Abstract

Increasingly, Flow Cytometry Shared Resource Facilities are asked to sort fixed cells for RNA isolation either in bulk or at the single cell level. With so many fixation methods in the literature, the Flow Cytometry Research Group (FCRG) decided to perform a systematic evaluation of the reported fixation methods to assess how the different fixatives affected the quality of RNA isolated from sorted cells. Based on the literature, four different common chemical fixatives were analyzed using the human cell line HL-60. The assessment included formaldehyde fixation, alcohol fixation (methanol and ethanol), zinc fixation, and three commercial reagents. Each method was tested at two separate shared facilities and for some methods different variations of the fixation procedure (i.e., time, temperature, and dilution) were also tested. The protocol involved fixing the cells first. The next day, fixed cells were sorted into lysis buffer. RNA isolated from the cells was assayed to determine purity, quality, and concentration. Each condition had sorted and unsorted samples. A NanoDrop was used for purity and a Bioanalyzer for quality and concentration. Few fixed samples (sorted or not) returned any intact RNA, pointing to the unreliability of many common fixation methods. Sorting did correlate with decreased RNA yield, although the cause has yet to be determined.

Fixation Protocols

1. Paraformaldehyde (PFA) (defined as methanol-free formaldehyde) was pelleted and resuspended to 1×10^6 cells/mL with PFA under the following conditions: 2% PFA at RT, 2% PFA on ice, 4% PFA at RT, and 4% PFA on ice. After a 20 min incubation, cells were washed with PBS-BSA.

2. Three alcohol-based fixatives were tested: 100% methanol, 70% ethanol, and 95.5 ethanol/acetic acid (EIOH:AA). For methanol-fixed samples, cells were pelleted, resuspended in 4 mL 100% methanol, and incubated on ice for 15 min. For ethanol-fixed samples, cells were pelleted, resuspended in 5 mL 70% ethanol, and incubated on ice for 30 min. For EIOH:AA-fixed samples, cells were pelleted, resuspended in 20 mL 95.5% EIOH:AA mix, incubated at -20°C for 15 min, centrifuged at 120 g for 10 min at 4°C, resuspended a second time in 20 mL 95.5% EIOH:AA, and incubated at -20°C for 15 min. All fixed samples were washed with PBS-BSA, resuspended in 2×10^6 cells/mL PBS-BSA, and stored for 24 h at 4°C.

3. Zinc-based – Samples were suspended in 1 mL Zinc Buffer [ZnBF: 0.1 M Tris-Cl, pH 7.8; 0.05% (v/v) calcium acetate; 17.16 mM zinc trifluoroacetate; 0.5% (v/v) zinc chloride] and stored overnight at -20°C in glycerol. Prior to sorting, cells were washed with PBS-BSA.

4. Commercial reagents – Cells were fixed with one of the following kits per manufacturer’s recommendations: BD Cytofix/Cytoperm (#554714) for 20 min at 4°C, eBioscience Intracellular Fix (#88-8424-00) for 20 min at RT, and FACSLyse (#540202) for 10 min at RT. All samples were washed twice with PBS-BSA.

RNA Testing

1. RNA extraction was performed using the RNasy Plus Micro Kit (Qiagen, Valencia, CA) per manufacturer’s recommendations.

2. RNA purity testing was performed with a NanoDrop.

3. RNA quality and yield testing was performed with the Agilent Bioanalyzer 2100 system.

Conclusions

- Highest RNA quality (as indicated by RIN) was attained from unfixed samples. Thus, our results indicate that all fixation methods had an affect on the quality and yield of the samples whether sorted or non-sorted.

- Our results indicate the need for identifying the method of fixation and the method of isolation of RNA for a specific cell type. Certain methods of fixation may work best for a particular cell type or tissue. Further processing of the sample for RNA isolation needs to take into account the fixation method. Determining these factors prior to sorting would be highly recommended by the FCRG.

- Our results indicate that a significantly lower RNA yield from sorted cells should be expected.

References