Rapid Oligonucleotide Synthesis Cycle Optimization to Reduce Reagent Consumption while Maintaining High Average Stepwise Yields and Maximizing Reproducibility

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ABSTRACT: Development of an optimal oligonucleotide synthesis cycle is a very time-consuming yet critical task, both for conducting R&D work as well as preparing an instrument for High-Throughput (HTP) manufacturing. Many dozens of variables must be identified and controlled across experiments to ensure each new set of data can be compared to data generated from another synthesis. Even with the use of multiple replicates and controls, controlling all critical variables can be extremely difficult, even for the most meticulous scientist. The ability to run multiple protocols simultaneously can eliminate most of those variables, allowing researchers to have much greater confidence in their results. A 16-channel High-Throughput (HTP) oligonucleotide synthesizer was developed with a dual emphasis on 1) enabling the user to conduct rapid protocol optimization as well as quick R&D experiments requiring multiple protocols and 2) robust HTP manufacturing of oligonucleotides. This synthesizer, the Shasta 1.0, was used to test 60 protocols, optimizing 11 variables, in just 4 runs. Both ASWY and reproducibility improved while reagent consumption was significantly reduced.

Introduction

When comparing oligonucleotide synthesizers that use closed columns containing solid support (e.g. ABI392/394s or K&A H-8/H-32s) vs. open columns in which reagents are dispensed from overhead nozzles (Mermade, Dr. Oligo, ABI3900), closed column systems tend to give more reproducible results and often provide higher purity oligos. The Shasta 1.0 synthesizer was designed to allow for reagents to flow through the use of brief pulses of positive pressure to either individual columns or wells in microtiter plate format (96- or 384-well). In designing this system, the primary goal was to create an open-column HTP instrument capable of providing both yields and reproducibility matching or surpassing those of closed-column systems. By integrating a work-flow consisting of design/hardware changes immediately followed by test syntheses, measuring both yields and reproducibility (as measured by the Coefficient of Variation), rapid iterations on synthesizer design were implemented, resulting in reproducible average stepwise yields (ASWYs) of 99.4-99.6%. However, the protocol that was used throughout most of its development period called for large volumes of reagents that would likely be cost-prohibitive in a manufacturing environment. We therefore conducted an experiment, testing 60 protocols over 4 runs, to reduce reagent consumption while maintaining high ASWY and minimizing variability.

Materials and Methods

Hardware: A Sierra BioSystems Shasta oligonucleotide synthesizer was used for all experiments. The Shasta—in its simplest configuration—is a high-throughput oligonucleotide synthesizer, capable of accepting 1 to 96 ABI-style synthesis columns or a 96-well synthesis plate. Alternative configurations allow for two 96-well plates (or 192 columns) or one or two 384-well plates. All experiments described here used 96-well plates from Biocomma (P/N MS96-0200) in the single-plate configuration.

The instrument dispenses from a set of 64 nozzles (4 per reagent), using pressurized Argon to provide an anhydrous positive-pressure environment. Reagents were held under a 9 psi Argon atmosphere while the internal reaction chamber pressure was held at 3 psi. Reagents are pulsed through the columns to the waste container, maintained at atmospheric pressure.

A typical synthesis cycle step consists of a Dispense step, in which reagent is dispensed to the column or well, followed by a Pulse step, in which the reagent is briefly pushed into the solid-support. A "Hold" step then allows the reagent to slowly pass through the synthesis column, typically for 25-120s, depending on the step. Finally, a Drain step pushes the remaining reagent through the solid-support. Columns/wells are divided into 16 independent Drain Groups, or "Banks" which can be pulsed or drained independently. The use of multiple nozzles for each reagent allows each bank to be treated independently of others, minimizing the potential for scheduling conflicts. When scheduling conflicts do occur, the speed at which reagents are dispensed ensures that no reagent can remain in contact with the solid-support for more than 12s longer than specified in the protocol.

A representative 10mer oligodeoxyribonucleotide of sequence 5'-dCATGTATGCT-3' was chosen as the primary test-oligomer to assess the ASWY and reproducibility of each hardware configuration or synthesis cycle tested. This 10mer was chosen because it is a typical oligo, incorporating all four standard DNA bases, yet is short enough to allow for facile HPLC and/or ESI LC-MS analysis, and thus a rapid development cycle turnaround time. Periodic testing with longer oligos and fluorescently labeled oligos has revealed that ASWYs for the 10mers are representative of longer oligos.

Optimization Experiments: Three 96-well plates were synthesized at 200 nmol scale using modifications to our standard synthesis cycle (the "Default Protocol"), detailed in **Table 1**. Each synthesis used 16 unique protocols, varying three or four variables relating to a single step of the standard oligo synthesis cycle (Deblock, Coupling, Capping, or Oxidation). The conditions of each protocol tested are described in **Tables 2a – 2c,** with the results presented in **Tables 3a-3c**. Fifteen test protocols were run per plate, using six replicates each, alongside a set of six positive control replicates (highlighted in yellow in **Tables 3a-3c**) that used the unmodified Default Protocol. This positive control had previously been used to demonstrate high

reproducibility and ASWYs when making 10mer to 80mer deoxyribonucleotides of various sequences.

Following the original three runs, a fourth experimental plate was designed to test 15 additional protocols, probing all four of the synthesis cycle steps, in an attempt to further reduce reagent consumption while maintaining high reproducibility (base synthesis cycle and modifications detailed in **Tables 4a** and **4b**).

Table 1. (right) Default Protocol conditions.

Tables 2a-c. (below) Variations of the Default Protocol assessed for effect on ASWY and reproducibility (%CV).

Step	# Steps	Time (s)	Vol. (uL)	Pulse (ms)
Deblock:	2	45	300	300
ACN	1	0	200	0
Couple:	2	40	120+90	15
Cap	1	30	200	300
Ox	2	30	100	200
ACN	1	0	200	0
Cap	1	30	200	300
ACN	1	0	200	0

Bank	Steps	Time	Vol.	Pulse
1	1	60	200	0
2	1	60	200	1
3	1	60	300	0
4	1	60	300	1
5	1	90	200	0
6	1	90	200	1
7	1	90	300	0
8	1	90	300	1
9	2	30	150	0
10	2	30	150	1
11	2	30	200	0
12	2	30	200	1
13	2	45	150	0
14	2	45	150	1
15	2	45	200	0
16	2	45	200	1

Table 2a: Deblock

Bank	Steps	Time	Vol.	Pulse
1	1	45	126	0
2	1	45	126	1
3	1	45	210	0
4	1	45	210	1
5	1	90	126	0
6	1	90	126	1
7	1	90	210	0
8	1	90	210	1
9	2	45	126	0
10	2	45	126	1
11	2	45	210	0
12	2	45	210	1
13	2	90	126	0
14	2	90	126	1
15	2	90	210	0
16	2	90	210	1

Table 2b: Coupling

Bank	Order	Vol.	Time
1	CO	120	25
2	CO	120	40
3	CO	200	25
4	CO	200	40
5	COC	120	25
6	COC	120	40
7	COC	200	25
8	COC	200	40
9	COOC	120	25
10	COOC	120	40
11	COOC	200	25
12	COOC	200	40
13	OC	120	25
14	OC	120	40
15	OC	200	25
16	OC	200	40

Table 2c: Ox/Cap

Cleavage and Analysis: All syntheses were followed by an automated diethylamine treatment protocol (10% DEA in ACN) on the synthesizer to remove cyanoethyl groups prior to the cleavage/deprotection step. Average Stepwise Yield (ASYW) and the Coefficient of Variation were calculated for each condition tested. All oligos were analyzed by HPLC and/or ESI LC/MS following gas-phase cleavage and deprotection using NH_3(g) at 65 °C for 3.25 h. Oligos were eluted from the synthesis plate in 400µL dH_2O and transferred to an Agilent 1100 series HPLC autosampler. 1-2µL of sample was injected and gradient-eluted from the HPLC column using ACN in a 100 mM solution of hexylammonium acetate ion-pairing reagent. The flow stream was directed to an Agilent G1315B DAD spectrophotometer and chromatograms were acquired at 260 nm. Selected samples were analyzed further by a Waters Micromass LCT Premier ESI mass spectrometer. ASWY was calculated from the HPLC DAD peak areas. Peak areas from all oligonucleotides present in the samples were exported to Excel, from which ASWY and %CV were calculated:

$ASWY = 100 \times (\%FLP/100)^{(1/n)}$, where n = number of couplings

$\%CV = 100 \times Stdev_{ASWY}/Average_{ASWY}$

Results

Plate 1 "Deblock": The first run focused on the Deblock step and varied the reaction time, volume of deblock, the number of dispense steps, and the presence or absence of a 100ms "pulse step." Results are detailed in **Table 3a**. Previous data showed that—for primer-length oligos—the harmful effects of 3% TCA did not increase depurination levels relative to 3% DCA when using 2x25s TCA reaction times in conjunction with the use of dmf-dG. The two best conditions from Plate 1, in terms of both ASWY and variability, used two deblock steps, shorter reaction time (2x30s), and a single 100ms pulse to push the reagent into the CPG bed. Both the 200µL dispense volume as well as the 150µL volume appeared ideal in generating oligos at 99.4% ASWY reproducibly. *Notably, the 2x150 µL dispense represents a 50% decrease in reagent consumption* relative to the original protocol.

Condition	# Steps	Time (s)	Vol (uL)	Pulse	Average	%CV
10	2	30	150	1	99.42	0.02
12	2	30	200	1	99.40	0.02
11	2	30	200	0	99.37	0.07
15	2	45	200	0	99.36	0.03
14	2	45	150	1	99.36	0.03
8	1	90	300	1	99.34	0.08
16	2	45	300	1	99.33	0.09
13	2	45	150	0	99.33	0.04
9	2	30	150	0	99.32	0.03
7	1	90	300	0	99.28	0.07
6	1	90	200	1	99.25	0.17
4	1	60	300	1	99.25	0.09
5	1	90	200	0	99.14	0.17
3	1	60	300	0	99.10	0.13
2	1	60	200	1	98.72	0.24
1	1	60	200	0	95.68	3.68

Table 3a. Conditions, ASWY, and %CV for Deblock protocols.

Plate 2 "Coupling": The second run focused on the Coupling step and varied the reaction time, volume of activator and phosphoramidite, the number of dispense steps, and the presence or absence of a 15ms "pulse step." Results are detailed in **Table 3b**. Earlier optimization experiments demonstrated that we could reduce phosphoramidite concentration to 50 mM if reaction times were increased or the number of coupling steps was doubled, so all reactions described here use *50 mM phosphoramidite*. Those experiments furthermore indicated that a 3:4 phosphoramidite/activator ratio provides optimal results when using 250 mM 5-(ethylthio)-1*H*-tetrazole (ETT) as activator with 50 mM phosphoramidite. Conditions 6 and 8 appear to provide the highest yields, but only condition 8 had low variance. Both conditions use a single 90s reaction time with a single 15ms pulse, but condition 6, using 126µL reaction volume displayed high variability while condition 8, with a 210µL reaction volume, was much more consistent. We suspect that the lower volume in condition 6, in conjunction with the pulse and the longer reaction time may have caused some wells to drain to dryness prior to the end of the step. Additional coupling conditions using shorter reaction times with both the low and high volumes were tested further on Plate 4.

Condition	# Steps	Time (s)	Vol (uL)	Pulse	Average	%CV
6	1	90	126	1	99.45	0.15
8	1	90	210	1	99.42	0.03
3	1	45	210	0	99.40	0.13
12	2	45	210	1	99.40	0.03
4	1	45	210	1	99.40	0.09
11	2	45	210	0	99.38	0.07
10	2	45	126	1	99.37	0.03
7	1	90	210	0	99.36	0.04
5	1	90	126	0	99.36	0.21
15	2	90	210	0	99.35	0.12
9	2	45	126	0	99.35	0.13
16	2	90	210	1	99.33	0.08
13	2	90	126	0	99.29	0.07
1	1	45	126	0	99.26	0.07
2	1	45	126	1	99.24	0.23
14	2	90	126	1	99.23	0.22

Table 3b. Conditions, ASWY, and %CV for Coupling protocols. Default protocol highlighted in yellow.

Plate 3 "Cap/Ox": The third optimization experiment varied the Cap/Ox order, the number of each step, the volume, and reaction time. Results are detailed in **Table 3c**. Nearly all reactions displaying both high ASWY and low %CV used protocols in which the Oxidation step(s) were sandwiched between two Cap steps. Both the Cap/Ox and Ox/Cap formats tended to provide higher variability in yields, though a single Ox/Cap condition using 100µL Oxidizer for 30s and one pulse with a Cap step using 200µL Capping reagents for 25s with a single pulse also provided 99.4% ASWY with %CV=0.03%. Further refinements of the Cap/Ox/Cap cycle were investigated on the fourth experimental plate as well.

Condition	Steps	Vol (uL)	Time (s)	Average	%CV
10	COOC	120	40	99.55	0.02
7	COC	200	25	99.54	0.03
8	COC	200	40	99.54	0.04
15	OC	200	25	99.54	0.03
11	COOC	200	25	99.54	0.07
9	COOC	120	25	99.54	0.09
12	COOC	200	40	99.54	0.05
6	COC	120	40	99.51	0.03
14	OC	120	40	99.51	0.08
13	OC	120	25	99.49	0.05
16	OC	200	40	99.44	0.11
4	CO	200	40	99.35	0.33
5	COC	120	25	99.10	1.41
3	CO	200	25	99.00	1.80
2	CO	120	40	99.00	0.86
1	CO	120	25	98.90	2.57

Table 3c. Conditions, ASWY, and %CV for Ox/Cap protocols.

Plate 4: Plate 4 focused on the best reaction conditions from the previous three plates, attempting to further reduce reagent consumption. Reactions followed a "Basic Refined Protocol" (**Table 4a**) and had certain steps replaced by the more restrictive conditions detailed in **Table 4b**. Amongst the four protocols investigating optimal deblock conditions, all conditions tested provided both good ASWY and low CV, with the exception of the lowest volume tested, 100µL. Condition 2, in which 125µL was dispensed twice, followed by a 75ms pulse and 30s reaction time for each step, provided the highest yields with the greatest reproducibility (%CV = 0.03%). *This represents a 58% decrease in deblock use (250µL vs 600µL), relative to the original protocol.* Of the reactions probing coupling conditions, all conditions tested provided high ASWY (99.5%) and low CVs (0.02-0.03%), so we will continue forward using the smaller volume (85+65µL activator/phosphoramidite) at the shortest reaction time (50s). Future studies will focus on reducing this volume further.

Step	# Steps	Time (s)	Vol. (uL)	Pulse (ms)
Deblock	2	30	150	100
ACN	1	0	200	0
Couple	1	90	120+90	15
Cap	1	30	120	75
Ox	2	30	100	200
ACN	1	0	200	0
Cap	1	30	120	75
ACN	1	0	200	0

Table 4a. Basic Refined Protocol.

Of the protocols varying the Cap and Oxidation steps, all protocols using the Cap/Ox/Ox/Cap sequence provided oligos with 99.5% ASWY. Interestingly, those using smaller volumes (conditions 11, 12, 14, 15) had the greatest reproducibility, each with %CVs of 0.02%. Notably, the lowest volumes tested for both Oxidizer and Capping reagents (60µL Ox, 60µL Cap) performed very well and will likewise be used in our future standard protocol. Further experiments will be performed to determine if lower volumes may be used for both reagents.

Condition	Steps	# Steps	Time (s)	Vol (uL)	Pulse (ms)	Average	%CV
12	Cap/Ox	1/2/1	30/30	80/120	100/200	99.54	0.02
14	Cap/Ox	1/2/1	30/30	100/60	200/50	99.53	0.02
11	Cap/Ox	1/2/1	30/30	60/120	50/200	99.52	0.02
15	Cap/Ox	1/2/1	30/30	100/80	200/100	99.51	0.02
16	Cap/Ox	1/2/1	30/30	100/100	200/200	99.51	0.05
13	Cap/Ox	1/2/1	30/30	100/120	200/200	99.46	0.08
6	Couple	1	60	120/90	15	99.53	0.03
9	Couple	1	60	85/65	15	99.51	0.02
10	Couple	1	70	85/65	15	99.51	0.03
8	Couple	1	50	85/65	15	99.51	0.03
5	Couple	1	50	120/90	15	99.50	0.02
7	Couple	1	70	120/90	15	99.49	0.02
2	Deblock	2	30	125	75	99.51	0.03
4	Deblock	2	30	175	100	99.48	0.04
3	Deblock	2	30	150	100	99.47	0.04
1	Deblock	2	30	100	50	99.33	0.04

Table 4b. Modifications to "Basic Refined Protocol". Rows using the unmodified Refined protocol are highlighted in yellow.

Discussion and Conclusions

Traditionally, when performing synthesis cycle optimization, experiments are plagued by run-to-run variability issues when all the relevant variables are not perfectly controlled. By removing this major source of variability and being able to test up to 16 different protocols on the same plate in parallel, the vast majority of these variables can be eliminated and the resulting data can be decision-making, even when running fewer replicates. In just one week, this simple set of experiments quickly allowed us to significantly reduce reagent consumption while maintaining good coupling yields and increasing reproducibility—notably, testing 60 different protocols in just 4 runs. Deblock volume was dropped from 600µL to 250µL, Coupling reagents dropped from 120+90µL to 85+65µL for Activator/Phosphoramidite (50 mM phosphoramidite), Capping reagent was reduced from 100µL to 60µL and Capping reagent was likewise reduced to 60µL from 200µL. By using 50 mM phosphoramidites, we can effectively use the equivalent of 32.5µL of 100 mM phosphoramidite while maintaining good ASWYs. Furthermore, 3 of 4 reagents gave reproducible results at the lowest volumes tested, suggesting further reductions will likely be possible.

As any oligonucleotide chemist who has attempted to increase ASWY beyond 99.5% will attest, measuring statistically significant differences between two protocols can be extraordinarily difficult. Although the rapid method described here is a very efficient "first pass" at quickly determining high-yielding conditions, we acknowledge that many of the conditions tested were not statistically significantly different (as measured by a 95% CI). As we attempt to further push up ASWYs, it is likely we will need to use 12 or even 24 replicates to ensure meaningful decisions can be made—which is still preferable to using 96 replicates and having to manage run-to-run variability issue. However, it did not escape our notice that on Plate 4, representing protocols slightly modified after only one round of optimization, all but one condition resulted in oligos with 99.5% ASWY, while Plates 1-3 displayed ASWYs ranging from 95.6% to 99.5%, thus demonstrating the utility of the parallel protocol optimization procedure described here.

ASSOCIATED CONTENT

This is the first Application Note of many that will relay developments of the Shasta as further refinements are integrated, both to the physical hardware, as well as any synthesis cycles that have been developed. In the future, a series of 'Application Notes' will be provided free of charge at https://sierrabio.com/category/sierra-bio-systems-news/application_notes.

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Author Contributions

‖G.P.M. conducted all of the synthesis cycle optimization experiments, analytical chemistry, and prepared the manuscript. M.C.H. and B.R.E. designed the Shasta hardware and M.H. assembled and fine-tuned it.

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ABBREVIATIONS

ACN: Acetonitrile; ASWY: Average Step-wise Yield; %CV: Percent Coefficient of Variation; DAD: Diode Array Detector; DEA: *N*,*N*-diethylamine; dmf-dG: 5'-Dimethoxytrityl-*N*-dimethylformamidine-2'-deoxyGuanosine,3'-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite; ESI LC-MS: Electrospray Ionization Liquid Chromatography-Mass Spectrometry; ETT: 5-(Ethylthio)-1*H*-tetrazole; HPLC: High Performance Liquid Chromatography; HTP: High-Throughput; ms: millisecond; s: second; 95% CI: 95% Confidence Interval.