Video Article

Increasing cDNA Yields from Single-cell Quantities of mRNA in Standard Laboratory Reverse Transcriptase Reactions using Acoustic Microstreaming

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Abstract

Correlating gene expression with cell behavior is ideally done at the single-cell level. However, this is not easily achieved because the small amount of labile mRNA present in a single cell (1-5% of 1-50pg total RNA, or 0.01-2.5pg mRNA, per cell [1]) mostly degrades before it can be reverse transcribed into a stable cDNA copy. For example, using standard laboratory reagents and hardware, only a small number of genes can be qualitatively assessed per cell [2]. One way to increase the efficiency of standard laboratory reverse transcriptase (RT) reactions (i.e. standard reagents in microliter volumes) comprising single-cell amounts of mRNA would be to more rapidly mix the reagents so the mRNA can be converted to cDNA before it degrades. However this is not trivial because at microliter scales liquid flow is laminar, i.e. currently available methods of mixing (i.e. shaking, vortexing and trituration) fail to produce sufficient chaotic motion to effectively mix reagents. To solve this problem, micro-scale mixing techniques have to be used [3,4]. A number of microfluidic-based mixing technologies have been developed which successfully increase RT reaction yields [5-8]. However, microfluidics technologies require specialized hardware that is relatively expensive and not yet widely available. A cheaper, more convenient solution is desirable. The main objective of this study is to demonstrate how application of a novel "micromixing" technique to standard laboratory RT reactions comprising single-cell quantities of mRNA significantly increases their cDNA yields. We find cDNA yields increase by approximately 10-100-fold, which enables: (1) greater numbers of genes to be analyzed per cell; (2) more quantitative analysis of gene expression; and (3) better detection of low-abundance genes in single cells. The micromixing is based on acoustic microstreaming [9-12], a phenomenon where sound waves propagating around a small obstacle create a mean flow near the obstacle. We have developed an acoustic microstreaming-based device ("micromixer") with a key simplification; acoustic microstreaming can be achieved at audio frequencies by ensuring the system has a liquid-air interface with a small radius of curvature [13]. The meniscus of a microliter volume of solution in a tube provides an appropriately small radius of curvature. The use of audio frequencies means that the hardware can be inexpensive and versatile [13], and nucleic acids and other biochemical reagents are not damaged like they can be with standard laboratory sonicators.

Video Link

The video component of this article can be found at http://www.jove.com/video/3144/

Protocol

1. Micromixing an RT Reaction

1. Before performing an RT reaction with micromixing, equilibrate the micromixer to the desired temperature of the RT reaction.
    1. Place the micromixer inside a 37°C (or the temperature recommended by the RT supplier) incubator for at least 1 hour prior to the onset of the RT reaction.
2. Set up the RT mix according to the reverse transcriptase supplier's (e.g. MMTV-RT from Promega, Omniscript from Qiagen) instructions, except use single-cell amounts of input total RNA (e.g. 0.1-1pg/μl), instead of the ng-μg amounts recommended by suppliers.
    1. We use standard sterile, nuclease-free 200μL thin-walled PCR tubes.
3. Position RT tubes securely into the micromixer as soon as they are ready for mixing. The micromixer should remain inside the 37°C incubator for the duration of the RT reaction.
4. Select the appropriate frequency and amplitude of micromixing. In this example we use 150Hz.
5. Switch the micromixer on and leave it on for the entire duration of the RT reaction (60 minutes).
6. Switch the micromixer off and take the RT tubes out once the RT reaction is complete.
7. Place the RT tubes on ice and proceed as normal with further applications such as quantitative Polymerase Chain Reaction (qPCR or real-time PCR).

2. Representative Results:

The benefit of micromixing in the context of RT reactions is an increase in cDNA yield relative to when other mixing methods (e.g. shaking, vortexing or trituration) are used. This can be seen, for example, upon subsequent analysis of the cDNA using quantitative Polymerase Chain Reaction (qPCR or real-time PCR). Figure 2A illustrates the effects of micromixing for the first 5 minutes (blue, "micromixed5") or the entire 60 minutes (red, "micromixed60") compared with trituration of the RT reaction immediately prior to the RT incubation (black, "trituration"). In this case the volume of the preceding RT reaction was 25µL and it contained 2.5pg of total RNA. qPCR traces using primers designed to detect cDNA representing Nurr-1 mRNA are shown above, and the means ±SEMs of the number of cycles to reach 50% maximum fluorescence in 3 repeats of this same experiment are plotted below. The asterisk denotes a statistically significant difference (one-way ANOVA with Tukey multiple comparisons). Note that micromixing for the first 5 minutes of the preceding RT reaction produced a non-significant improvement over trituration whereas micromixing for the entire 60 minutes of the preceding RT reaction produced a significant improvement over trituration, the magnitude of which was equivalent to approximately 15 qPCR cycles, on average.

The benefit of micromixing can also be seen in melting curve analysis of the subsequent qPCR products obtained. In the experiment illustrated in Figure 2B, micromixing for the entire 60 minutes of the preceding RT reaction resulted in a consistent qPCR signal in duplicate samples (Figure 2Ba, 2 red traces), whereas micromixing for the first 5 minutes or trituration only produced inconsistent qPCR traces (Figure 2Ba, 2 blue traces and 2 black traces, respectively). The grey traces are negative controls ("neg.") in which cDNA was left out of the qPCR reaction. When the qPCR products from this experiment were denatured by ramping their temperature (i.e. melting curve analysis) it was apparent that appropriate PCR products (i.e. an amplicon that was identical to that obtained when much higher concentrations of mRNA were reverse transcribed and amplified by qPCR, data not shown) were present only following RT reactions that had been micromixed for 60 minutes (Figure 2Bb, 2 red traces). All the others (i.e. micromixed5, trituration and negative controls) generated alternative amplicon products (e.g. primer dimers).

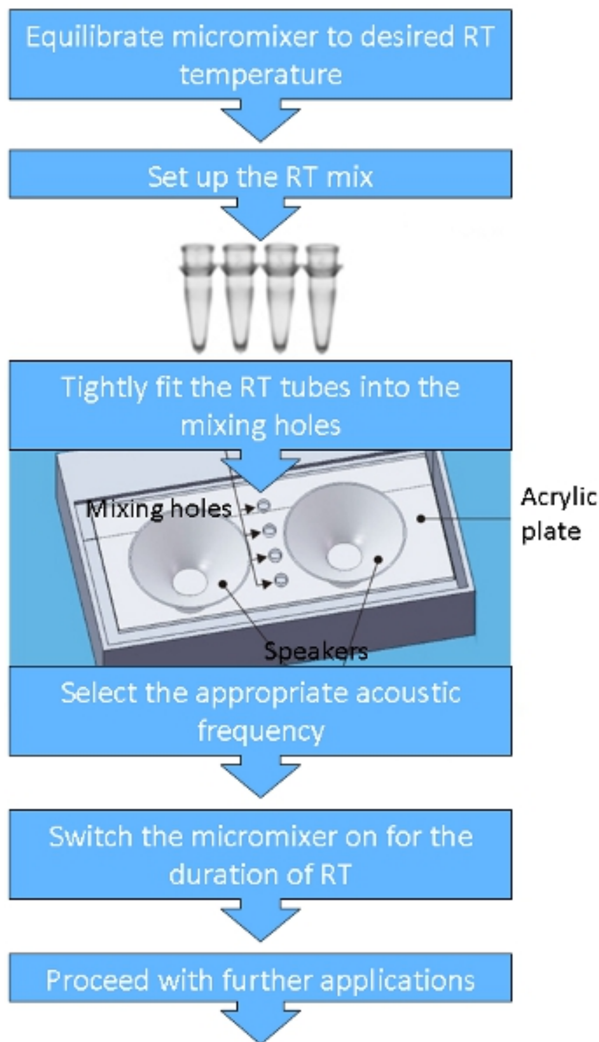

Figure 1. Flow diagram of the protocol for applying micromixing to standard laboratory RT reactions. More detailed descriptions of the physical principle involved, the micromixer and how to build an in-house micromixer are provided in references [13,14]

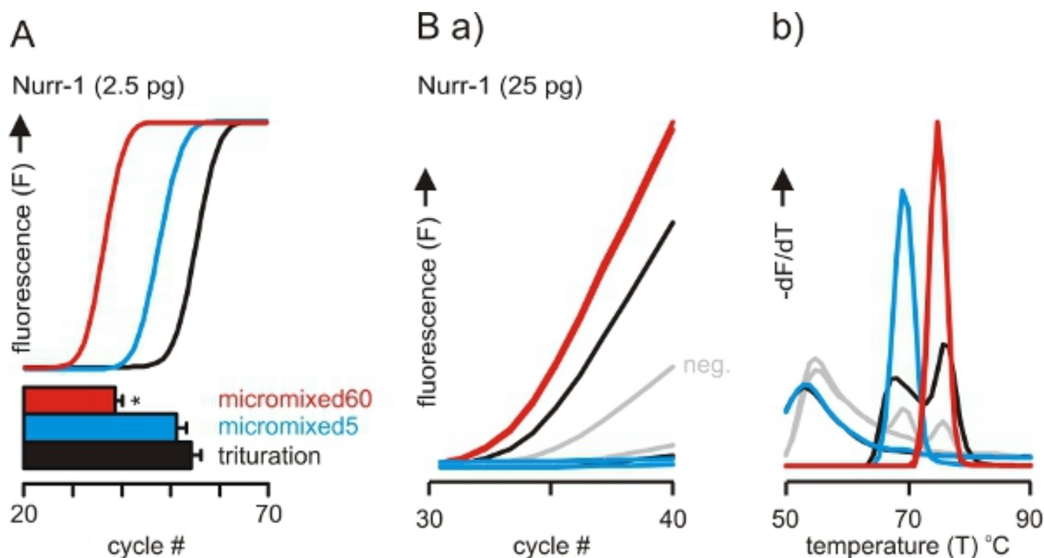

Figure 2. Representative qPCR data illustrating the benefits of micromixing applied to preceding RT reactions comprising single-cell quantities of mRNA in otherwise standard laboratory RT reactions. **A** Example qPCR traces detecting cDNA representing mRNA encoding the Nurr-1 gene. The preceding RT reactions contained 2.5pg of total RNA in a volume of 25μL and were performed in an incubator at 37°C for 60 minutes. RT reactions were mixed by trituration of the RT mix prior to incubation (black traces, "trituration"), micromixed during the first 5 minutes of the RT reaction (blue traces, "micromixed5") or micromixed for the entire 60 minutes of the RT reaction (red traces, "micromixed60"). Means ± SEMs of the number of qPCR cycles required to reach 50% maximum fluorescence are plotted below for n=3 repeats of this experiment. Asterisk indicates a significant difference (one-way ANOVA with Tukey multiple comparisons). **B** Another qPCR experiment examining Nurr-1 cDNA product, this time following RT reactions containing 25pg of total RNA in a volume of 25μL. qPCR traces **(a)** as well as melting curve analysis of the qPCR products obtained **(b)** are shown for the different mixing conditions. Negative controls in which no cDNA was included in the preceding RT reaction are also included (gray traces, "neg."). RT reactions with much higher concentrations of mRNA were also performed and produced melting curve peaks (data not shown) identical to those in **(b)** for micromixed60 (red traces) samples.

Discussion

The method of application of micromixing to standard laboratory RT reactions described here can, of course, involve mRNA harvested via any method (e.g. cell lysis, laser capture microdissection). It can also comprise any brands or types of RT reagents, any temperature (within the tolerance of the materials of the micromixer), and any period of time. For example, we have observed improved cDNA yields from RT reactions comprising random hexamer or oligo-dT primers. It might also be applied to other biochemical transformations (e.g. DNA hybridization [15]) conforming to the following constraints. The main constraint of the current technique is the reaction must occur within a small (microliter) drop of solution that forms a curved liquid-air interface (e.g. a meniscus). We routinely use volumes of 10 or 25μL in 200μL thin-walled PCR tubes, producing menisci with radii of curvature of 4.7 or 6.9mm, respectively. This is a general requirement for a vortex flow to be set up in the drop, which elongates the interface between different solutions and allows diffusion to take place over shorter timescales. This constraint therefore applies to all situations in which micromixing might be used. Indeed the use of small volumes can be an advantage because often the amount of input material (e.g. RNA, DNA) is limited, plus significant savings can be made on other reagents. In the specific context of improving RT reaction yields, the reaction must also comprise a limiting amount of mRNA. We have shown that micromixing standard laboratory RT reactions comprising single-cell amounts of input RNA increases their cDNA yield by approximately 10-100-fold over standard methods of mixing (shaking, vortexing or trituration), including for genes other than Nurr-1 illustrated here [14]. We have also shown this improvement decreases as the concentration of input RNA increases [14]. Presumably this is because at higher RNA concentrations, the increased rate of diffusion in the absence of micromixing ensures more mRNA is converted to cDNA before it degrades.

The micromixing method will benefit from high-fidelity transmission of the acoustic signal through the reaction vessel to the reaction solution. We helped achieve this by careful machining of the mixing holes in the acrylic plate overlying the speakers (Figure 1) such that they were tapered to precisely fit the 200μL thin-walled PCR tubes used. This maximized the contact between the tubes and the acrylic plate and therefore maximized transmission of the acoustic signal. One also needs to ensure that the reaction tubes are securely held within the mixing holes because they can come out during mixing as a result of the mechanical vibrations.

Our definition of acoustic microstreaming is possibly broader than some phenomenologically-based definitions. However, there is a good review by Riley [16] that classifies these flows fundamentally, rather than phenomenologically. The nonlinear term in the Navier-Stokes equation has to be large enough so that at second order there is a mean flow. Since that term is a velocity times a velocity gradient, anything we do to make the length scale over which the velocity varies sufficiently small (i.e. make the velocity gradient sufficiently large) can give rise to a microstreaming flow. Our acoustic microstreaming-based device ("micromixer") enables acoustic microstreaming at audio (i.e. low power) frequencies by ensuring the system has a liquid-air interface with a small radius of curvature [13]. In contrast, "acoustic streaming" is often defined by a nonlinear term that is made large by a large velocity (i.e. high power). In our case, a small drop in a well is made to oscillate; the surface tension of the drop, together with its viscosity, may play a role in creating a mean flow useful for mixing [13]. Experiments have also shown that large (10-100μm) particles can be moved within larger vibrating drops than we use, however this is probably by a different mechanism specific to particles [17].

In conclusion, the application of micromixing to standard laboratory RT reactions comprising single-cell amounts of RNA is an effective way to dramatically increase their cDNA yield. This improvement is over and above what can be achieved using currently available methods of mixing microliter volumes (i.e. shaking, vortexing or trituration). Although there are many circumstances in which microfluidics-based devices performing RT would be a great advance, in other circumstances, including our own, micromixing alone provides significant and adequate benefit without the need for further specialized equipment and methods.

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Disclosures

The authors have nothing to disclose.

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References

1. Livesey, F.J. *Brief Funct Genomic Proteomic.* **2** (1), 31 (2003).
2. Aumann, T.D., Gantois, I., Egan, K. et al. *Exp Neurol.* **213** (2), 419 (2008).
3. Ottino, J.M., & Wiggins, S. *Philos Transact A Math Phys Eng Sci.* **362** (1818), 923 (2004).
4. Losey, M.W., Jackman, R.J., Firebaugh, S.L. et al. *J Microelectromech. Syst.* **11**, 709 (2002).
5. Bontoux, N., Dauphinot, L., Vitalis, T. et al. *Lab Chip.* **8** (3), 443 (2008).
6. Marcus, J.S., Anderson, W.F., & Quake, S.R. *Anal Chem.* **78** (9), 3084 (2006).
7. Marcus, J.S., Anderson, W.F., & Quake, S.R. *Anal Chem.* **78** (3), 956 (2006).
8. Warren, L., Bryder, D., Weissman, I.L. et al. *Proc Natl Acad Sci U S A.* **103** (47),17807 (2006).
9. Wang, S.S. , Jiao, Z.J., Huang, X.Y. et al. *Microfluidics Nanofluidics.* **6**, 847 (2009).
10. Ahmed, D., Xiaole, M., Juluri, B.K. et al. *Microfluidics Nanofluidics.* **7** (5), 727 (2009).
11. Paxton, W.F., O'Hara, M.J., Peper, S.M. et al. *Anal Chem.* **80**, 4070 (2008).
12. Wixforth, A. *J. Assoc. Lab. Autom.* **11**, 399 (2006).
13. Petkovic-Duran, K., Manasseh, R., Zhu, Y. et al. *Biotechniques.* **47** (4), 827 (2009).
14. Boon, W.C., Petkovic-Duran, K., White, K. et al. *Biotechniques.* **50** (2), 116 (2011).
15. Liu, R.H., Lenigk, R., Druyor-Sanchez, R.L. et al. *Anal Chem.* **75** (8), 1911 (2003).
16. Riley, N. *Ann Rev Fluid Mech.* **33**, 43 (2001).
17. Whitehill, J., Neild, A., Ng, T.W. et al. *Applied Physics Letters.* **96** (5), 053501 (2010).