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Short communication

High salt buffer improves integrity of RNA after fluorescence-activated cell sorting of intracellular labeled cells

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ABSTRACT

Over the past years, massive progress has been made in the ability to collect large-scale gene expression data from a limited sample size. Combined with improvements in multiplex flow cytometry-based techniques, this has made it possible to isolate and characterize specific cellular subtypes within heterogeneous populations, with a great impact on our understanding of different biological processes. However, sorting based on intracellular markers requires fixation and permeabilization of samples, and very often the integrity of RNA molecules is compromised during this process. Many attempts have been made to improve the quality of nucleic acids from such samples, but RNA degradation still remains a limiting factor for downstream analyses. Here we present a method to isolate high quality RNA from cells that have been fixed, permeabilized, intracellularly labeled and sorted. By performing all incubation steps in the presence of a high salt buffer, RNA degradation was avoided and samples with remarkable integrity were obtained. This procedure offers a straightforward and very affordable technique to retrieve high quality RNA from isolated cell populations, which increases the possibilities to characterize gene expression profiles of subpopulations from mixed samples, a technique with implications in a broad range of research fields.

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1. Introduction

Awareness regarding the importance of cellular heterogeneity in complex tissues and samples is increasing, and much knowledge would be gained would it be possible to obtain high quality RNA from cells isolated from heterogeneous populations, for example from primary tissue. Flow cytometry based techniques are powerful tools for isolation of cells, but depend heavily on labeling of extracellularly facing antigens for retrieval of intact RNA. Intracellular labeling of fixed and permeabilized cells allows for separation of cell populations using fluorescence-activated cell sorting (FACS). The necessary fixation and permeabilization steps before intracellular antibody staining however has profound effects on RNA integrity (Russell et al., 2013). The single stranded nature of RNA molecules renders them sensitive to degradation, and crosslinking resulting from fixation has well known detrimental effects on RNA integrity and often precludes downstream analyzes such as gene arrays and quantitative real-time PCR (QPCR) (Auer et al., 2003; Fleige and Pfaffl, 2006). Several attempts have been made to improve protocols in order to isolate RNA from sorted cells with acceptable quality, however, RNA integrity values remain problematically low (Diez et al., 1999; Nishimoto et al., 2007; Iglesias-Ussel et al., 2013). Different RNA-preserving fixation solutions has also been developed, but these are often costly and not compatible with antibody labeling (Zaitoun et al., 2010).

Here we present a method to obtain high quality RNA from intracellularly labeled and sorted cells in a straightforward and affordable way. We show that the presence of a high-salt buffer protects RNA from degradation during antibody staining and sorting steps, which enables the collection of high quality RNA from cells sorted based on intracellular markers.

2. Materials and methods

2.1. Cell culture

786-O and Jurkat cells were cultured in DMEM high glucose (Thermo scientific) supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin solution (Thermo scientific) at 37 °C and 5% CO_2.

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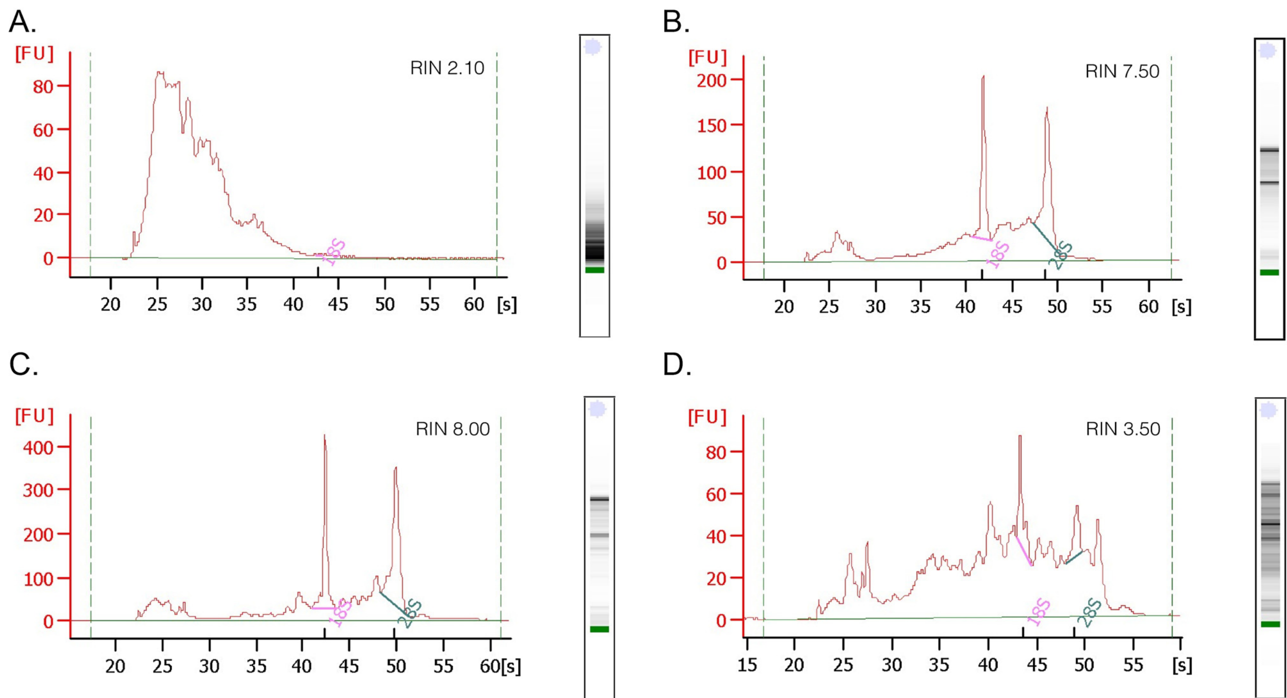

Fig. 1. (A) Electropherograms from Bioanalyzer showing the poor integrity of RNA isolated from cells after fixation, permeabilization, antibody labeling and sorting. Fixation and permeabilization alone (B), as well as fixation and permeabilization followed by sorting (C) results in RNA of high quality. (D) Degraded RNA from cells isolated after fixation, permeabilization, and antibody labeling.

2.2. Fixation and permeabilization

At 90% confluency cells were trypsinized and counted. Cells were fixed in 4% paraformaldehyde solution (PFA) for 10 min at room temperature. Fixed cells were kept on ice for 1 min before permeabilization by addition of ice-cold 100% methanol to a final concentration of 90%. Cells were subsequently incubated on ice for 30 min and stored at $-20\,^{\circ}$C.

2.3. Antibody staining

Fixed and permeabilized cells kept in methanol were centrifuged for 5 min at $1000 \times g$ at $4\,^{\circ}$C. Supernatant was removed and cells were washed in PBS or 2 M NaCl in PBS to remove the remaining methanol. Cells were incubated in blocking buffer (0.5% BSA in PBS, with or without 2 M NaCl for 10 min in room temperature. Staining with primary conjugated anti-human cytokeratin 7/8 antibody (Alexa Fluor 647, clone CAM5.2, BD Biosciences) was performed for 20 min at room temperature in blocking buffer. Excess antibody was washed away using blocking buffer. Antibody intensity was analyzed on a FACS Calibur (BD Biosciences).

2.4. Fluorescence activated cell sorting

For cell sorting a FACSAria Cell Sorter (BD Biosciences) was used. As a precaution to avoid RNA degradation cells were collected in 4 M NaCl and 1% BSA in PBS.

2.5. RNA isolation and quality control

RNA was isolated using Qiagen's FFPE kit according to the manufacturer's protocol. For all samples the highest recommended volume of PKD buffer (240 μL) was used to avoid interference from remnants of high salt buffer. RNA integrity was assessed with Agilent RNA 6000 Nano kit and Agilent 2100 Bioanalyzer (Agilent Technologies).

2.6. cDNA synthesis and quantitative real-time PCR

cDNA synthesis was performed using MultiScribe Reverse Transcriptase enzyme and random hexamers (Applied Biosystems). Quantitative real-time PCR was performed with SYBR Green PCR Master Mix on a 7300 Real-Time PCR System (Applied Biosystems). The comparative Ct method was used to quantify relative RNA levels and three separate housekeeping genes (HMBS, RPL13A, YWHAZ) were used for normalization. Primer sequences used were CK8 forward: 5′-AGGGCTGACCGACGAGAT-3′, reverse: 5′-CACCACAGATGTGCTCGAGA-3′, HMBS forward: 5′-GGCAATGCGGCTGCAA-3′, reverse: 5′-GGGTACCCACGCGAATCAC-3′, RPL13A forward: 5′-CCTGGAGGAGAAGAGGAAAGAGA-3′, reverse: 5′-TTGAGGACCTCTGTGTATTTGTCAA-3′, YWHAZ forward: 5′-ACTTTTGGTACATTGTGGCTTCAA-3′, reverse: 5′-CCGCCAGGACAAACCAGTAT-3′.

3. Results and discussion

3.1. RNA integrity is lost during antibody incubation of fixed and permeabilized cells

The detrimental effects on RNA integrity caused by formalin crosslinking and antibody incubation steps have made it difficult to obtain RNA of adequate quality from complex samples where fixation and intracellular labeling is required before cell sorting. To illustrate this problem, we performed the procedure in the human renal carcinoma cell line 786-O. Cells were fixed in 4% paraformaldehyde, permeabilized using methanol and labeled with a primary Alexa 647 conjugated cytokeratin 7/8 antibody (CK7/8), that should result in intracellular staining of these cells. Positive cells were collected using FACS and RNA was isolated. The quality of the obtained RNA was determined on a Bioanalyzer. The RNA integrity number, or RIN value, obtained from the Bioanalyzer gives a measurement of the degree of degradation that has occurred, where a RIN value of 10 corresponds to intact RNA (Schroeder et al.,

2006). As shown in Fig. 1A, the process of fixation, permeabilization, antibody labeling and sorting resulted in RNA with a RIN value as low as 2.1. We next evaluated the effect of each of these steps on RNA integrity. Fixation and permeabilization alone had no negative effect (Fig. 1B); also sorting of fixed and permeabilized cells could be performed without compromising RNA quality (Fig. 1C). However, RIN-values of RNA from cells that were fixed, permeabilized and incubated with antibody were considerably lower (Fig. 1D), indicating that the blocking and antibody incubation steps had a detrimental effect on RNA integrity.

3.2. Incubation in high salt buffer preserves RNA integrity

It has previously been reported that antibody incubation in a high salt buffer improves the quality of RNA obtained after laser micro-dissection of specifically stained cell populations (Brown and Smith, 2009). This method yielded RNA of better quality than the commercially available RNA preserving agent RNAlater, and enabled prolonged antibody incubation times without compromising RNA integrity. We therefore set out to test whether this method could also be applicable on cells isolated by FACS. In order to test this method, we performed blocking and antibody labeling in the presence of 2 M NaCl. RIN-values of RNA isolated from cells incubated under these conditions were markedly improved, as illustrated in Fig. 2A. Also when the cells were sorted after fixation, permeabilization, blocking and antibody labeling at room temperature in high salt buffer, RNA with RIN values as high as 7.9 were obtained (Fig. 2B). These results indicate that the high salt buffer protects RNA from fragmentation, resulting in distinctly improved RNA quality. No difference in total RNA yield was observed whether or not the high salt buffer was used.

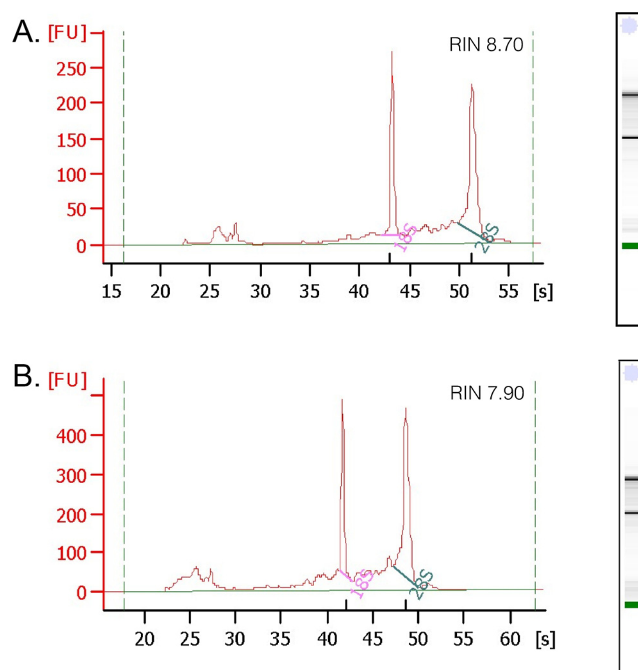

Fig. 2. Antibody incubation of fixed and permeabilized cells performed in the presence of high salt buffer results in RNA of high quality (A), also when cells are sorted after labeling (B).

3.3. High salt buffer do not have major impact on antibody binding efficiency

Antibody binding to specific epitopes can be sensitive to changes induced by fixation and other procedures. To assure that the high salt buffer did not compromise the specificity or affinity of the antibody, 786-O cells were stained with the CK7/8 antibody in PBS or high salt buffer, and the intensity of the staining was analyzed by flow cytometry. 786-O cells stained in PBS buffer were positive for CK7/8, as expected. Antibody incubation in high salt buffer resulted in a slight decrease in staining intensity, but the same proportion of cells were positive, and the signal was still well separated from the signal of unstained cells (Fig. 3A and B). In contrast to 786-O cells, human T lymphocyte Jurkat cells do not express CK7/8, and should thus be negative for this antibody. As expected, all Jurkat cells were negative for this antibody regardless of whether the antibody incubation occurred in PBS or high salt buffer, indicating that high salt buffer did not induce unspecific staining (data not shown).

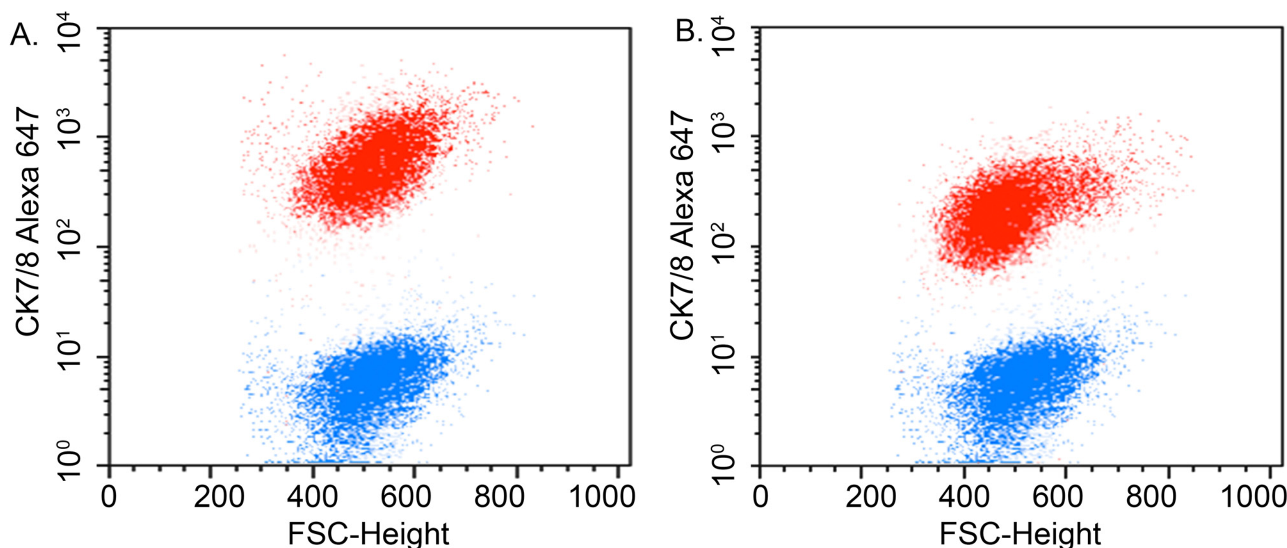

Fig. 3. Flow cytometry results showing the intensity of CK7/8 Alexa 647 antibody staining of positive (red) 786-O cells, where antibody incubation was performed in PBS (A) or high salt buffer (B), compared to unstained cells (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

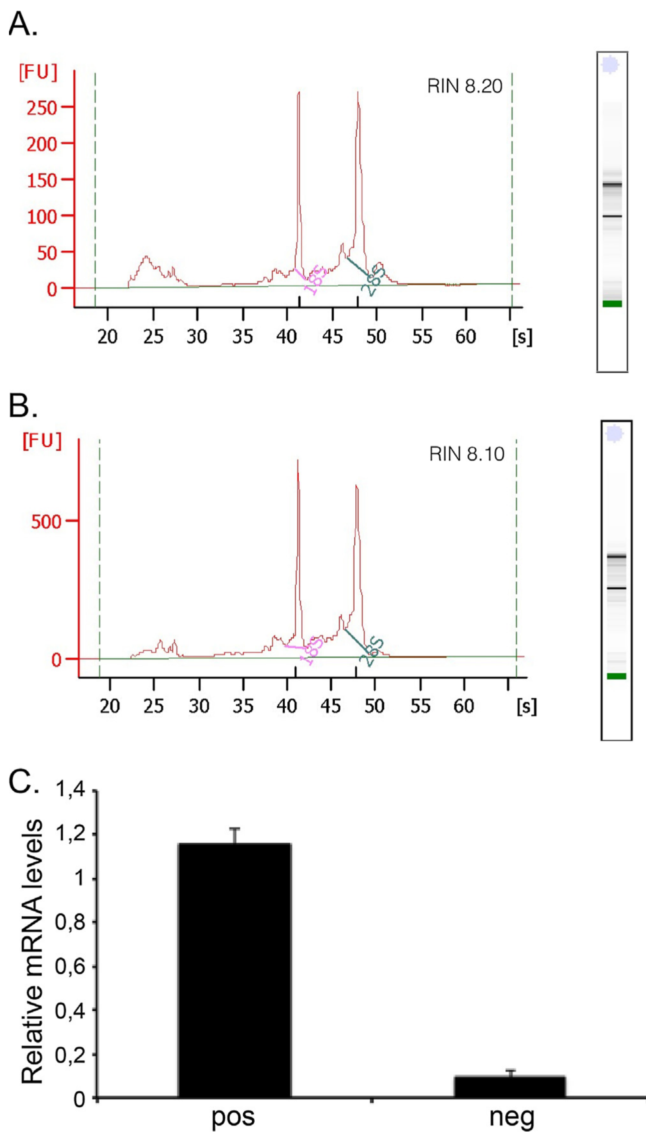

Fig. 4. Electropherograms of RNA isolated from a mixture of 786-O and Jurkat cells after fixation, permeabilization and CK7/8-antibody labeling followed by sorting based on CK7/8 positivity. Both CK7/8-positive (A) and negative (B) populations have high RIN values. (C) QPCR results showing expression of CK8 in the population that stained positive for this antibody, while negative cells express very low levels of this marker.

3.4. *High salt buffer enables preparation of high quality RNA from a specific cell population identified and collected by FACS after intracellular antibody labeling*

To illustrate possible applications of this improved method, we prepared a mixture of the two different cell types, 786-O and Jurkat cells. Cells were fixed, permeabilized and stained with the CK7/8 antibody in the presence of high salt buffer as described above. Using FACS, positive (786-O) and negative (Jurkat) cells were separated and RNA was isolated from both populations (Fig. 4A and B). Expression of cytokeratin 8 was analyzed in these samples using QPCR, confirming the separation of the two populations (Fig. 4C). This experiment also validates that the quality of the isolated RNA is sufficient for cDNA synthesis and QPCR reactions.

An additional advantage using this protocol is the possibility to store samples before analysis by FACS, enabling collection of large datasets that can be analyzed in parallel. It also allows for samples to be shipped to other locations for analysis, something that can be useful in cases of multi-centric collaborations or when the FACS analysis is performed at distant core facilities.

4. Conclusion

This improved method opens up for low-cost FACS based cell sorting of different subtypes of cells from complex populations, using intracellular labeling of cells. The protocol results in high quality RNA suitable for further downstream characterization by a broad repertoire of molecular biology platforms.

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