APPLICATION NOTE

# High-throughput Microliter-Sized Cell-Free Transcription-Translation Reactions for Synthetic Biology Applications Using the Echo® 550 Liquid Handler

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## ABSTRACT

Cell-free transcription-translation systems (TXTL) are becoming reliable tools to rapidly prototype biochemical systems programmed with synthetic gene circuits. Here we scale down TXTL reactions to 0.5 µL using the Echo® 550 Liquid Handler, and compare the results to 5 µL reactions prepared using manual methods. Using reaction condition optimization and gene circuit prototyping, we demonstrate that data obtained via miniaturization with the Echo 550 system is equivalent or superior to corresponding manual methods at larger volumes. Miniaturization reduces the cost per reaction up to a factor of 10. By implementing the Echo system we also reduce reaction setup time and work load on the individual researcher.

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## INTRODUCTION

Cell-free transcription-translation (TXTL) systems have become powerful tools to help understand complex biochemical systems by executing DNA programs in test tube reactions. These systems have recently been developed to study biological network prototyping, biosynthesis, artificial cell systems, and functional membrane protein production.[1] In vivo assays often take days to weeks to run due to cell transformation and cultures. With TXTL, gene circuits can be prototyped in a matter of hours using a fluorescence readout. While TXTL has decreased the time of the design-build-test cycle, the throughput capacity and cost per reaction remains a bottleneck. TXTL reactions are typically assembled at volumes on the order of 10 µL. Commercial TXTL kits are expensive, and the cost per reaction can be very high.

TXTL reactions are complex, being comprised of a dissimilar collection of reaction components, including salts, energy buffers, DNA, and molecular crowding agents. In the all *E. coli* system used in this article, transcription is initially driven by the endogenous *E. coli* RNA polymerase and sigma factor 70. The numerous component variations make it impractical to screen TXTL reactions manually. The sheer number of potential reactions makes effective implementation and efficient use of reagents critical, while maintaining reproducibility and achieving high-throughput capability.

We used the Labcyte Echo 550 Liquid Handler to miniaturize the volume of TXTL reactions by as much as 10 fold, significantly reducing the use of precious reagents, and thus the cost per reaction. The Echo 550 Liquid Handler is an acoustic droplet ejection (ADE) dispenser, which uses focused sound pulses to deliver 2.5 nL droplets of reagent from source plates to assay plates. The 2.5 nL droplets can be dispensed hundreds of times per second to easily assemble reactions of one to two microliter volumes with no contact from tips or pipettes during the dispense of concentrated reagents for assay assembly. We found that more reactions can be assembled in five-fold less time, and there is no need to pipette reactions into an assay plate for measurement because the Echo system dispenses directly into the final assay plate. Echo Liquid Handlers transfer reagents from any well on a source plate into any well of a destination plate quickly and reliably, freeing the scientist from complicated plate maps otherwise required for complex reaction set up.

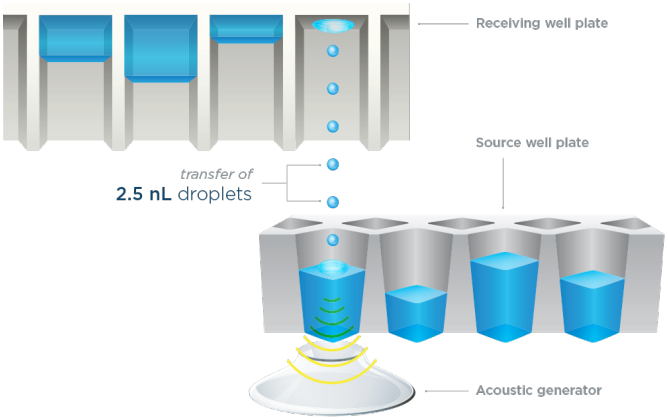



**FIGURE 1:** Acoustic droplet ejection (ADE) technology for liquid transfers.

The ability to dispense in 2.5 nL increments also affords the user high precision granularity in assembling TXTL reactions that is unsurpassed by any other technology. In a typical 5-10 µL TXTL reaction, manual methods of assembly require that no single component compose less than 5 % of the reaction volume to prevent pipetting error from becoming statistically significant. With the Echo system dispensing as little as 2.5 nL, not only is the overall volume of the reaction reduced, but a single component can compose as little as 0.125 % of the total 2 µL reaction volume. This allows the scientist to add and vary more components during the reaction assembly, enabling a broader space to be investigated in a way that was previously cost-prohibitive or logistically impossible.

## METHODS

TXTL reactions were assembled either manually by micropipette and divided into duplicates, or by using the Labcyte Echo 550 Liquid Handler and 384-well polypropylene source plates to make triplicate reactions. Water, 180 mM magnesium glutamate, 20 % w/v PEG 8000, 100 nM plasmid DNA, 500 mM maltodextrin, and a mixture comprised of *E. coli* lysate and an amino acid mix were dispensed individually using the Echo 550 BP2 (Buffer-Protein) fluid class. 3 M potassium glutamate and an energy buffer were dispensed using the Echo 550 CP (Crystallography-Protein) fluid class. All reactions were assembled similar to previously published methods[1] into Costar 96-well v-bottom plates (CLS3357) for incubation at 29°C and fluorescence reading in the BioTek Synergy Neo plate readers. The fluorescent reporter in our tests is deGFP, a version of eGFP with truncations at the N and C terminus, modified for more efficient production in our *E. coli* TXTL system. A pre-packaged version of this cell-free system, where only DNA and water must be added to complete a reaction, is available from Arbor Biosciences (myTXTL).

# RESULTS

## General Effect of Reaction Miniaturization

It is important to weigh the effects of TXTL reaction miniaturization and assembly using the Echo 550 Liquid Handler on both the cost and, most importantly, the scientific results. The lowest reaction volume possible that will eliminate manual pipetting error is 10 uL. From a 10 µL reaction, it is possible to accurately and precisely pipette as low as 1-2 µL into a well of an assay plate for reading, but this represents too many unnecessary replicates. For our work, we chose to divide the 10 µL mix into two wells of 5 µL for assay technical replicates. Using the Echo system allowed us to directly assemble triplicate reactions with little to no effect on the final data. Despite a dead volume associated with acoustic dispensing, we found that this volume is recoverable for reuse, minimizing the loss of reagents and the overall cost of the program.

To determine if the implementation of the Echo 550 Liquid Handler for TXTL reaction miniaturization was reasonable, we performed a simple volume reduction test (Figure 2). In one assay plate, we tested the same reaction assembled at 0.5, 1, 2, 3 and 5 µL final volumes using the Echo system. Each TXTL reaction contained 5 nM P70a-deGFP plasmid as this DNA concentration maximizes GFP expression. The expressed reporter is a version of eGFP with truncations at the N and C terminus, which has been modified for more efficient production under an *E. coli* sigma 70 promoter (Addgene plasmid #40019). All reactions were assembled by the Echo system to avoid manual pipetting bias between volumes, and were composed of 90 mM potassium glutamate, 4 mM magnesium glutamate, 30 mM maltodextrin, and 2 % PEG 8000.

We see efficient protein synthesis for TXTL reaction volumes between 0.5-5 µL, with the 2 µL reaction volume giving the highest endpoint protein yield, at 70 µM GFP. This is consistent with results published previously.[1] However, reaction volumes as low as 0.5 µL give similar protein synthesis rates for the first four hours of the reaction, making the 0.5 µL volume useful for preliminary testing. Using the smaller volume TXTL reactions saves reagents and costs, while also saving time in reaction assembly. The rate of protein synthesis in larger reaction volumes (3 and 5 µL) is non-linear and slower than that in smaller volumes, though smaller reactions stop expressing sooner. As TXTL reactions are very sensitive to oxygen, at smaller volumes oxygen could diffuse more quickly throughout the entire volume. The 3 and 5 µL reactions could benefit from active mixing during the fluorescence kinetics to improve oxygenation and the rate of protein expression, though that is outside the scope of this work and it would not benefit from the reduced costs of the smaller reaction volumes.

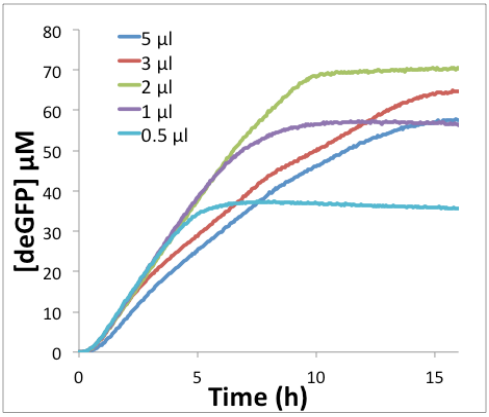



**FIGURE 2:** The direct effect of miniaturization on TXTL reactions at 0.5, 1, 2, 3, and 5 µL. Reactions were all dispensed using the Echo 550 Liquid Handler to create the same conditions and incubated with 5 nM P70a-deGFP plasmid (Addgene plasmid #40019) at 29°C.

Assembling reactions using the Echo 550 Liquid Handler is significantly faster than doing the same experiment using manual methods. Using the Echo system, a single well of a 96-well plate can be completed over four times faster than a well of the same design done manually, when preparation, mixing reagents, and plating into the well are considered (Figure 3A). Repeating the same experiment is even faster using the Echo system as the repeat experiment can be done eight times faster, almost entirely due to the ability to re-use the reagent plate (Figure 3B).

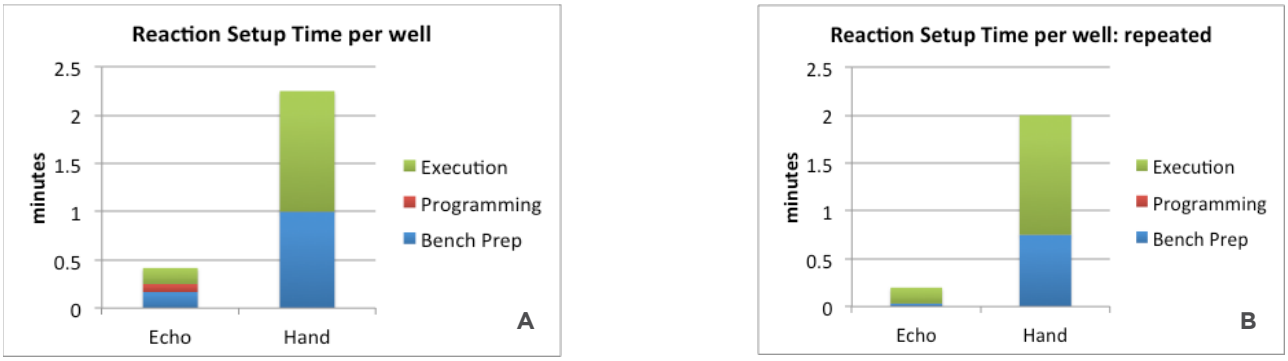



**FIGURE 3:** TXTL reaction assembly timing. A) Time necessary to assemble a unique TXTL reaction using the Echo 550 Liquid Handler vs. manual methods. A full assay plate is done in 40 minutes with the Echo system and in 216 minutes manually. B) Time necessary to repeat a TXTL reaction using the Echo 550 Liquid Handler vs. manual methods. A repeated assay plate is done in 19 minutes with the Echo and in 192 minutes manually.

## Reaction Conditions Optimization

For maximum protein synthesis rate from any expression plasmid, it is critical to optimize the reaction conditions. To better see the differences in GFP expression rates and levels caused by varying reagent conditions during optimization, we used 2 nM P70a-deGFP plasmid (Addgene plasmid #40019) to not impart production stress the TXTL system. There are many different components that make up a TXTL reaction, including potassium glutamate, magnesium glutamate, PEG, maltodextrin, energy buffer, and amino acid mix. Certain components may also be coupled, which increases the parameter space needed to explore to optimize the concentration for each component. This makes reaction optimization very tedious and time consuming when done manually. With the Echo 550 Liquid Handler, once the parameters are defined, the reaction assembly is done quickly and concerns around human error are eliminated.

Figure 4A shows kinetics for two concentrations of PEG for reactions assembled to final volumes of 2 µL when using the Echo 550 Liquid Handler, or 5 µL manually. The maximum protein synthesis rate for reactions assembled using the Echo system is greater than that achieved manually; furthermore, reactions assembled using the Echo system reach this synthesis rate more quickly and sustain this rate for longer. We posit this is due to the oxygenation effects described above.

Figure 4B shows endpoint expression for increasing concentrations of both potassium glutamate and PEG8000 from reactions assembled using the Echo 550 Liquid Handler. The coupled reagent optimums are in the range of 2-3 % PEG8000 and 40-80 mM potassium glutamate. 2-4 mM magnesium glutamate was also found to be an optimum in this reaction (data not shown). This represents 32 wells of data capable of being produced from a single 96-well plate when reactions were dispensed with the Echo system. This is an example of how a reaction can easily and effectively be optimized by simultaneously varying multiple components utilizing the Echo system. Assembling reactions manually and varying more than one component at once is typically not reasonable based upon time constraints and operator skill.

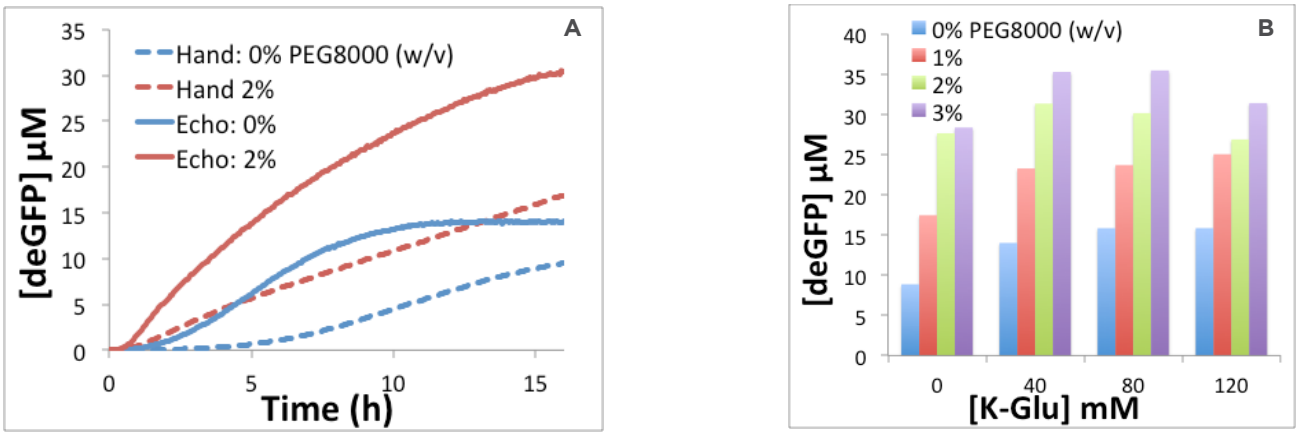
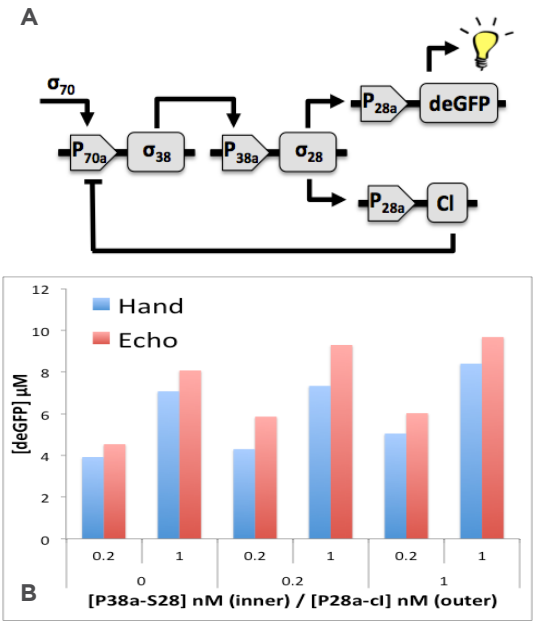



**FIGURE 4:** Natively Cell-free reaction component optimization with 2 nM P70a-deGFP plasmid (Addgene plasmid #40019). A) deGFP fluorescence kinetics while varying PEG8000 concentration (% w/v) in reactions assembled using the Echo 550 Liquid Handler and manual methods. B) Endpoint (16 h) deGFP synthesis varying PEG8000 (% w/v) and potassium glutamate (mM) in reactions assembled using the Echo 550 Liquid Handler.

## Gene Circuit Prototyping

To represent a more complicated gene circuit that might be explored, we tested a multiple-stage cascade with delayed feedback, with no protein degradation (Figure 5A). Similar to optimizing reaction conditions, it is important to vary the concentrations of each plasmid construct in a gene circuit to either maximize protein synthesis, or investigate the response of the circuit to different gene stoichiometry. To verify that reactions assembled using the Echo 550 Liquid Handler behave similarly to those assembled manually, we tested increasing concentrations of P38a-S28 and P28a-cI, while holding P70a-S38 and P28a-deGFP constant at 1 nM (Figure 4B). All reactions contain 2 % PEG8000, 80 mM potassium glutamate, and 4 mM magnesium glutamate. The endpoint (16 h) deGFP expression for reactions assembled using the Echo system at 2 µL were slightly higher than those assembled manually at 5 µL, and the relative ratio was nearly identical across the range of plasmid concentrations. This suggests that results acquired by either method of reaction assembly are comparable.

To examine the broader behavior of the circuit with respect to DNA concentrations, we used the Echo 550 Liquid Handler to assemble 2 µL reactions to 0, 0.2, and 1 nM for each of P70a-S38, P38a-S28, and P28a-cI, while holding P28a-deGFP constant at 1 nM (Figure 5C). This amounts to a total of 84 wells (27 reactions + 1 background reaction, all in triplicate). Increasing the concentration of P38a-S28 increased the expression of deGFP in the range of concentrations tested. The circuit was very



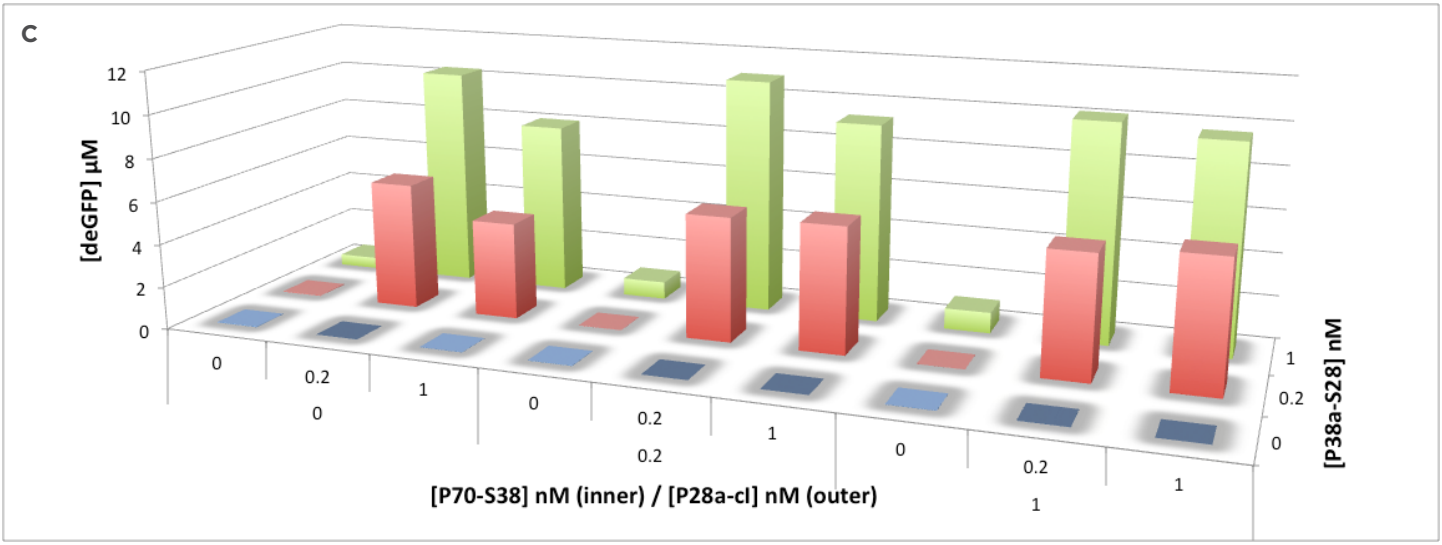




**FIGURE 5:** Prototyping a gene circuit: multiple stage cascade with delayed feedback. A) Circuit diagram is shown. B) Endpoint deGFP fluorescence for reactions assembled using the Echo 550 Liquid Handler and manually, with 1 nM P28a-deGFP, 1 nM P70a-S38, and indicated concentrations of P38a-S28 and P28-cI. C) Endpoint deGFP fluorescence for reactions assembled using the Echo 550 Liquid handler, with 1 nM P28a-deGFP, and indicated increasing concentrations of the other three circuit plasmids.

sensitive to an increase of P70a-S38 from 0 to 0.2 nM, however, a further increase from 0.2 to 1 nM saw no increase in deGFP expression. This suggests that only a very small concentration of S38 is required to turn on the transcription of deGFP. An excess of S38 will not increase deGFP expression because it is a competitor to S28 for RNA polymerase and the P38a promoter may be quickly saturated with S38-RNA polymerase holoenzyme.[1] Varying the concentration of P28a-cI repressor resulted in no change in the deGFP expression. The cI protein represses transcription from P70a promoter, therefore repressing the expression of S38. However, a small amount of S38 can be expressed prior to the repressor, because the repressor is expressed in the third stage of the cascade. The Echo 550 Liquid Handler facilitates the characterization of gene circuits in TXTL by accelerating the preparation of reactions, allowing the rapid and simple dispensing of the 28 conditions in triplicate for our analysis.

## CONCLUSION

We have shown that with the Echo 550 Liquid Handler we can effectively miniaturize TXTL reactions, reducing the volume per well in a 96-well assay plate by 2-fold (5 uL final volume) to 20-fold (0.5 uL final volume) over the standard 10 µL manual reaction assembly. The 2 µL reactions are the optimum volume for extended reactions and endpoint protein synthesis. The rate and endpoint deGFP production of reactions assembled using the Echo system are comparable to those done manually indicating that it is a viable tool to use with TXTL systems. The Echo system allows for a greater variation of reaction components and an ease of optimization of multiple reaction components simultaneously. More conditions can be assembled at lower volumes than possible manually, with equivalent or superior results. Finally, the Echo system significantly reduces the work load on the individual researcher, while also decreasing the probability of errors. The reaction assembly time was reduced by a factor of four in a single experiment, and further by a factor of eight when the experiment was repeated. The Echo 550 Liquid Handler improves TXTL reaction assembly. We have demonstrated savings in reagents and time, as well as increases in the number of investigation parameters possible while providing equivalent or superior results to those achieved with manual assembly.

## ACKNOWLEDGEMENTS

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## REFERENCES

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