TECHNICAL NOTE

Miniaturization and Rapid Processing of TXTL Reactions Using Acoustic Liquid Handling

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ABSTRACT

Cell-free transcription-translation (TXTL) systems are a versatile platform for production of recombinant proteins within synthetic biology. Because *in vitro* protein synthesis is decoupled from cellular functions, the experimental cycle time can be greatly reduced while the application range is expanded. The Arbor Biosciences™ myTXTL® technology is a commercially available cell-free system capable of consistent and highly-efficient protein output. Using the Labcyte® Echo® 525 Liquid Handler in combination with the myTXTL Master Mix provides a comprehensive solution for gene expression projects that require rapid high-throughput processing, flexibility, and reproducibility in a low cost assay. This experiment uses the 25 nanoliter granularity of the Echo 525 Liquid Handler to optimize different gene expression systems within the myTXTL system at a three-fold reduced volume.

MOVING LIQUIDS *with* SOUND

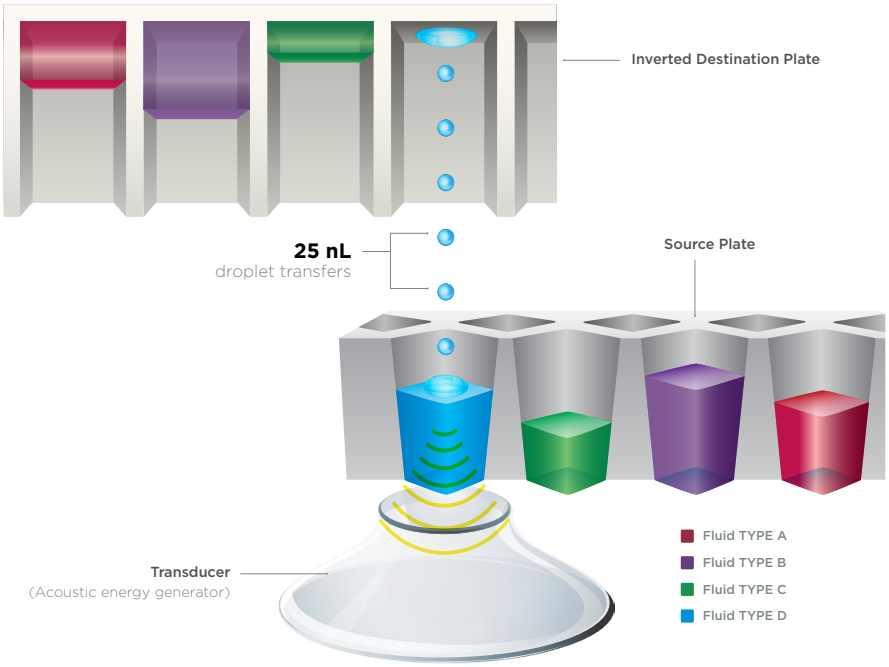
INTRODUCTION

Transcription-translation (TXTL) systems have gained popularity in many scientific fields ranging from protein engineering to synthetic biology for high-throughput screening applications. In general, TXTL involves the addition of a nucleotide template to an *in vitro* reagent mix comprising the TXTL machinery and essential building blocks, by which the DNA is transcribed into RNA, and the RNA is translated into its encoded protein. The practical use of cell-free TXTL systems has progressed rapidly for a variety of applications. Currently, the fields of bioengineering[1,2], metabolic engineering[3], genetic circuit design[4–6], enzyme evolution[7], and a variety of other applications are being interrogated by TXTL systems.

Much of the recent progress is driven by the commercial availability of reliable, ready-to-use cell-free systems. Home-made extracts require a laborious preparation procedure and often lack reproducible performance. Arbor Biosciences' myTXTL technology allows for the consistent high-yield production of a protein output from circular or linear DNA input (**FIGURE 1**). This system is based upon *E. coli* cytoplasmic extract entirely relying on the endogenous core RNA polymerase and the transcription factor sigma 70 (σ70), and an effective energy regeneration system. Within a few minutes, gene products can be directly applied to functional analysis with or without prior purification.

TXTL offers several benefits over traditional *in vivo* gene expression. As cell viability can be ignored completely, otherwise toxic proteins can now be produced. Also, due to the open-reaction setup of *in vitro* systems, the addition of co-factors (enzyme engineering), detergents and membrane-formation supporting reagents (membrane protein production) or expensive building blocks (site-specific protein labeling using non-natural amino acids) is particularly easy. Finally, the greatly shortened time to complete a design-build-test cycle – being hours as opposed to multiple days as for cell-based assays – leads to cost-reduction and dramatically increases sample throughout. TXTL immensely benefits from state-of-the-art DNA synthesis technology promising to deliver high-quality, error-free circular and linear DNA templates within days at decreasing costs.

FIGURE 1: Typical workflow of *in vitro* protein production using myTXTL.



In this study, we will be showing the utility of myTXTL coupled with the Echo® 525 Liquid Handler for high-throughput applications. The Echo Liquid Handler uses a transducer to acoustically dispense in 25 nL increments (**FIGURE 2**). The Echo 525 Liquid Handler transfers reagents contamination-free, without sacrificing their quality or concentration to tubing, tips, or nozzles. All of this can be accomplished while maintaining an extraordinary flow rate of up to 5000 nL/s. The nanoliter granularity of the Echo system enables greatly lower reagent cost at the highest level of precision and accuracy.

FIGURE 2: The Echo system transducer rapidly moves between wells on the source plate while the destination plate also moves, allowing rapid transfer from any well to any well for multiple fluid types.

OVERVIEW

Prior work has demonstrated the ability of the Echo® 550 Liquid Handler to work with lab-generated TXTL reagents in a miniaturized reaction with protein production comparable to larger scale manually arrayed reactions[8]. This study seeks to build upon those results with commercially available myTXTL Master Mix, showcasing sampling of TXTL reactions for two expression systems (**FIGURE 3**) and miniaturization of the reaction from 12 µL to 4 µL.

As an example for gene expression controlled by the endogenous *E. coli* TXTL machinery, the plasmid P70a-deGFP encoding an engineered version of the enhanced green fluorescent protein (**FIGURE 3 A/Left**) was titrated into the myTXTL Master Mix for optimizing protein output using the 25 nL granularity of the Echo 525 Liquid Handler. A standard curve prepared with purified deGFP allowed quantification of deGFP produced in TXTL.

Alternatively, gene expression in myTXTL was optimized for the popular T7 promoter system (**FIGURE 3 B/Right**). Therefore, RNA polymerase derived from bacteriophage T7 needs to be provided to the myTXTL reaction as it is not part of the endogenous *E. coli* machinery. Either addition of T7 RNA polymerase protein or co-expression from a DNA template demands fine tuning of reagent concentrations for maximum protein yield. Here, the Echo 525 system with its 25 nL granularity facilitated an utmost degree of flexibility, accuracy and experimental resolution.

In this study, we demonstrate the ability to process Arbor Biosciences' myTXTL system and input DNA in low volumes using the Labcyte Echo Liquid Handler. This lowers overall cost by reducing assay time and reagent consumption, while minimizing the risk of contamination and errors.

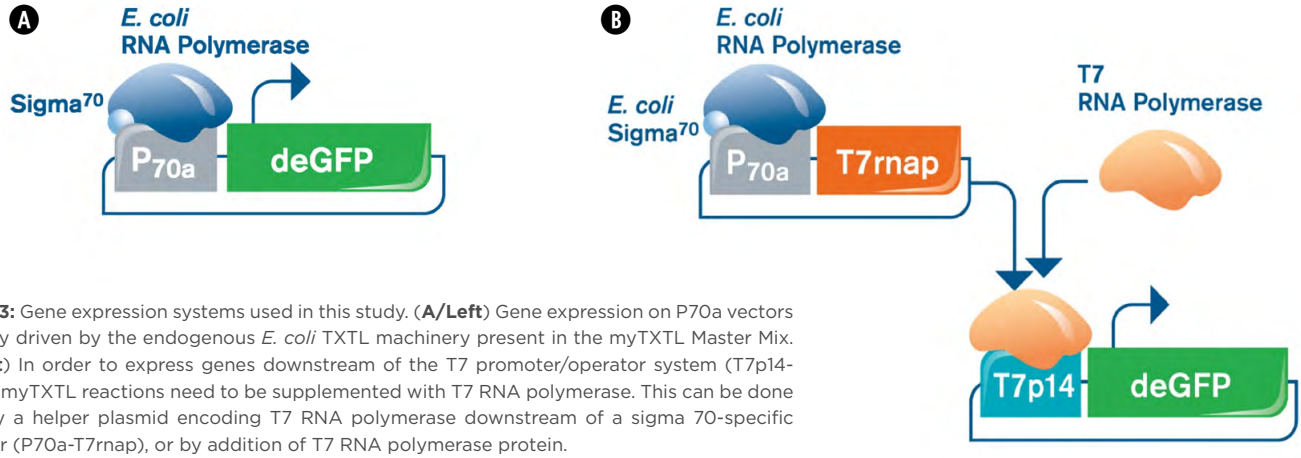


FIGURE 3: Gene expression systems used in this study. (**A/Left**) Gene expression on P70a vectors is entirely driven by the endogenous *E. coli* TXTL machinery present in the myTXTL Master Mix. (**B/Right**) In order to express genes downstream of the T7 promoter/operator system (T7p14-deGFP), myTXTL reactions need to be supplemented with T7 RNA polymerase. This can be done either by a helper plasmid encoding T7 RNA polymerase downstream of a sigma 70-specific promoter (P70a-T7rnap), or by addition of T7 RNA polymerase protein.

METHODS

I. Preparation of a deGFP Standard Curve at Various Volumes

In order to determine the amount of the fluorescent model protein produced in myTXTL, deGFP standards in the range of 0–17.5 nM were prepared using the *Direct Dilution* option of the Echo 525 Liquid Handler. Aliquots of a recombinant deGFP stock solution (Arbor Biosciences) and myTXTL Sigma 70 Master Mix or Hank's Balanced Salt Solution (HBSS), respectively, were transferred from Echo qualified 384-well polypropylene microplates into black walled 384-well assay plates using the 384PP_Plus_AQ_BP calibration on all reagents. The deGFP standard was tested at the 1, 3, 6, 12 µL volumes with 0, 2.5, 5, 7.5, and 10 nM final concentration of protein. After transfer, the assay plate was sealed with a foil seal, and centrifuged at 1500 RCF for 30 seconds. A Labcyte MicroClime® Environmental Lid hydrated with sterile water was then placed on top to prevent evaporation during the incubation period.

Fluorescence signal was recorded in a PHERAstar FS (BMG Labtech) plate reader during incubation with an excitation wavelength of 485 nm and an emission wavelength of 520 nm for 16 h and 29°C at an interval of 5 min using the bottom reading setting. This protocol was kept consistent throughout the study. The bottom optic read was used to account for possible differences in the height of the reaction over time. Prior to each read, the assay plate was subjected to linear shaking at 400 RPM for 30 seconds. The internal gain value of the fluorescence reader was set manually to 249, which was 50% of the maximum value of the standard curve.

A deGFP standard curve with higher resolution was generated at the 4 µL volume in order to more accurately quantitate the proceeding experimental results. This was accomplished using the HBSS along with the purified deGFP which were transferred using the 384PP_Plus_AQ_BP calibration in 4 µL total volume. After transfer, the assay plate was sealed with a foil seal, and centrifuged at 1500 RCF for 30 seconds. A MicroClime Lid hydrated with sterile water was then placed on top prior to loading in the PHERAstar FS for the same protocol described above.

II. *In vitro* Gene Expression Driven by the Endogenous *E. coli* TXTL Machinery

A single plasmid system was tested in myTXTL to determine the robustness of the assay at the lower volume of 4 µL. A typical myTXTL reaction is comprised 75% of myTXTL Master Mix and 25% nucleotide template, which was also applied to miniaturized reactions assayed in this study. Two different stock solutions of P70a-deGFP, 1 and 20 nM, were prepared to cover the desired plasmid concentration range between 6.25 and 2000 pM. The 384PP_Plus_AQ_BP calibration was used on the Echo 525 Liquid Handler to dispense all of the reagents shown in **TABLE 1**. The variable amounts of input DNA were normalized using 10 mM Tris/HCL pH 8.0. The order of addition for the reagents was important for assay reproducibility. The Tris buffer backfill was added first, followed by the plasmid, and the myTXTL added last. After transfer, the assay plate was sealed and centrifuged. A hydrated MicroClime Lid was then placed on top prior to loading in the PHERAstar FS.

Final P70a-deGFP Conc. (pM)	**Reagent**	**Volume** (nL)
2000	P70a-deGFP (20 nM)	400
	myTXTL Master Mix	3000
	Tris/HCL Buffer (10 mM pH 8)	600
1750	P70a-deGFP (20 nM)	350
	myTXTL Master Mix	3000
	Tris/HCL Buffer (10 mM pH 8)	650
1500	P70a-deGFP (20 nM)	300
	myTXTL Master Mix	3000
	Tris/HCL Buffer (10 mM pH 8)	700
1250	P70a-deGFP (20 nM)	250
	myTXTL Master Mix	3000
	Tris/HCL Buffer (10 mM pH 8)	750
1000	P70a-deGFP (20 nM)	200
	myTXTL Master Mix	3000
	Tris/HCL Buffer (10 mM pH 8)	800
750	P70a-deGFP (20 nM)	150
	myTXTL Master Mix	3000
	Tris/HCL Buffer (10 mM pH 8)	850
500	P70a-deGFP (20 nM)	100
	myTXTL Master Mix	3000
	Tris/HCL Buffer (10 mM pH 8)	900
125	P70a-deGFP (20 nM)	25
	myTXTL Master Mix	3000
	Tris/HCL Buffer (10 mM pH 8)	975
100	P70a-deGFP (1 nM)	400
	myTXTL Master Mix	3000
	Tris/HCL Buffer (10 mM pH 8)	600
87.5	P70a-deGFP (1 nM)	350
	myTXTL Master Mix	3000
	Tris/HCL Buffer (10 mM pH 8)	650
76	P70a-deGFP (1 nM)	300
	myTXTL Master Mix	3000
	Tris/HCL Buffer (10 mM pH 8)	700
62.5	P70a-deGFP (1 nM)	250
	myTXTL Master Mix	3000
	Tris/HCL Buffer (10 mM pH 8)	750
50	P70a-deGFP 1 nM)	200
	myTXTL Master Mix	3000
	Tris/HCL Buffer (10 mM pH 8)	800
37.5	P70a-deGFP (1 nM)	150
	myTXTL Master Mix	3000
	Tris/HCL Buffer (10 mM pH 8)	850
25	P70a-deGFP (1 nM)	100
	myTXTL Master Mix	3000
	Tris/HCL Buffer (10 mM pH 8)	900
6.25	P70a-deGFP (1 nM)	25
	myTXTL Master Mix	3000
	Tris/HCL Buffer (10 mM pH 8)	975

TABLE 1: Acoustic transfer scheme for setting up 4 µL myTXTL reactions with P70a-deGFP as template. Reagents were transferred from an Echo Qualified 384-well Polypropylene 2.0 Plus Microplate into an opaque Greiner 384-well assay plate using the 384PP_Plus_AQ_BP calibration. All reactions were set up in quadruplicate.

III. *In vitro* Gene Expression Driven by the T7 Promoter and Exogenous T7 RNA Polymerase

In myTXTL, gene expression from a T7 promoter system requires exogenous addition of T7 RNA polymerase. This was conducted by the acoustic transfer of recombinant T7 RNA polymerase (Thermo Fisher Scientific) to the 4 µL myTXTL reactions. The plasmid concentration ranges found in the previous experiment using σ70 controlled deGFP were employed here as well. For the plasmid T7p14-deGFP, a range of 125 to 2000 pM final concentration was interrogated. The plasmid was transferred from an Echo Qualified 384-Well Polypropylene 2.0 Plus Microplate using the 384PP_Plus_AQ_BP calibration.

The 384PP_Plus_AQ_GP calibration was used to transfer a range of 3 to 0.00625 units of recombinant T7 RNA polymerase from an Echo Qualified 384-Well Polypropylene 2.0 Plus Microplate. The myTXTL Master Mix and Tris buffer were dispensed from an Echo® Qualified Reservoir using the 6RES_AQ_BP calibration due to a more favorable dead volume for the amount needed.

Each of the exact volumes transferred acoustically can be seen in **SUPPLEMENTAL TABLE 1***. Conditions were tested in quadruplicate. For this assay, the order of reagents during reaction assembly was maintained similar to the single plasmid addition procedure. Any Tris buffer backfill was added first, followed by the plasmid, then the T7 RNA polymerase, and the myTXTL Master Mix added last. The myTXTL reagent volume was kept the same at 75% of the input volume. After transfer, the assay plate was sealed, centrifuged and covered with a hydrated MicroClime Lid, and fluorescence signal was recorded with a PHERAstar FS plate reader at identical settings as described before.

*To view Supplemental Table 1, go to **www.labcyte.com/g129-table1**

IV. *In vitro* Gene Expression Driven by the T7 Promoter System with Co-expression of T7 RNA Polymerase from a DNA Template

T7 RNA polymerase can also be generated within the myTXTL reaction by co-expression from P70a-T7rnap plasmid. To find the optimal ratio for this two-plasmid system leading to maximum protein yield, a concentration matrix of P70a-T7rnap and T7p14-deGFP was assayed. The T7p14-deGFP concentration range used was kept between 125–2000 pM as in the previous experiment, whereas the T7 RNA polymerase producing P70a-T7rnap plasmid was added at the lower range of 0.05 to 12 pM based on preliminary testing. Each of the exact volumes transferred acoustically are displayed in **SUPPLEMENTAL TABLE 2****. The plasmids were acoustically transferred from an Echo Qualified 384-Well Polypropylene Microplate using the 384PP_Plus_AQ_BP calibration.

Again, the myTXTL Master Mix and Tris buffer were dispensed from an Echo Qualified Reservoir using the 6RES_AQ_BP calibration. Conditions were tested in quadruplicate. As before, the order of addition for the reagents was important for assay reproducibility. The Tris buffer backfill was added first, followed by both plasmids, and the myTXTL added last. The myTXTL reagent volume was maintained at 75% of the input volume. After transfer, the assay plate was sealed, centrifuged and covered with a hydrated MicroClime Lid, and fluorescence signal was recorded with a PHERAstar FS plate reader at identical settings as described before.

To view Supplemental Table 2, go to **www.labcyte.com/g129-table2

RESULTS AND DISCUSSION

I. Preparation of a deGFP Standard Curve at Various Volumes

When working in reaction volumes of single digit µL scale, accuracy during reaction setup as well as during assay readout are critical for reproducibility. Automated liquid handling systems are therefore perfect tools to generate reliable and valuable data. Here, we used the 25 nL granularity of the Echo 525 system to prepare deGFP standard concentration series at volumes between 1 and 12 µL, in order to visualize the highly precise acoustic transfer technology, but also to quantify deGFP produced by myTXTL in subsequent experiments (**FIGURE 4**). Data points of all assayed reaction volumes followed a linear regression curve over the analyzed deGFP concentration range correlating with high accuracy during reagent transfer. Additionally, no difference in fluorescent signal was found between standard curves that were setup with myTXTL Sigma 70 Master Mix and HBSS, respectively. The Labcyte MicroClime Environmental Lid was required to prevent evaporation of the solution over time. The MicroClime Lid was saturated with water to allow for a humidity curtain around the Greiner 384-well assay plate while also permitting oxygen diffusion needed for TXTL in general, but also to promote maturation of deGFP fluorescence.

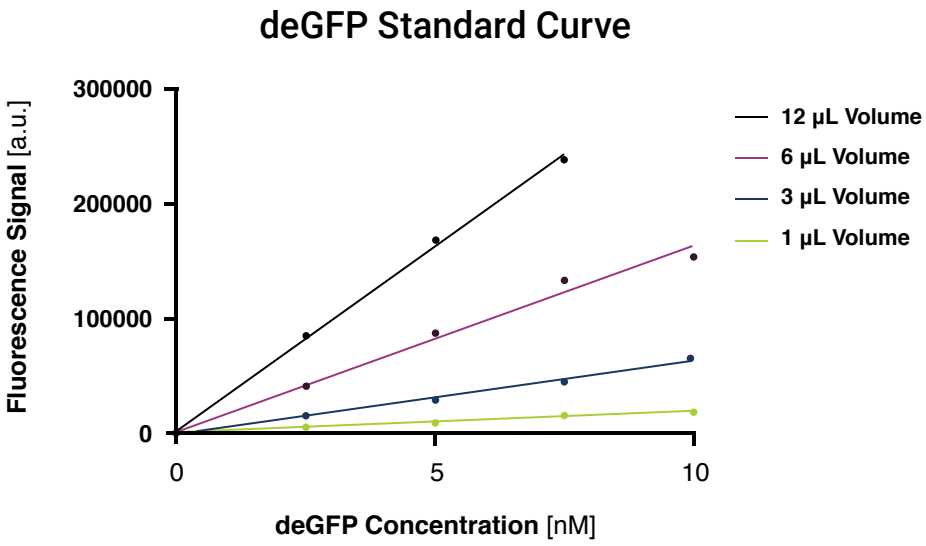


FIGURE 4: Effect of miniaturization on fluorescence signal of deGFP standards. deGFP concentration series at different volumes were prepared using the *Direct Dilution* function of the Echo 525 Liquid Handler and directly transferred into myTXTL Master Mix in an opaque 384-well plate for fluorescent reading. deGFP fluorescence was excited at 485 nm on a BMG Labtech PHERAstar® FS plate reader and fluorescence emission was recorded at 520 nm respectively.

Upon visual observation, only at volumes of 4 µL or more the wells of the 384-well assay plate seemed to be covered with liquid completely. Consequently, the 4 µL reaction volume was chosen for all subsequent *in vitro* gene expression experiments, as it constitutes the best compromise between reagent consumption and assay conditions in this particular study. Therefore a more granular standard curve was obtained (**FIGURE 5**). The focal plane, gain, and temperature from the PHERAstar FS plate reader were then kept the same throughout the remaining experiments to allow quantification of deGFP produced in myTXTL. These results provided a consistent comparison for deGFP quantification.

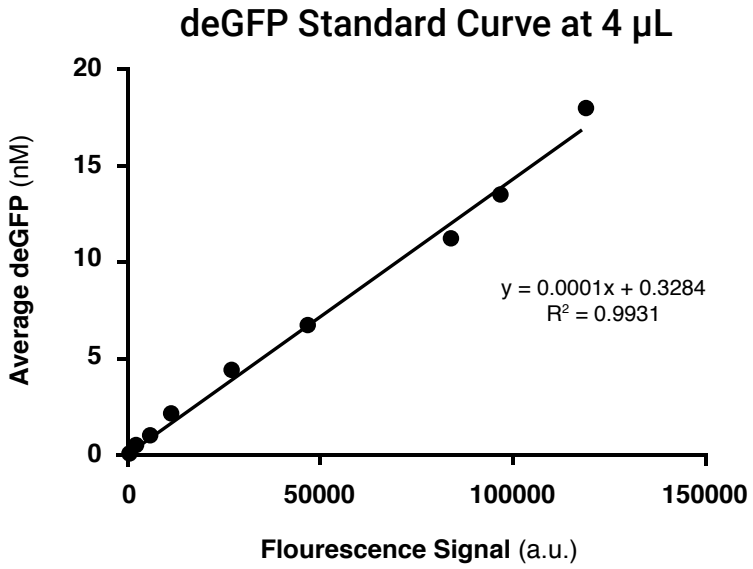


FIGURE 5: deGFP standard curve at 4 µL volume with HBSS as diluent recorded on BMG Labtech PHERAstar® FS plate reader (λEx = 485 nm, λEm = 520 nm). deGFP produced in myTXTL during this study was quantified according to the displayed equation, which resulted from linear regression fit. Each point was done in quadruplicate and the curve had an average percent CV of 4.05%.

II. *In vitro* Gene Expression Driven by the Endogenous *E. coli* TXTL Machinery

Gene expression in myTXTL relies on the endogenous TXTL machinery of *E. coli*, which consists of the core RNA polymerase and transcription factor σ70. Here, the P70a-deGFP plasmid encoding the strong constitutive lambda phage promoter PL, which is specific to σ70 transcription factor, was used to exemplify high-throughput optimization of *in vitro* protein production from a simple single plasmid system using the Echo 525 system (**FIGURE 6**). In this miniaturized reaction volume of 4 µL (3-fold lower than a standard myTXTL reaction), the production of the model protein deGFP linearly increased within the P70a-deGFP concentration range of 500 pM to 2000 pM. At lower plasmid concentrations, TXTL of deGFP only occurs at background level, whereas higher plasmid concentrations would likely have further increased the deGFP yield. For assay reproducibility, the order in which each myTXTL reaction component was transferred into the destination plate (Tris/HCL buffer, plasmid and myTXTL Master Mix) was found to be extremely important.

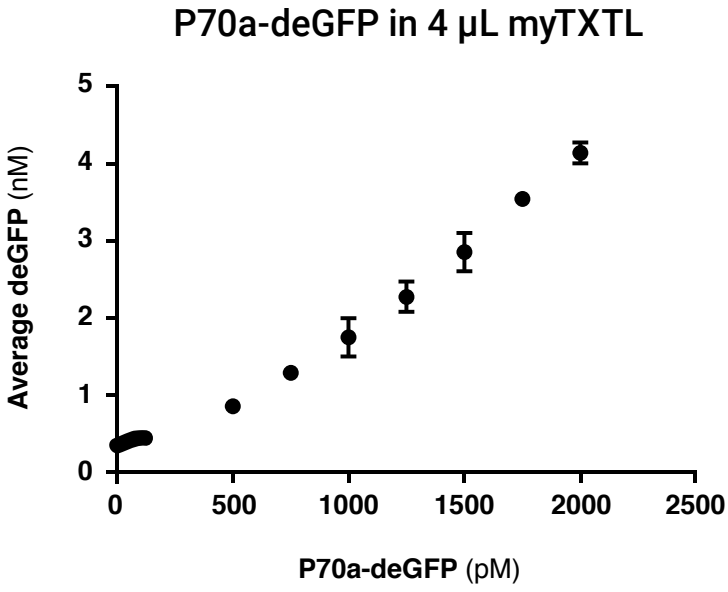


FIGURE 6: Gene expression from P70a-deGFP plasmid into 4 µL total reaction volume of myTXTL. Readings were taken on a BMG Labtech PHERAstar FS (λEx = 485 nm, λEm = 520 nm) after 12.5 h of incubation at 29°C. Each point was done in quadruplicate and had an average percent CV of 7.60%.

III. *In vitro* Gene Expression Driven by the T7 Promoter and Exogenous T7 RNA Polymerase

To decouple deGFP production from T7 RNA Polymerase production, we decided to exogenously add the enzyme to a T7 RNA polymerase-controlled plasmid (T7p14-deGFP) in 4 µL total volume myTXTL (**FIGURE 7**). Relevant concentrations of the T7p14-deGFP plasmid were determined to be between 125 pM and 2000 pM based on the results from the σ70 controlled plasmid. The maximum volume of exogenous T7 RNA polymerase possible was also added into the system. The deGFP-specific fluorescence signal was converted to deGFP concentration using the standard curve generated with purified deGFP (**FIGURE 5**). deGFP production occurred very rapidly reaching a plateau already after only 2.3 h. A noticeable peak deGFP production at 0.78 nM was observed at 1 Unit T7 RNA polymerase and 2000 pM T7p14-deGFP. The rapid drop off observed in protein production might have resulted from a decrease in T7 RNA polymerase activity over time or an adverse effect from a component such as glycerol contained in the T7 RNA polymerase storage buffer. The results shown in **FIGURE 7** demonstrate the requirement for assay optimization when working with a cell-free system. The ability of the Echo 525 Liquid Handler to transfer accurately and precisely multiple fluid types with a large dynamic range of volume enabled this optimization.

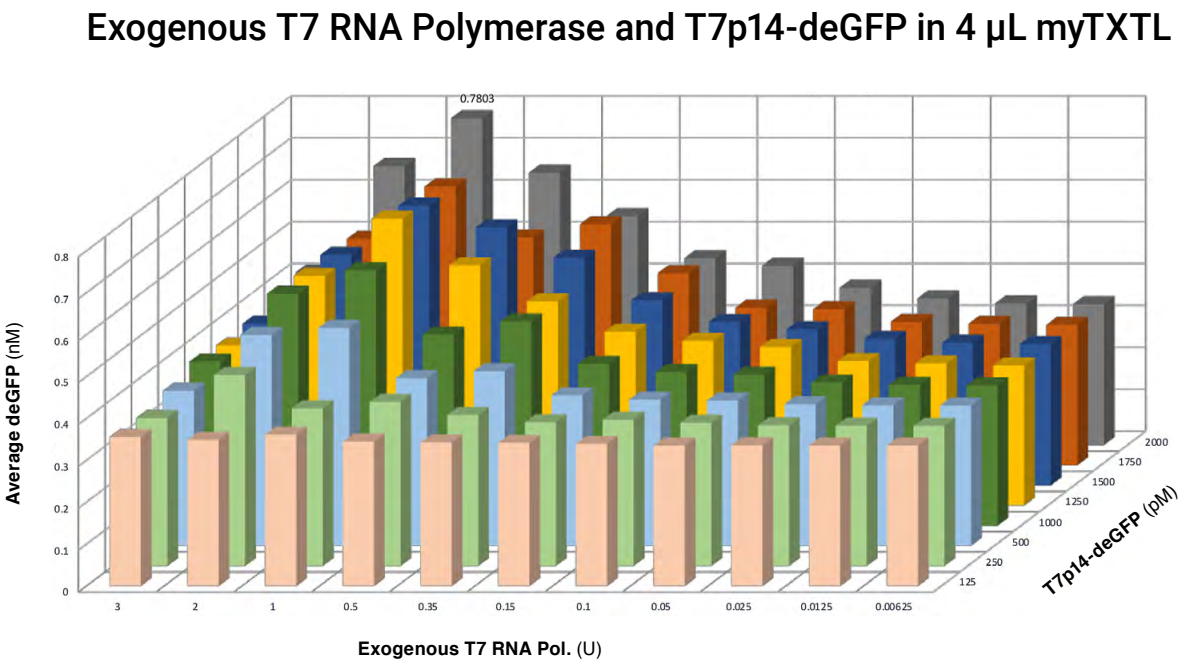


FIGURE 7: Multi-variable titration of the T7p14-deGFP plasmid and exogenous T7 RNA polymerase into 4 µL myTXTL reactions after 2.3 h of incubation. Concentrations of produced deGFP calculated from the protein specific fluorescence signal which was acquired on a BMG Labtech PHERAstar FS (λEx = 485nm, λEm = 520 nm) are displayed. Each point was done in quadruplicate and the curve had an average percent CV of 14.8%. The GFP production at 1 unit of T7 RNA polymerase was higher than additional amounts of added enzyme. Peak production was found to be at 1 Unit of T7 RNA polymerase and 2000 pM T7p14-deGFP plasmid at 0.78 nM deGFP.

IV. *In vitro* Gene Expression Driven by the T7 Promoter System with Co-expression of T7 RNA Polymerase from a DNA Template

Apart from adding T7 RNA polymerase exogenously, gene expression in myTXTL from a T7 promoter can be accommodated by a mini gene circuit, consisting of a sigma 70-driven T7 RNA polymerase expressing plasmid (here: P70a-T7rnap) in the presence of a T7 promoter plasmid encoding the gene of interest (here: T7p14-deGFP; **FIGURE 3**). This circuit requires optimization with respect to the ratio between the plasmids for maximum production of protein of interest due the their competition for the TXTL machinery and resources. Applying the concentration range of T7p14-deGFP previously tested and an about 100-fold lower concentration range for the P70a-T7rnap plasmid, the kinetic of deGFP production was followed over 16 h. Again, the use of MicroClime Lids proved crucial to the progression of the reaction over many hours as the low volumes evaporated without the lid. During the 16 h incubation period, deGFP yield increased for each reaction condition in the initial 7.5 h, after which no additional deGFP was produced (**FIGURE 8**). At the plasmid concentrations of 1250 pM T7p14-deGFP and 12 pM P70a-T7rnap, the deGFP concentration was 18.95 nM. This is a dramatically larger concentration in comparison to the results from exogenous T7 RNA polymerase addition seen above in **FIGURE 7**. This could be due to the continuous replenishment of T7 RNA polymerase in the two-plasmid circuit system compared to the one-time addition. The high level of protein production with minimal input DNA demonstrates the potential of the myTXTL platform.

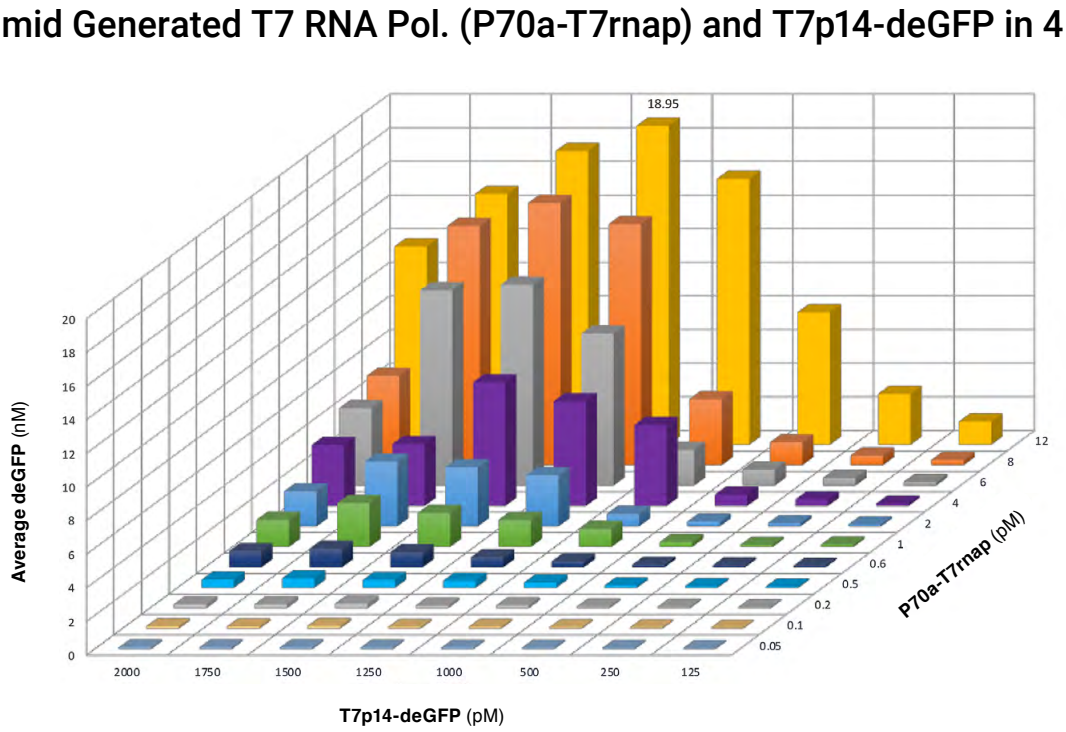


FIGURE 8: Multi-variable titration of the T7p14-deGFP and P70a-T7rnap plasmids into 4 µL myTXTL reactions after 7.5 h of incubation. Readings were taken on a BMG Labtech PHERAstar FS (λEx = 485 nm, λEm = 520 nm). Each point was done in quadruplicate and the plot had an average percent CV of 14.51%. Peak production was found to be at 1250 pM T7p14-deGFP and 12 pM P70a-T7rnap with an average deGFP concentration of 18.95 nM.

CONCLUSION

In this study, we demonstrated the accurate and precise processing of Arbor Biosciences' myTXTL Cell-Free Expression system in single digit microliter volumes by the Labcyte Echo 525 Liquid Handler. High-throughput liquid handling of myTXTL Master Mix allows assays for hundreds to thousands of gene template constructs and to investigate many experimental setups in parallel within a matter of hours. Meanwhile, efficient *in vitro* gene expression systems, like myTXTL, provide reduced total reaction volumes, and therefore reagent cost, while still generating sufficient protein for downstream analysis and functional assays. The assay optimization run on the Echo 525 Liquid Handler delivered robust results at lowered reagent consumption of 3-fold and saved preparation time of many days compared to a manual reaction setup. The ability of the Echo Liquid Handler to assemble gene constructs from modular pieces coupled with myTXTL's capability to accept linear DNA templates for gene expression gives rise to a new era of protein engineering.

MATERIALS

Equipment	Manufacturer
Echo® 525 Liquid Handler	Labcyte Inc.
PHERAstar FS®	BMG Labtech
Centrifuge 5430	Eppendorf
MixMate®	Eppendorf

Consumables	Manufacturer	Part No.
Echo® Qualified 384-well Polypropylene 2.0 Plus Microplate	Labcyte Inc.	PPL-0200
Echo® Qualified Reservoir	Labcyte Inc.	ER-0050
MicroClime® Environmental Lid	Labcyte Inc.	LL-0310
384-Well Microplate, Polystyrene, F-Bottom, μCLEAR®, Black-Walled	Greiner	781096

Reagents	Manufacturer	Cat. No.
myTXTL® Sigma 70 Master Mix Kit	Arbor Biosciences	507096
P70a-deGFP	Arbor Biosciences	502117
P70a-T7map HP	Arbor Biosciences	502134
T7p14-deGFP HP	Arbor Biosciences	502136
Purified recombinant deGFP	Arbor Biosciences	n/a
HBSS with Calcium and Magnesium, No Phenol Red	Gibco	14025126
T7 RNA Polymerase	Thermo Fisher Scientific	EP0111

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