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Next generation 1536-well oligonucleotide synthesizer with on-the-fly dispense

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ABSTRACT

Here we report the development of our Next Generation Automated Multiplexed Oligonucleotide Synthesizer (NG-1536-AMOS), capable of producing 1536 samples in a single run using a multi-well filtered titer plate. With the potential to synthesize up to 3456 samples per plate, we converted the BioRAPTR Flying Reagent Dispenser into an open-well system where spent reagents are drained to waste under vacuum. During synthesis, reagents are delivered on-the-fly to each micro-titer well at volumes ≤5 μl with plate speeds up to 150 mm/s. Using gas-phase cleavage and deprotection, a full plate of 1536 60mers may be processed with same-day turnaround with an average yield per well at 3.5 nmol. Final product at only $0.00277/base is eluted into a low-volume collection plate for immediate use in downstream application (e.g. Biomek FX for versatile sample handling). Also, crude oligonucleotide quality is comparable to that of commercial synthesis instrumentation, with an error rate on the NG-1536-AMOS platform of 1.53/717 bases. Furthermore, mass spectral analysis on strands synthesized up to 80 bases showed high purity with an average coupling efficiency of 99.5%.

1. Introduction

The Stanford Genome Technology Center (SGTC) has been at the forefront of oligonucleotide DNA synthesis automation since the early 1990s with its development of the first 96-well Automated Multiplexed Oligonucleotide Synthesizer (96-AMOS) (Lashkari et al., 1995). Capable of generating 6000 25-base primers per month on a single machine, it made large-scale projects such as sequencing the *Saccharomyces cerevisiae* and human genomes timely and economically feasible (Dietrich et al., 1997; Lander et al., 2001).

While industry costs at the time for low-throughput column synthesis were $2.00–3.00 per base (Hager et al., 1999; Goforth, 2002), 96-AMOS was synthesizing DNA 10–15-fold cheaper within the first year of production. Currently at $0.06 per base, this platform remains competitive with other column and titer plate-based synthesizers that have since been introduced (Cheng et al., 2002; Livesay et al., 2002; Rayner et al., 1998; Lebl et al., 2007; Gan, 1990; Jensen et al., 2012).

However, with today's multiplexed, small volume assays (Hardenbol et al., 2003; Dahl et al., 2007), there is a greater demand for even higher throughput and lower cost oligonucleotide synthesis. As such high density synthesis arrays and micro-fluidic devices (Gao et al., 2004; Livesay et al., 2002; Lee et al., 2010; Kong et al., 2007) offer mass production at a fraction of the cost. And though array densities can be as high as 4 million features, low yield and heterogeneous oligonucleotide pooling may present a challenge for specific downstream applications, in particular synthetic biology (Kim et al., 2012; Borovkov et al., 2010; Ma et al., 2012a,b; Quan et al., 2011; Mueller et al., 2009).

In contrast to the microarray which may be limited to 10^6 molecules per feature, multi-well/column platforms generally give very high yields (>25 nmol); but this is often a wasteful overproduction of sample. As a consequence, excess product is either disposed of or kept in deep-well collection plates (volumes up to 1.2 ml/well) for long term storage thus taking up valuable freezer space.

Focusing on the strengths of both the array and multi-well/column platforms, we describe the development of our Next Generation AMOS capable of producing 1536 samples on a single titer plate (NG-1536-AMOS). Based on the original 96-AMOS, we combined its technology with that of the commercial BioRAPTR Flying Reagent (non-contact) Dispenser (BioRAPTR FRD, Beckman Coulter), intended for ultra high-throughput delivery (up to 3456 wells) of aqueous buffers, diluents and dye solutions (Yasgar et al., 2012; Auld et al., 2008).

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Using on-the-fly dispense, NG-1536-AMOS delivers reagents ≤5 μl per well at plate speeds up to 150 mm/s. Following each reaction step, vacuum is applied to draw spent reagents to waste two rows (96 wells) at a time. This feature allows for either full or partial plate synthesis as well as synthesis of varying length strands simultaneously, something not possible with top pressure drain systems (e.g. 96-AMOS).

NG-1536-AMOS has several other advantages which include (i) post-synthesis amplification is not required (on average, yield per well is 3.5 nmol), (ii) product is eluted into shallow-well collection plates for maximum space-saving storage, and (iii) collection plates are fully compatible with existing robotic devices such as the Biomek FX for versatile sample handling (e.g. cherry picking and for creating combinatory oligonucleotide libraries). Also, fragments used in synthetic biology to construct genes de novo can be swapped without having to re-synthesize the entire plate/array.

In addition to mass spectral confirmation of 80-base strands synthesized on our NG-1536-AMOS, we sequence-verified 40-base fragments used to assemble the green fluorescent protein (GFP) gene. With error rates comparable to commercial synthesis methodologies, to date we have processed several 1536-well titer plates in our general synthesis production lab. Moreover, with implementation of a modified gas-phase cleavage and deprotection protocol (based on previous work (Jensen et al., 2010a)), it is possible to process an entire plate (60 bases/well) with same-day turnaround for immediate use in downstream application (Hardenbol et al., 2003; Dahl et al., 2007; Cobb et al., 2013; Weber and Fussenegger, 2012).

2. Materials and methods

2.1. Instrument design and setup

The basic principles of the original 96-AMOS platform were applied to the BioRAPTR FRD to create our custom next generation oligonucleotide synthesizer. The BioRAPTR FRD was converted into an open reaction well system (all modifications to the original platform were designed in-house (SGTC) and fabricated by Nevarez Machining and SGTC)). The 16-nozzle dispense head (Fig. 1I and II) is stationary while the titer plate (Fig. 1III) moves below in the *X* and *Y* directions during reagent delivery. Argon is used to (i) pressurize the bottles (9 psi), (ii) provide for an inert gas layer over the reaction wells and (iii) allow for vacuum-assisted low pressure reagent drain-to-waste.

2.2. Drain block design

To manage reagent flow-through to waste during synthesis, the titer plate (NG-1536-AMOS) is seated over a 1536-well drain block (Supplementary Figure 1). Sealed with a 0.5 mm silicone gasket (or comparable), the bottom drip directors of the titer plate extend inside individual drain holes (Supplementary Figure 2) where each two rows (96 wells) feed into a single channel. From here, valves control the vacuum on each two-row block allowing spent reagents to drain to waste over a given time (e.g. 1 s) according to a specified protocol step (a synthesis protocol file is a list of reagent dispenses, reaction hold times and drain steps repeated for each cycle/base addition).

2.3. Instrument specifications

The key features of this platform include (i) plate speeds 80–150 mm/s (accuracy within 45 μm at 80 mm/s) with on-the-fly dispense and repeatability (%CV) of ±5 μm, (ii) 0.1 μl and ≥0.8 μl accuracy of ±15% and ±10%, respectively, (iii) dispense range from 0.1 μl to 60 μl, and (iv) pre-defined variable plate configurations (96, 384, 1536 and 3456-well).

2.4. Titer plate and solid support

Custom titer plates with 1536-well configuration were designed in-house (SGTC) and molded (Zeonor 1420 R 4%) by Plastic Design Corporation. Custom 1.2 mm × 2.0 mm synthesis plugs (1000 Å controlled pore glass (CPG) with UnyLinker) were manufactured by Chemgenes Corporation, and pressure fitted into the wells (Fig. 2).

2.5. NG-1536-AMOS user interface (UI)

Based on the original 96-AMOS UI (Jova Solutions), software for our next generation platform is a multifaceted design giving the end-user complete control over how the instrument functions (Supplementary Figure 3). Here, synthesis is initiated by uploading the protocol and sequence files (*.PRO, and *.SEQ), then selecting the START button (below "protocol file", Supplementary Figure 3B). The protocol file is displayed in real-time, highlighting the current step. Indicator bars show the current step, cycle and total synthesis progress. A 1536-well grid also displays which bases are currently being synthesized for all active well positions.

2.6. Oligonucleotide synthesis

All samples were generated in-house (SGTC) with our NG-1536-AMOS platform. General synthesis steps (e.g. similar to Applied Biosystems AB3900 synthesizer protocol) were followed using trichloroacetic acid (TCA) (3% in dichloromethane) (American International Chemicals (AiC)), acetonitrile, also referred to as the "Wash" reagent (AiC), 0.02 M oxidizing solution (Sigma–Aldrich), capping reagents A/B (Glen Research), 0.10 M solutions of dA, dG, dC and dT phosphoramidites (Thermo Scientific), 0.25 M 5-Ethylthio-1H-tetrazole (Glen Research), used as an activator during nucleoside phosphoramidite coupling steps, and argon gas (Praxair).

2.7. Post-synthesis

Inside a small aluminum box (150 mm × 115 mm × 75 mm, Supplementary Figure 4A), the titer plate (with fully synthesized oligonucleotides) is placed top down over a trough (Supplementary Figure 4B) filled with 50 ml ammonium hydroxide (28–30% NH_4OH, Fischer Scientific). A cover then seals the chamber, and under elevated pressure (12.75 psi) and temperature (70 °C) for 4 h, gaseous ammonia cleaves and deprotects oligonucleotides embedded within the solid support matrix. This procedure is adapted from of our prior work studying universal linker-bound oligonucleotide cleavage and dephosphorylation in the gas-phase (Jensen et al., 2010a). The reaction chamber is then removed from the incubator and allowed to cool to room temperature. A release valve at the bottom of the apparatus is opened to vent residual ammonia under the fume hood. The titer plate is then seated over a low volume collection plate (Greiner Bio-one, 18 μl capacity per well) with drip directors positioned inside the collection wells. Five μl of deionized water are added to the wells, then fully processed samples are eluted from the synthesis plugs through centrifugation (e.g. 1 min at 1000 RPM). This process can be repeated to maximize yield.

2.8. Oligonucleotide quality analysis

To validate the mass (Daltons) of 80-base strands (Supplementary Table 1) generated on our NG-1536-AMOS platform, Electrospray Ionization Mass Spectrometry (ESI MS) was carried