

Facilitating Ultra-High Throughput Synthetic Biology Discovery via RCA, Colony PCR and Massively Multiplexed Plasmid and Amplicon Sequencing

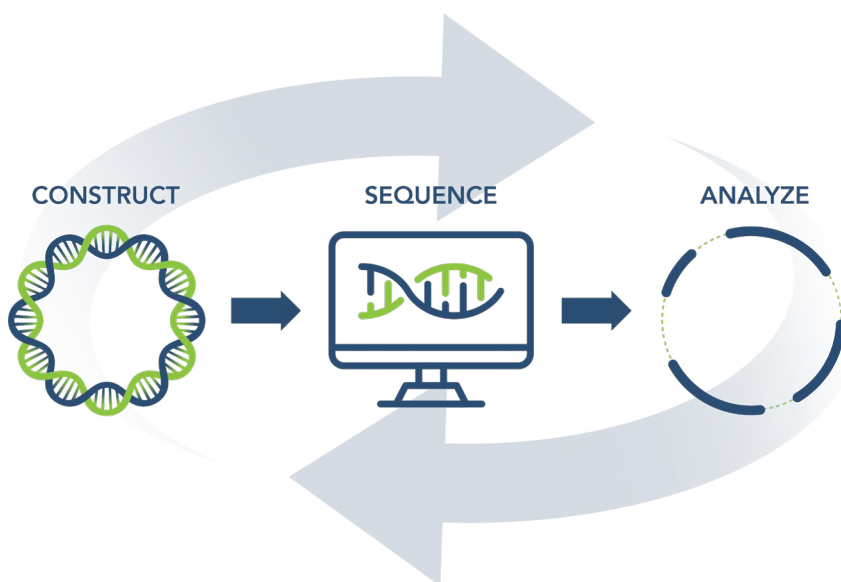
Introduction

The incorporation of synthetic biology approaches such as Design-Build-Test-Learn (DBTL) iterative cycles within therapeutic development and industrial-scale protein engineering have necessitated breakthroughs in highly multiplexed molecular biology. Rapid gene synthesis and assembly methods have enabled construction of thousands of new sequences, and new sequencing instruments provide significant capacity for verifying these constructs. However, in order to take advantage of these improvements, laboratories must find a way to break the bottleneck between synthesis and sequencing. How can a lab create sequencer-ready libraries from samples in a manner that matches sequencing capacity without building out a large infrastructure of people or hardware?

Verification of plasmid sequences following synthesis is a crucial quality control step that can create a bottleneck in synthetic biology pipelines. RCA and direct PCR from colonies (i.e. colony PCR) can eliminate cumbersome miniprep plasmid purification. The use of ExpressPlex HT NGS library preparation downstream of RCA or colony PCR is uniquely enabling for sequencing of thousands of plasmids using a single Illumina run in less than 24 hours.

In this technical note, we detail scalable methods that include colony picking, liquid handling, rolling circle amplification (RCA) or colony PCR, DNA sequencing and bioinformatic analysis that can dramatically shorten the typical synthetic biology DBTL cycle from weeks to hours.

Integration of ExpressPlex™ HT, an auto-normalizing, one-step library preparation method that provides 6,144 indexes in a convenient 384-well, assay-ready configuration (384 wells x 16 plates) is highlighted.



Why RCA or Colony PCR?

- ▶ Rolling circle amplification (RCA) is an isothermal amplification technique widely used in clinical, research and pharmaceutical industries mostly for its simplicity, time saving, cost effectiveness and high throughput capability. RCA generates a concatemer containing tens to hundreds of tandem repeats that are complementary to the circular template (Figure 1). The polymerase Phi29 is commonly used in this process because of its excellent processivity and strand displacement synthesis capabilities. Due to the nature of RCA, a simple heat block is sufficient to perform the method.

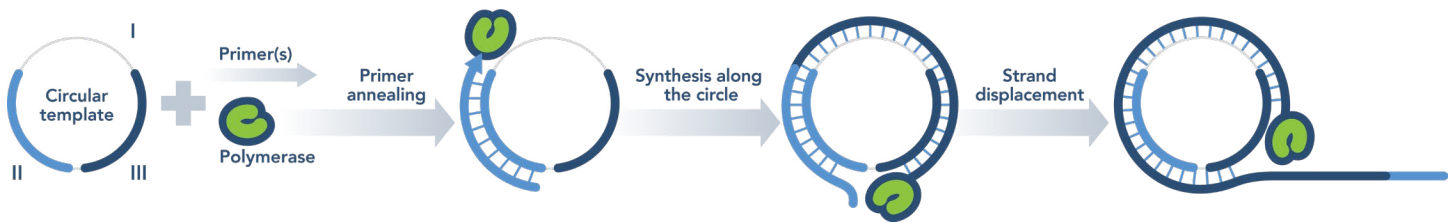


Figure 1: RCA process

- ▶ Colony PCR allows rapid screening of colonies generated after a transformation step, bypassing plasmid DNA purification (Figure 2). It's a convenient high throughput and inexpensive solution for verifying the sequence of cloned constructs. Individual clones can either be lysed in water with a short heating step or added directly to the PCR reaction and lysed during the initial heating step. Colony PCR will result in only sequencing the region of interest as the primers are designed to amplify only the DNA insert in a vector DNA, as compared to full plasmid amplification and sequencing with RCA.

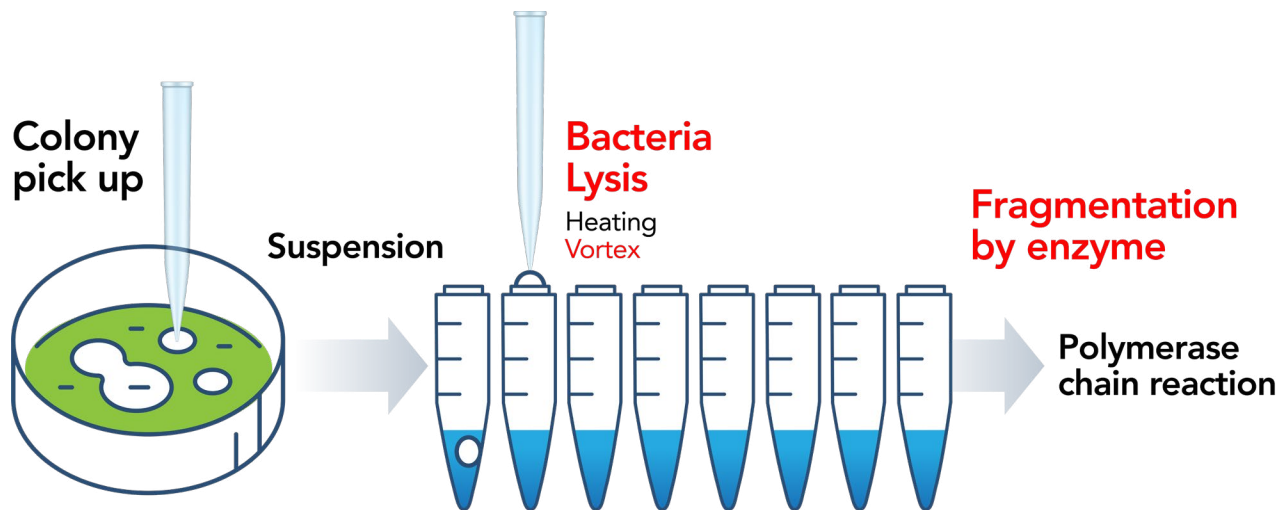


Figure 2: Colony PCR process

To exploit the full potential of RCA and Colony PCR in plasmid sequence verification, it's necessary to pair those techniques with a NGS library prep method that can match their throughput, ease of workflow, and cost. ExpressPlex™ HT is a library preparation kit designed specifically to meet the needs of ultra high throughput plasmid and amplicon sequencing including high efficiency, speed and automation readiness (Figure 3). Key features include:

- Conveniently formatted in a 384 wells PCR plate
- Up to 6,144 barcodes available (combinatorial dual indices)
- Everything included: no complex supply chain management of barcodes and primers
- Ready to go: no dispensing of reagents. Just add your DNA
- With an easy 90 minutes workflow, the ExpressPlex HT method is readily automatable. Inexpensive liquid handlers such as the SPT Labtech Apricot® S3 or the Opentrons Flex™ instrument can be used for prep, freeing up larger, more complex instrumentation for higher complexity operations.

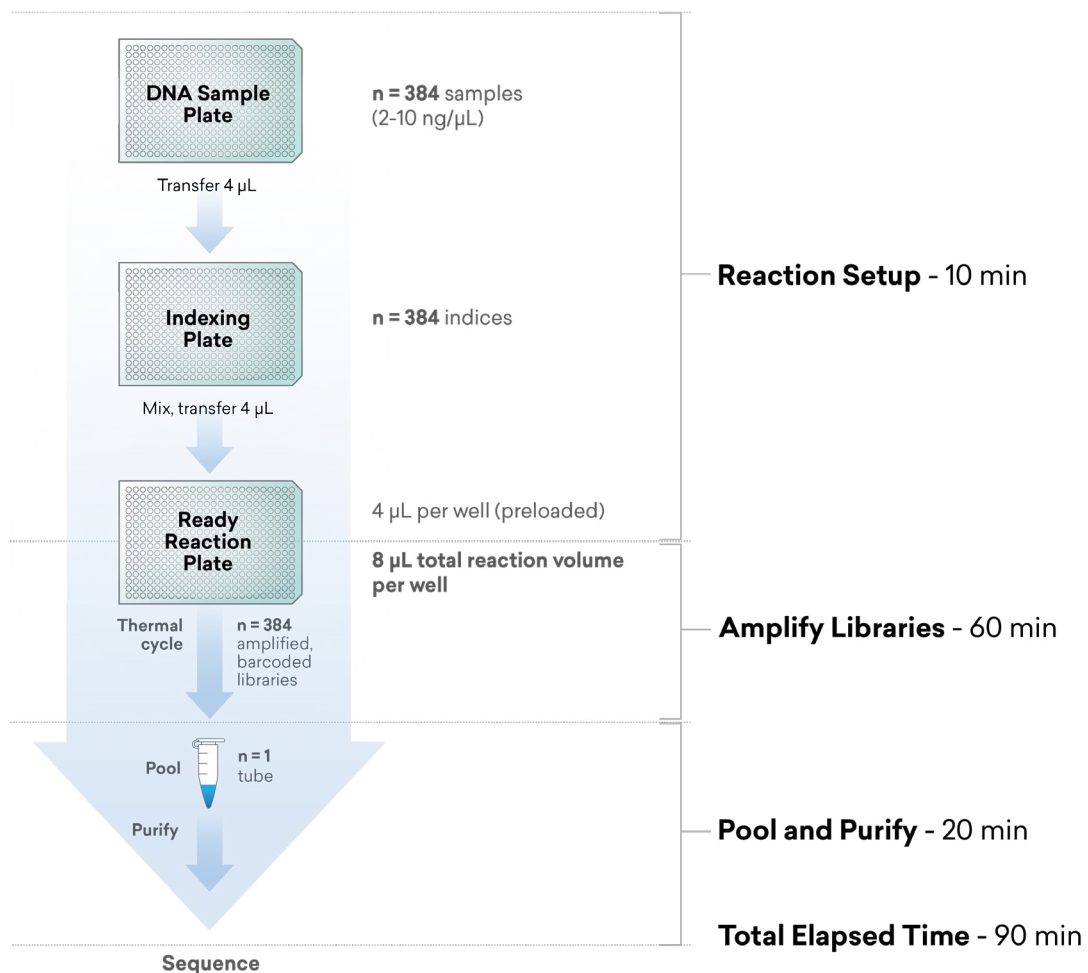


Figure 3: ExpressPlex HT Library Workflow

Materials and Methods

E.coli transformed with pDONR™xxx plasmids (Table 1) were plated on LB agar containing Kanamycin, incubated at 37°C for 24 hours. Twelve clones with different insert sizes were chosen for this experiment and colonies were randomly picked into four 96 wells plates containing 50µl of Ultrapure Water, to accommodate ExpressPlex HT 384 wells format (Figure 4).

Clone	Insert size / bp	PCR amplicon size / bp	Full plasmid size / bp	Vector
ScD00013271	5508	5746	8058	pDONR221
ScD00013266	5052	5290	7602	pDONR221
ScD00013267	5052	5290	7602	pDONR221
ScD00013263	4995	5233	7545	pDONR221
ScD00008904	4962	5200	7512	pDONR221
YpCD00016721	2016	5254	4566	pDONR221
VcCD00060963	1776	2014	4326	pDONR221
YpCD00013559	1719	1957	4269	pDONR221
YpCD00016351	1554	1792	4104	pDONR221
VcCD00025726	1539	1777	4089	pDONR221
YpCD00013828	1524	1762	4074	pDONR221
YpCD00013545	1512	1750	4062	pDONR221

Table 1: Plasmid constructs

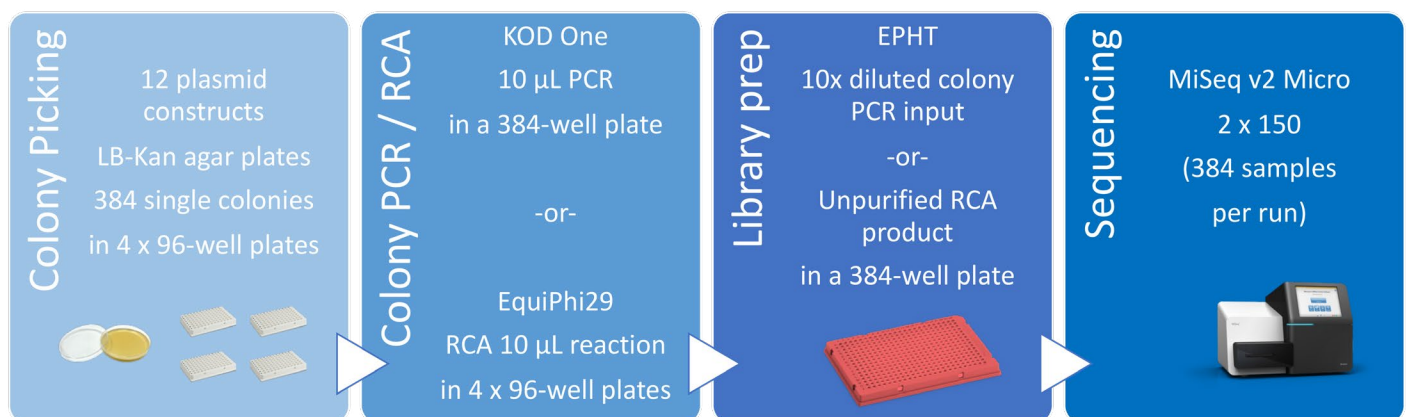


Figure 4: Plasmid verification workflow incorporating RCA/Colony PCR and ExpressPlex HT library preparation

All liquid transfer involved in ExpressPlex HT library preparation was performed using the Apricot S3 Liquid handler. The libraries were sequenced on the Illumina MiSeq™ on a v2 Micro kit 300 cycles, 2 X 150bp (384 samples). The 6,144 samples were run on the NextSeq™ 2000 on a P1 300 cycles kit.

Rolling Circle Amplification Protocol

Material	Supplier	Part Number
EquiPhi29™ DNA Polymerase	Thermo Fisher Scientific	A39390
Exo-Resistant random primers(500pM)	Thermo Fisher Scientific	SO181
dNTP Mix(25mM)	Thermo Fisher Scientific	NA
Focused Primer	IDT	NA
ECOR1	NEB	R0101S
DTT (0.1M)	Thermo Fisher Scientific	707265ML
Ultrapure Water	Invitrogen	10977-015
1M Tris-HCL pH 8.0	Invitrogen	15568-025

Reaction Setup

1. Pick one colony into each well of a labeled strip tube or plate containing 50µl of ultrapure water
2. Lyse cells at 95°C for 3 minutes. Keep lysate on ice
3. Verify concentration via Qubit™ and size via 2% E-gel™ (optional)
This is a safe stopping point. Samples can be stored at -20°C for up to 48hours, or proceed to the next step.
4. Prepare the following working solutions from stocks

Note: All reagents are diluted with 10mM Tris-HCl, pH8.0

2X Master Mix: For 20µl total reaction (10µl Mix + 10µl Lysate)

Reagent	Working Solution
dNTP	10mM
DTT	100mM
Focused Primers	12.5µM
Random Primer	0.78µM

Reagent	Volume per 20µl reaction
Ultrapure water	4.67µl
10X EquiPhi29 reaction buffer	2µl
10 mM dNTP	2µl
100mM DTT	0.2µl
12.5µM focus primers	0.08µl
0.78µM random primers	0.05µl
EquiPhi29 polymerase	1µl
Total volume	10µl

- Label a new 96 wells plate or strip tube and transfer 10µl of 2X Master Mix + 10µl of colony lysate to each well
- Pipette mix 10 times and run the RCA program:

Temperature	Time
42°C	3 hours
65°C	10 minutes
4°C	Hold

Safe stopping point. Samples may be stored at -20°C for up to 48 hours or proceed to ExpressPlex library protocol

Note: Dilution of RCA protocol before going into the library is optional. If the yield is too high, we recommend doing a global dilution

- Follow the ExpressPlex User Guide with 12 cycles amplification: <https://seqwell.com/resources/>

Colony PCR Protocol

Material	Supplier	Part Number
KOD One™ PCR Master Mix	TOYOBO	KMM-101
Ultrapure Water	Invitrogen	10977-015
pDONR221, pDONR201 FWD/ REV primers	IDT	NA
1M Tris-HCl, pH. 8.0	Invitrogen	15568-025

Reaction Setup

- Add 50µl of Ultrapure water and 1 colony of bacterial culture to each well of a labeled strip tube or plate
- Lyse cells at 95°C for 3 minutes. Keep lysate on ice

Safe stopping point. Samples may be stored at -20°C for up to 48 hours or proceed to the next step
- Prepare reagent working solutions from stock

Reagent	Volume for 10µl reaction	Volume for 50µl reaction
Ultrapure water	5µl	25µl
10X EquiPhi29 reaction buffer	1.2µl	6µl
10 mM dNTP	1.5µl	7.5µl
100mM DTT	2.3µl	11µl
Total	10µl	50µl

Note: All reagents are diluted in 10mM tris-HCl, pH 8.0. 50µl is the reaction volume standard

4. Run KOD One amplification program:

Temperature	Time
98°C	10 minutes
98°C	10 seconds
60°C	10 seconds
68°C	25 seconds
72°C	2 minutes
4°C	Hold

} 30-40 cycles

Note: If working with high yield samples (>10ng/μl) we advise to be on the lower end of the cycle range

5. Proceed to sample QC using Qubit and 2% E-gel

Safe stopping point. Store samples at -20°C for up to 48 hours, or continue with ExpressPlex library preparation

6. Perform a global dilution to accommodate ExpressPlex HT input range (8 – 40ng)

Note: Higher input may result in a large amount of untagged material (Multiple peaks), over estimating library yield. In this case, it's recommended to load the sequencer at 15pM instead of 12pM

7. Follow the ExpressPlex HT User Guide with 15 cycles of amplification: <https://seqwell.com/resources/>

Example Library Traces

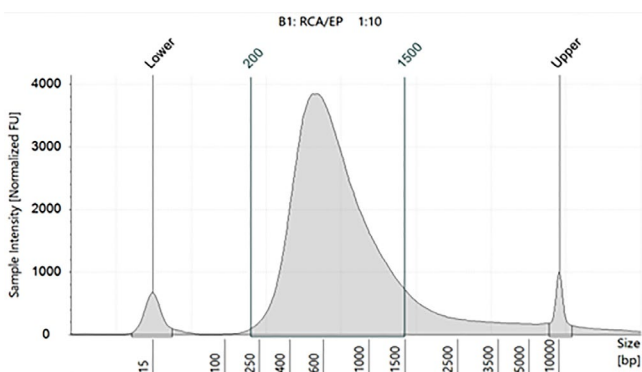


Figure 5a: RCA/EPHT library electropherogram, measured via Tape Station

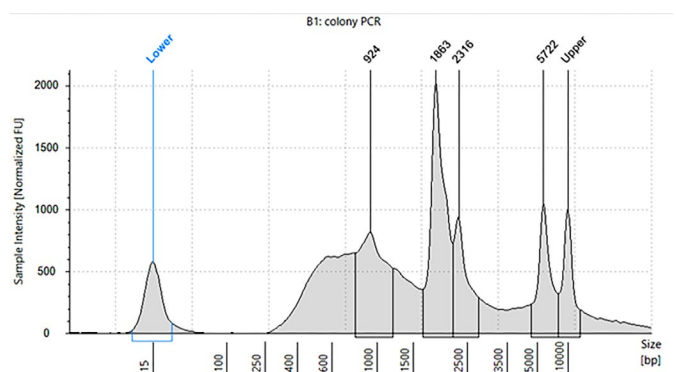


Figure 5b: Colony PCR/EPHT library electropherogram, measured via Tape Station

Note: Amplicons libraries (Colony PCR/EP) generated with ExpressPlex HT may have atypical appearance due to residual products, but successfully sequence without any issues

Sequencing Quality Control

In high throughput plasmid sequencing, key metrics generally assessed are

- Error-free assembly of the DNA insert
- High percentage alignment to the reference
- Coverage

These metrics were successfully measured in this experiment. The samples were assembled with our SNAP pipeline and verified using the Blastn. The assembly rate is greater than 95% for both colony PCR and RCA with an alignment rate of about 97% (Figure 6). Both methods showed Coverage uniformity across the entire length of each plasmid (Figure 7).

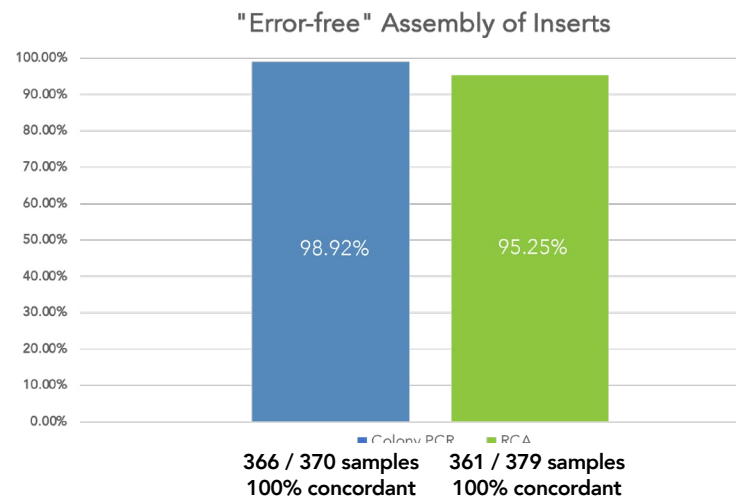


Figure 6: Assembly rate RCA / Colony PCR

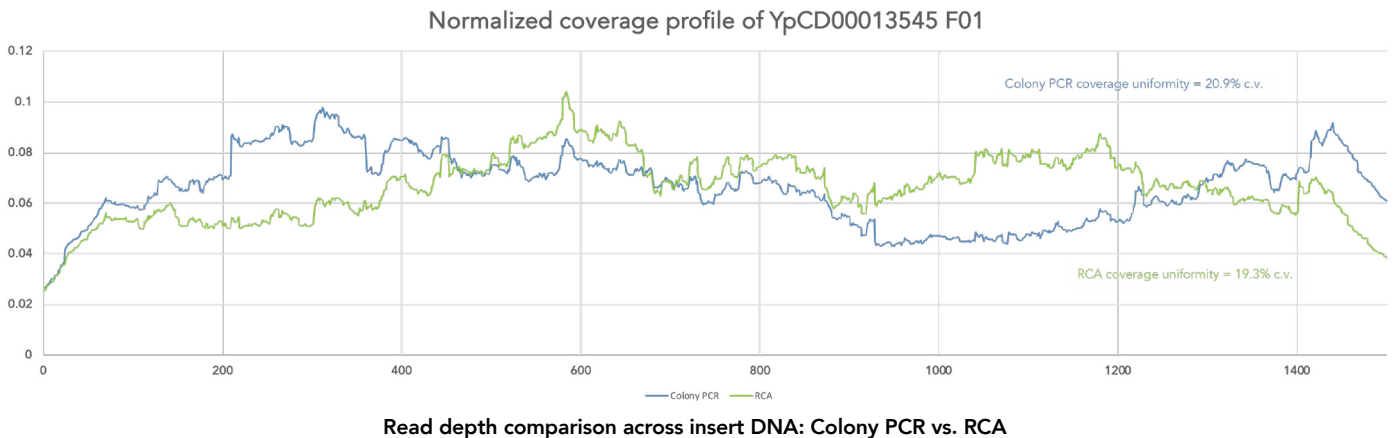


Figure 7: Normalized coverage across target inserts of 1.5kb for Colony PCR and RCA.

Summary

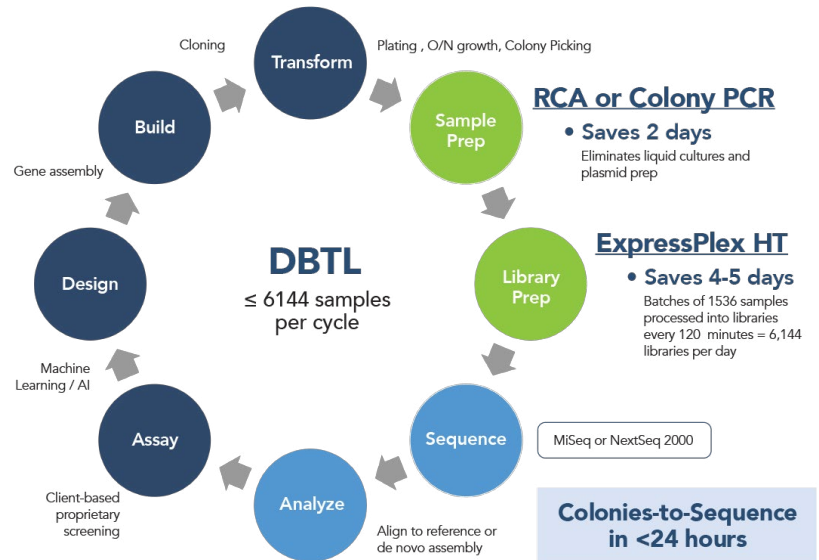
In synthetic biology, a verification method that is high throughput, cost-effective and reliable is essential. A streamlined workflow combining RCA or Colony PCR with ExpressPlex HT provides a highly effective pipeline to screen thousands of constructs in the same sequencing run, side-stepping cumbersome high throughput plasmid minipreps.

In an industrial setting, logistics and expenses can rapidly become a challenge in synthetic biology. Having a rapid workflow that is cost effective permits more time to analyze data and less time processing samples. This streamlined workflow provides a colony-to-sequence turnaround time of less than 24 hours.

We have demonstrated the robustness of this workflow across a large number of sample types, using different sized plasmids and inserts. We anticipate that these techniques can accelerate the DBTL iterative cycle by up to 4 to 5 days, which will greatly impact the efficiency of synthetic biology-driven initiatives and protein engineering.

Conclusions

The synthetic biology discovery process requires significant advances in overall throughput that matches the desired turnaround time. Less time spent processing samples on the bench allows more time to analyze data and get to the needed results. Combining RCA or colony PCR with the ExpressPlex library prep method results in the fastest possible DBTL cycle at a cost and cycle time that is competitive with Sanger sequencing.



Resources of Interest

- Rolling Circle Amplification as an Efficient Analytical Tool for Rapid Detection of Contaminants in Aqueous Environments. [Biosensors \(Basel\). 2021 Oct; 11\(10\): 352.](#)
- [OCTOPUS 3.0 – Next-day turnaround of full plasmid sequencing directly from colonies.](#)
- [ExpressPlex HT Product Brochure](#)
- [ExpressPlex HT User Guide](#)