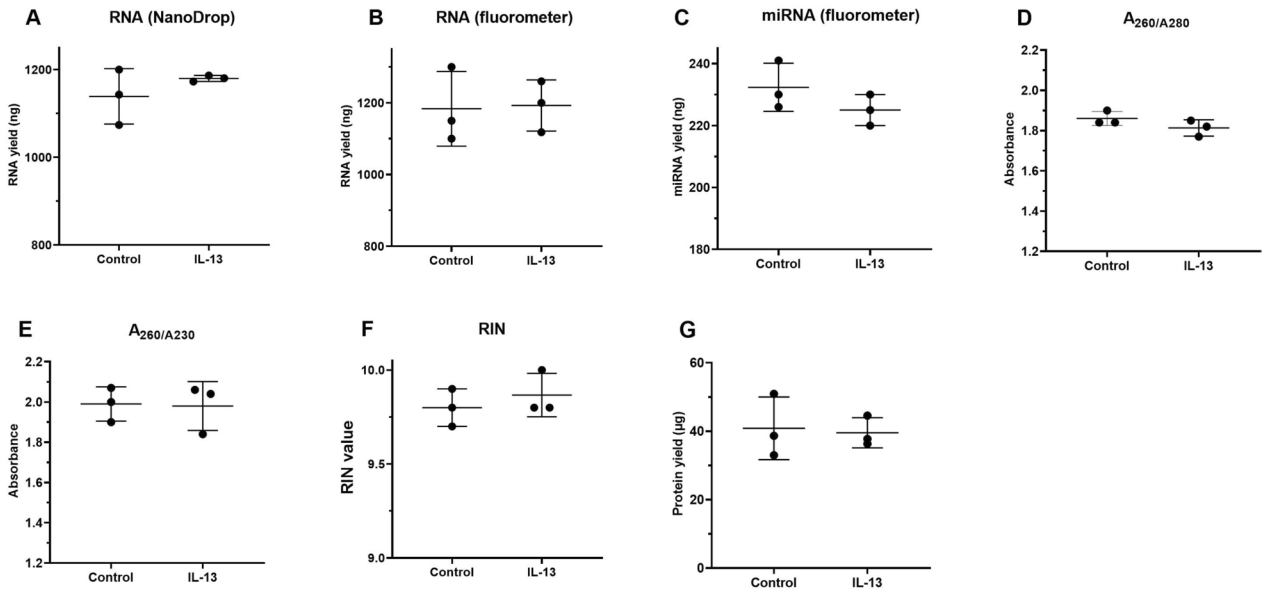

Fig. 13 Assessment of miRNA, RNA, and protein concentration, purity, and integrity between PCLS treated with IL-13 and untreated

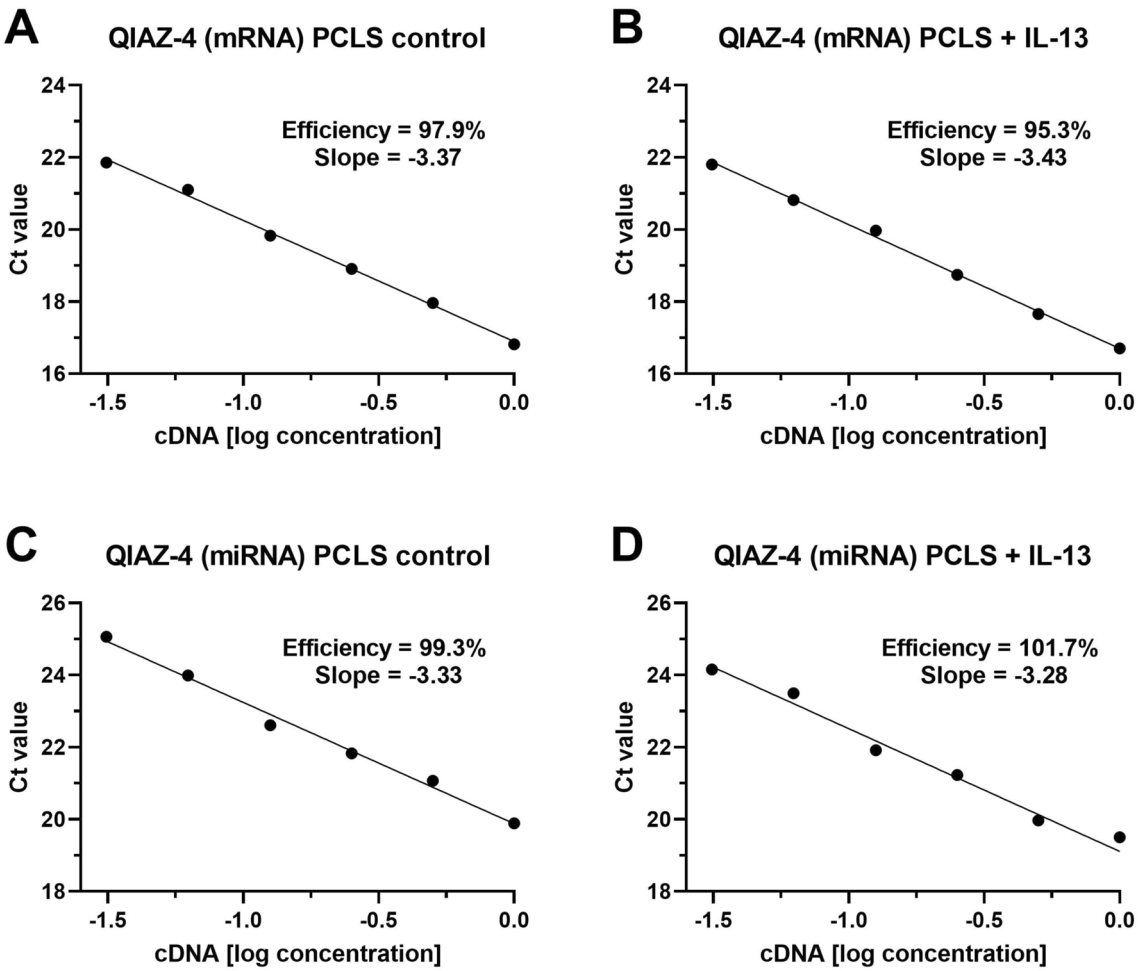

Fig. 14 Comparison of *GAPDH* and rno-miR-223-3p amplification efficiency between PCLS treated with IL-13 (**A**, **C**) and untreated (**B**, **D**)

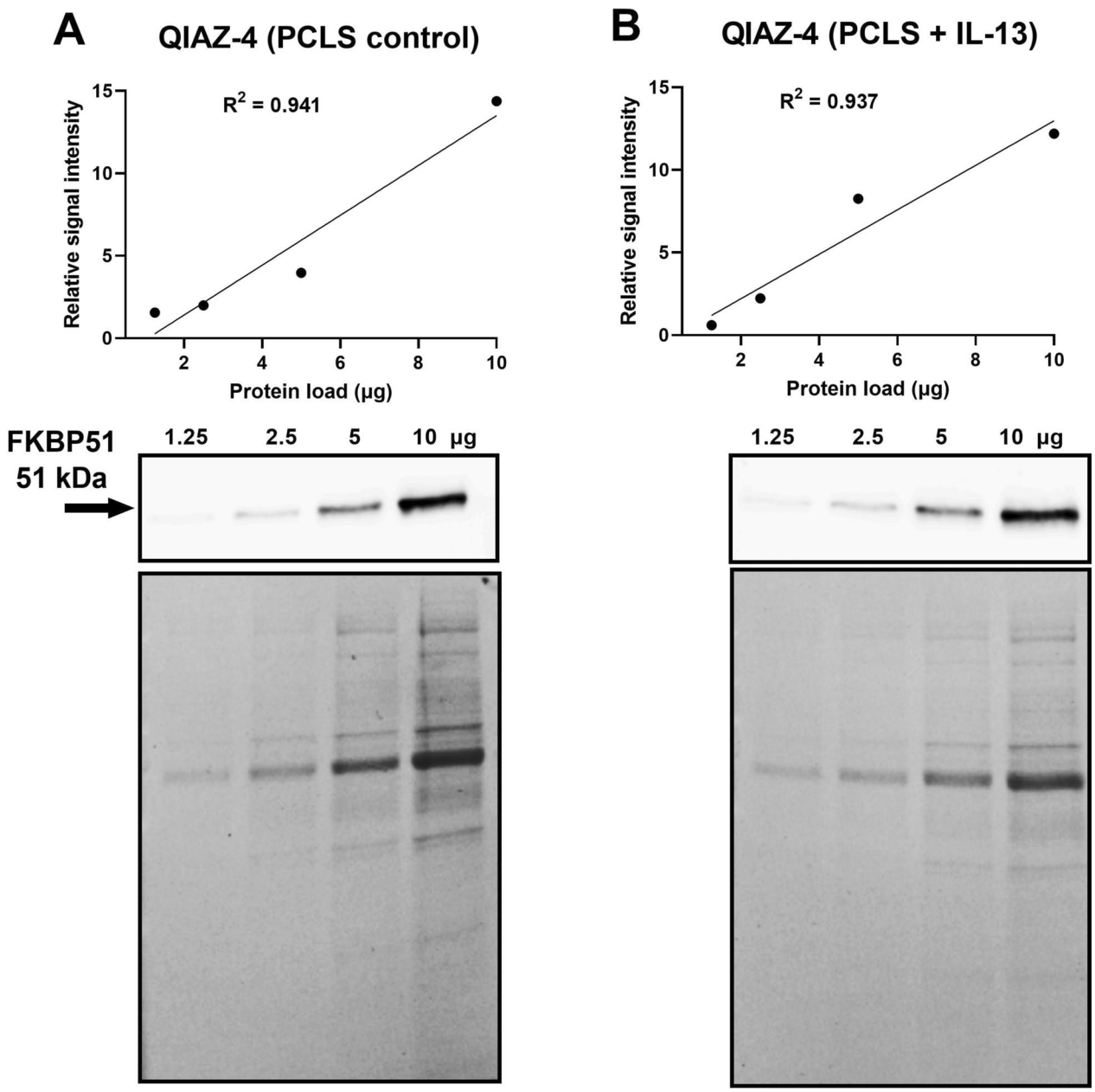

Fig. 15 Comparison of linear detection of FKBP51, total protein and normalized signal intensity between PCLS treated with IL-13 and untreated

for these two protocols were under the acceptable range, indicating degraded RNA.

To our knowledge, protein isolation from PCLS was not previously optimized in any study. Therefore, we first lysed PCLS in RIPA Lysis Buffer as an isolation control for QIAzol-based protocols. We found that precipitation with acetone for 3 h at −20 °C (QIAZ-P3, QIAZ-P4, QIAZ-P5) provided the highest protein yield, similar to that obtained with RIPA. Furthermore, the protein extracted with these protocols showed linear, specific detection of total protein as well as the level of FKBP51, highlighting the applicability of our protocol in proteomic analyses. In a general comparison of efficiency on native rat lungs, our protocols provided superior yield compared to the standard QIAzol protocol and results comparable to those obtained with RIPA buffer. We also demonstrated that QIAZ-4 can be successfully used in PCLS treated with proinflammatory cytokines such as IL-13 as an ex vivo model of allergic inflammation. In these experiments, QIAZ-4 was used to confirm the

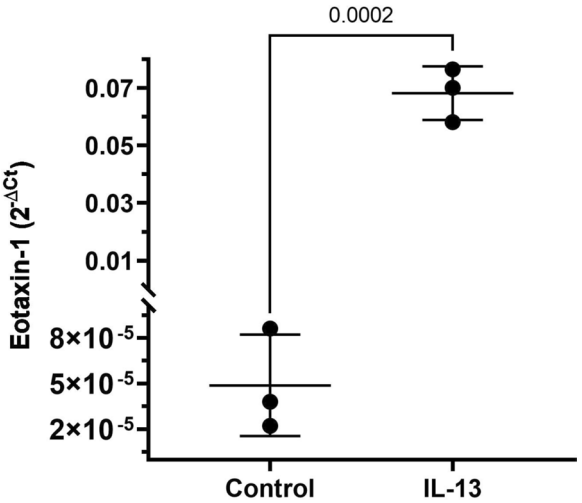

Fig. 16 Gene expression of Eotaxin-1 in PCLS treated with IL-13 as compared to untreated control

increased expression of Eotaxin-1, an eosinophilic chemoattractant, following IL-13 treatment that highlights the utility of our protocols in studies employing PCLS as a translational model of human respiratory diseases. Finally, our optimized protocol can be used for simultaneous DNA isolation, however, it requires cleaning up with a Monarch PCR & DNA Cleanup Kit (New England Biolabs) based on silica columns to reach acceptable purity (data not shown).

In summary, we developed a cost-effective protocol for the simultaneous isolation of high-yield and purity miRNA, RNA, and protein from agarose-rich samples/tissues suitable for downstream applications.

Abbreviations

PCLS	Precision-cut lung slices
LDH	Lactate dehydrogenase
PBS	Phosphate-buffered saline
GHCL	Guanidinium hydrochloride

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12931-024-03026-3.

Supplementary material 1.

Supplementary material 2.

Acknowledgements
Not applicable

Author contributions
All authors read and approved the final manuscript. WL, JN, KZ, MK: generated and cultured rat PCLS WL, JN, KZ, BN, KS, ZS: performed RNA isolation and real-time PCR analysis WL, KS, ZS: performed protein isolation and Western-blot analysis WL, AS: wrote and revised the manuscript.

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Availability of data and materials
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable (In accordance with local requirements, ethical approval was not required as animals were subjected to tissue sampling but not to experimental procedures).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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