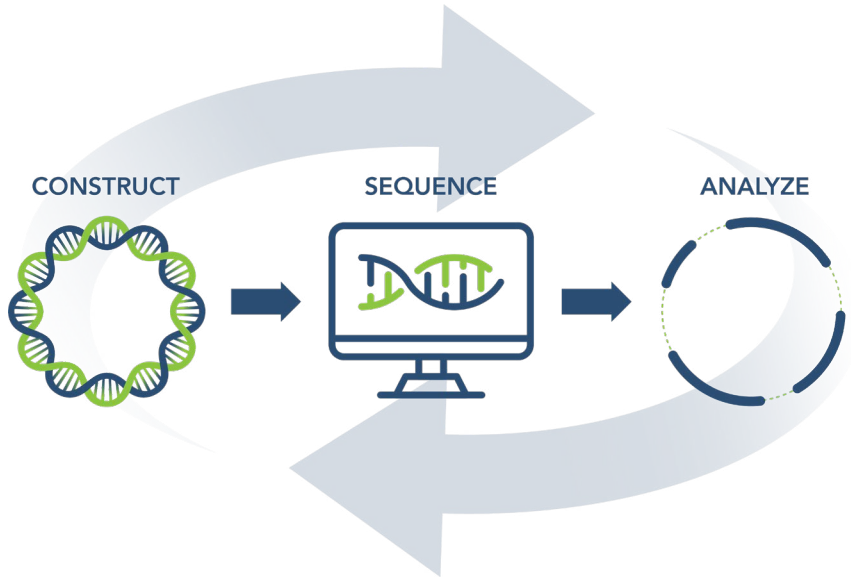
# Facilitating Ultra-High Throughput Synthetic Biology Discovery via RCA, Colony PCR and Massively Multiplexed Plasmid and Amplicon Sequencing

## 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.

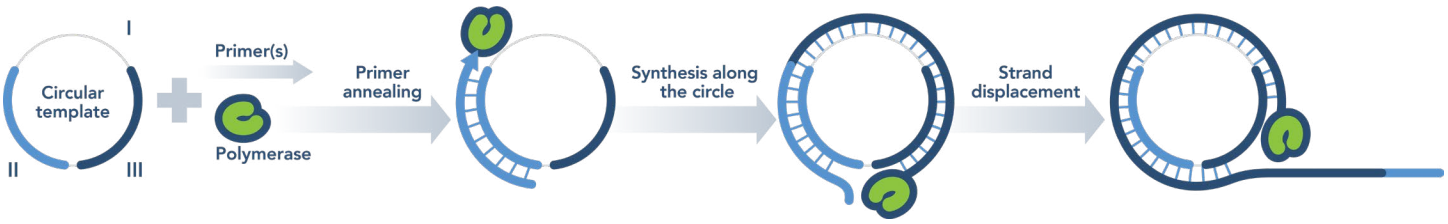



**Figure 1:** RCA process

- 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.

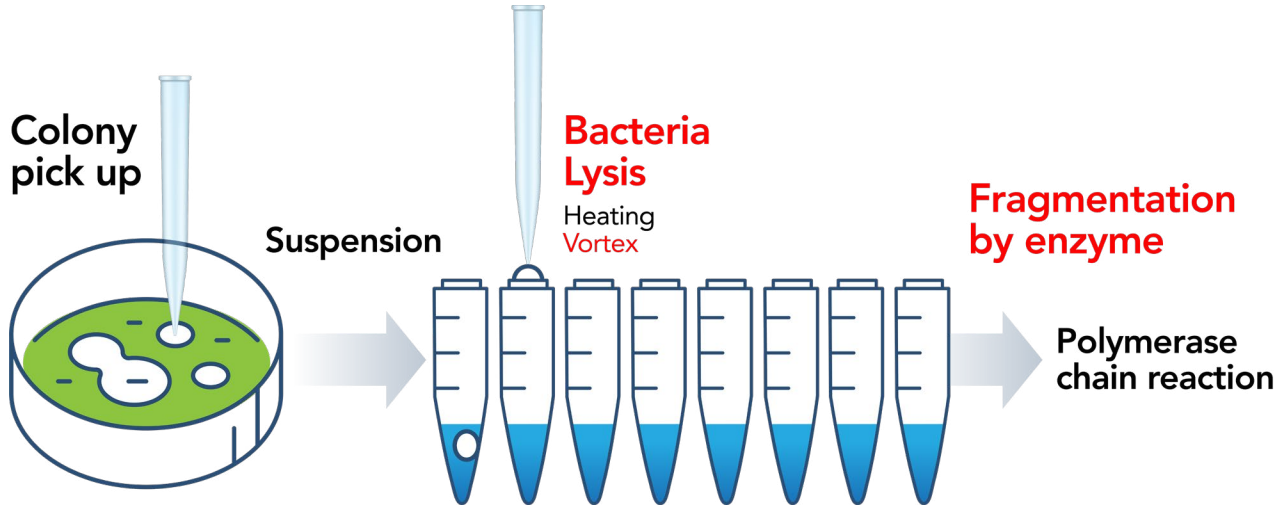



**Figure 2:** Colony PCR process

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-minute workflow, the ExpressPlex HT method is readily automatable.

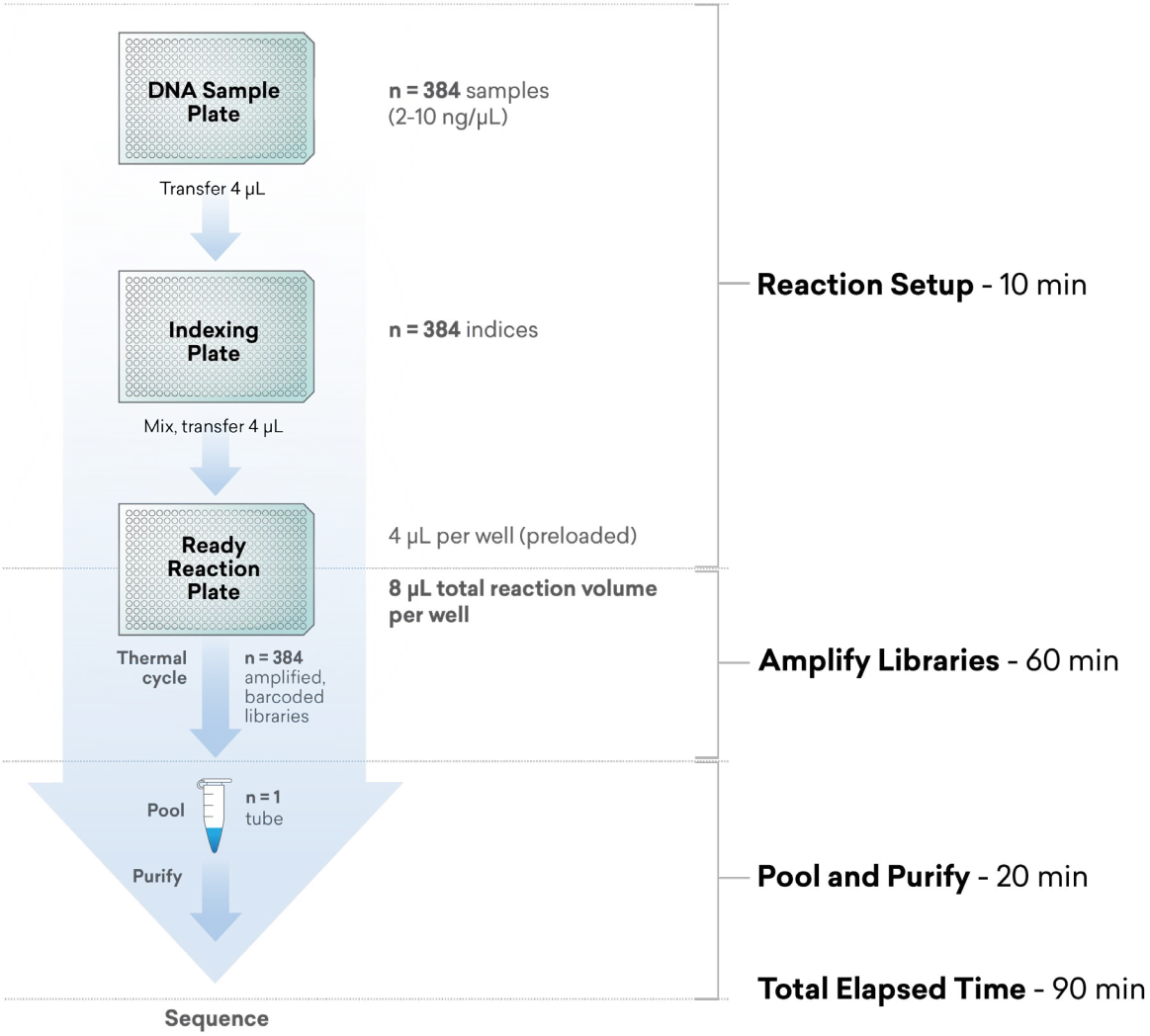



**Figure 3:** ExpressPlex HT Library Workflow

## Materials and Methods

*E.coli* transformed with pDONR221 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

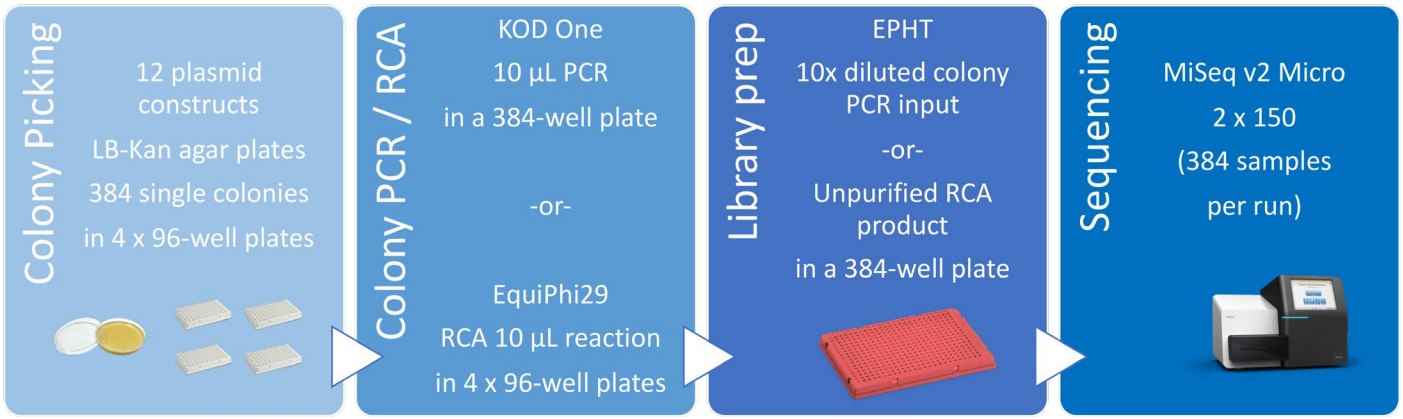



**Figure 4:** Plasmid verification workflow incorporating RCA/Colony PCR and ExpressPlex HT library preparation

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).

## Rolling Circle Amplification Protocol

| Material | Supplier | Part Number |
|---|---|---|
| EquiPhi29™ DNA Polymerase | Thermo Fisher Scientific | A39390 |
| Exo-Resistant Random Primers (500µM) | Thermo Fisher Scientific | SO181 |
| dNTP Mix (25mM) | Thermo Fisher Scientific | R1121 |
| 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. Add 50 µl of Ultrapure water and 1 colony of bacterial culture into each well of a labelled strip tube or 96-well plate. Vortex and briefly pulse-spin.
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 RCA Master Mix as follows:

## Master mix:

| Reagent | Volume for 1 reaction | Volume for 96 reactions |
|---|---|---|
| Ultrapure water | 4.67 µl | 448.32 µl |
| 10X EquiPhi29 reaction buffer | 2 µl | 192 µl |
| 10 mM dNTP* | 2 µl | 192 µl |
| 100 mM DTT* | 0.2 µl | 19.2 µl |
| 12.5 µM Focus primer** | 0.08 µl | 7.68 µl |
| 0.78 µM Random primer* | 0.05 µl | 4.8 µl |
| EquiPhi29 Polymerase | 1 µl | 96 µl |
| Total Volume | 10 µl | 960 µl |

*Diluted from stock in 10mM Tris-HCl pH 8.0.
**For optimal performance, primer titration with samples may be needed.

5. Label a new 96 wells plate or strip tube and transfer 10µl of RCA Master Mix and 10µl of colony lysate to each well.
6. Pipette mix 10 times and be careful not to introduce bubbles.
7. 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 product before going into the library preparation is optional. If the yield is too high, we recommend doing a global dilution.

8. Follow the ExpressPlex User Guide with 12 cycles amplification.

## Colony PCR Protocol

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

## Reaction Setup

1. Add 50µl of Ultrapure water and 1 colony of bacterial culture to each well of a labeled strip tube or plate.
2. 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.
3. Verify concentration via Qubit™ and size via 2% E-gel™ (optional).
4. Prepare reagent working solutions from stock:

| Reagent | Volume* |
|---|---|
| Ultrapure water | 11.5 µl |
| FWD/REV Primer Mix (5µM)** | 6 µl |
| Colony lysate | 7.5 µl |
| 1M Tris-HCl, pH. 8.0 | 25 µl |
| Total Volume | 50 µl |

*Volume can be miniaturized to 10 µl reaction.
**Diluted from Stock in 10mM Tris-HCL pH 8.0.

5. Pipette mix 10 times and be careful not to produce bubbles.
6. Place in thermocycler and run KOD One amplication program:

| Temperature | Time | |
|---|---|---|
| 98°C | 10 minutes | |
| 98°C | 10 seconds | 30-40 cycles |
| 60°C | 10 seconds | |
| 68°C | 25 seconds | |
| 72°C | 2 minutes | |
| 4°C | Hold | |

**Note:** Adjust the number of cycles according to the sample. Keep in mind that the concentration of Colony PCR products need to be >10 ng/µl for global dilution in downstream application.

7. 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 HT library preparation.

8. 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 in the library fragment trace), hence overestimating library yield. In this case, it's recommended to load the sequencer at 15pM instead of 12pM.

9. Follow the ExpressPlex HT User Guide with 15 cycles of amplification:

## Example Library Traces

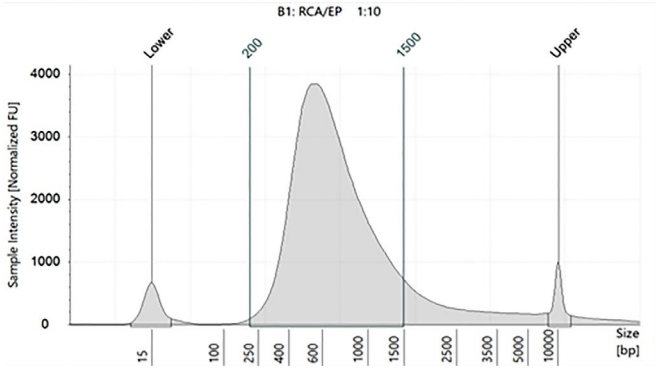



**Figure 5a:** RCA/EPHT library electropherogram, measured via Tape Station

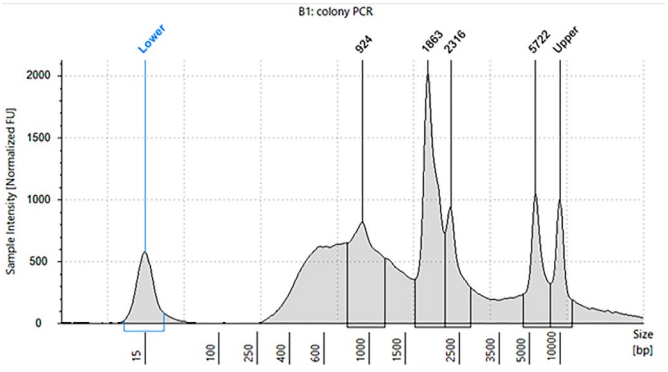



**Figure 5b:** Colony PCR/EPHT library electropherogram, measured via Tape Station

**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 include:

- 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 seqWell's SNAP pipeline and verified using BLASTN. The de novo assembly rate is greater than 95% for both colony PCR and RCA (Figure 6) with an alignment rate to the reference of about 97%. Both methods showed coverage uniformity across the entire length of each plasmid (Figure 7).

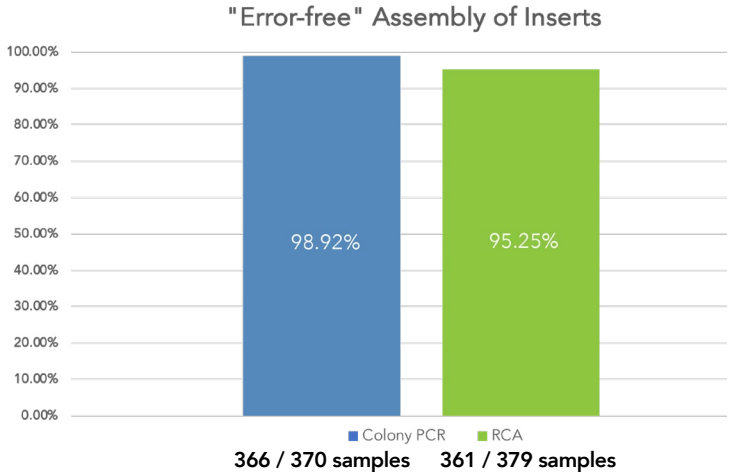



**Figure 6: De novo assembly rate for colony PCR and RCA.** 366 of 370 samples of colony PCR (98.92%) and 361 of 379 samples of RCA (95.25%) yielded "error-free" de novo assembly of inserts. 14 colony PCR samples and 5 RCA samples were omitted due to clone contaminations.

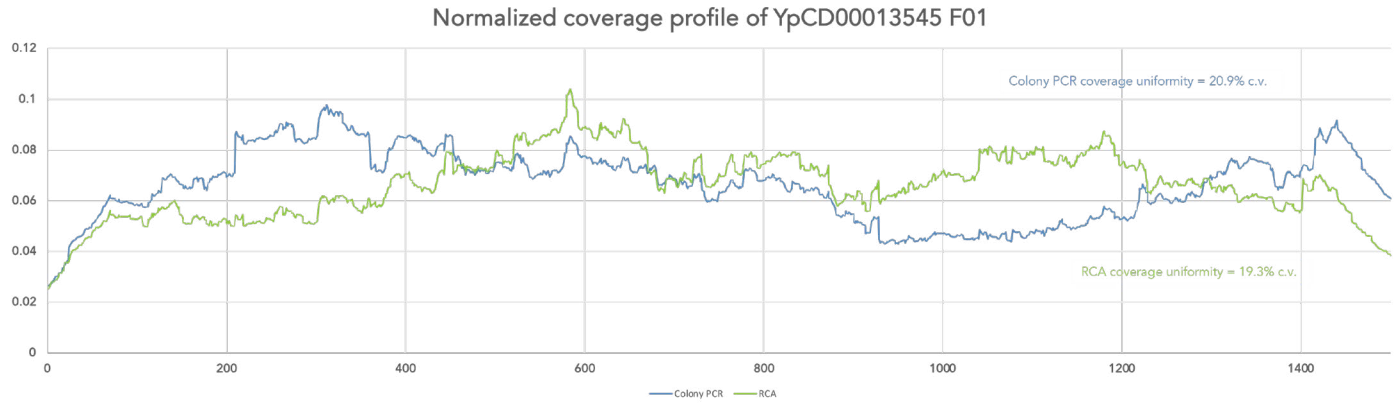



**Figure 7:** Normalized coverage across target inserts of 1.5kb for Colony PCR and RCA.

## Summary

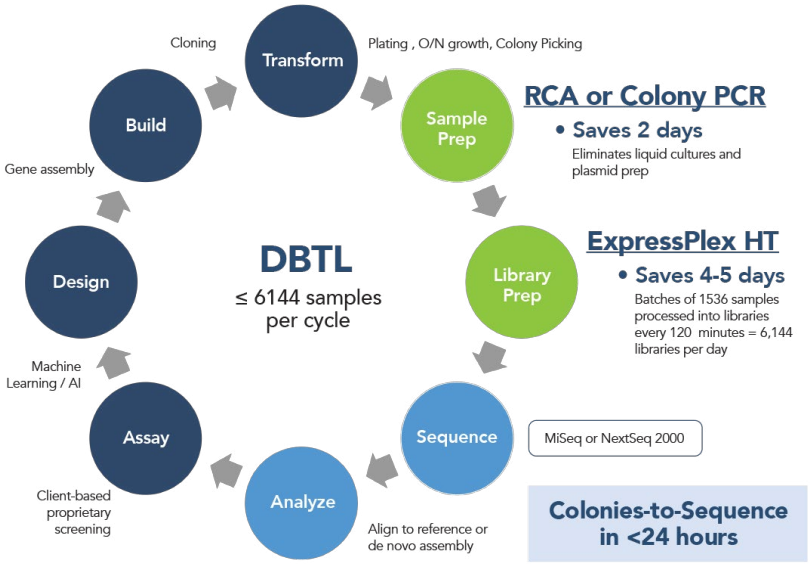
In synthetic biology, a verification method that is high throughput, cost-effective and reliable is essential. A streamlined workflow combining RCA or Colony PCR with ExpressPlex HT provides a highly effective pipeline to screen thousands of constructs in the same sequencing run, side-stepping cumbersome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