FULLY AUTOMATED PARALLEL OLIGONUCLEOTIDE SYNTHESIZER

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Design and construction of automated synthesizers using the tilted plate centrifugation technology is described. Wash solutions and reagents common to all synthesized species are delivered automatically through a 96-channel distributor connected to a gear pump through two four-port selector valves. Building blocks and other specific reagents are delivered automatically through banks of solenoid valves, positioned over the individual wells of the microtiterplate. These instruments have the following capabilities: Parallel solid-phase oligonucleotide synthesis in the wells of polypropylene microtiter plates, which are slightly tilted down towards the center of rotation, thus generating a pocket in each well, in which the solid support is collected during centrifugation, while the liquid is expelled from the wells. Eight microtiterplates are processed simultaneously, providing thus a synthesizer with a capacity of 768 parallel syntheses. The instruments are capable of unattended continuous operation, providing thus a capacity of over two millions 20-mer oligonucleotides in a year.
Keywords: Automated oligonucleotide synthesizer; Parallel syntheses; Array-based technologies; Oligonucleotides; Solid-phase syntheses; Combinatorial chemistry; Libraries; Genomics.

The oligonucleotide synthesis technology is of major strategic importance in the field of genomics. Commercially available single channel synthesizers cannot satisfy the demand of emerging technologies. Currently, there are several instruments for parallel synthesis of oligonucleotides such as: (i) a 96-channel instrument based on a microtiter plate format developed by scientists at Stanford University[1]; (ii) PolyPlex machine produced by the company GeneMachines[3]; or synthesizer using two microtiterplates for simultaneous synthesis of 192 oligonucleotides, developed at The University

of Texas Southwestern Medical Center at Dallas, and sold under name MerMade by company BioAutomation[4]. While these technologies meet the modest requirements of most experiments today, they are inadequate for the manufacturing needs looming in the very near future. Current synthesis technologies do not meet the need for manufacturing large numbers of oligonucleotides (tens of thousands to millions of sequences) cost-effectively. Our goal was to fill this gap and build the parallel (and economical) synthesizer capable of preparation of needed numbers of oligonucleotides.

This need for oligonucleotide synthesis technology is being driven by the increasing availability of sequence information. The initial draft of the human genome sequence was finished and results were published recently[2]. With this explosion in sequence information, there has also been the development of new technologies that take advantage of this information. Some of these technologies have a high dependence on the availability of oligonucleotides. For example:

a) Array-based technologies. These technologies allow parallel analysis of thousands of sequences at a time. The new oligonucleotide synthesis technology described in this article allows the full potential of different array technologies to be realized by allowing sequences to be represented on arrays rapidly and cost-effectively. The synthesis of arrays "on location" (ref.[5]) is too costly and does not allow preparation of long oligonucleotides.

b) Genome-wide polymerase chain reaction (PCR) (*e.g.* amplification of each predicted open reading frame or exon in a genome)

c) Systematic sequencing (*e.g.* contiguous overlapping).

In order to accelerate the synthesis of large single compound arrays, much effort is currently devoted to the implementation of high throughput synthesis automation (see *e.g.* ref.[6]). Numerous companies have recently started to develop and market instruments for the automated parallel solid-phase synthesis of compound arrays. Most of these instruments are based on the solid-phase synthesis technology[7], and use commercially available pipetting robots for the delivery of reagents and wash solutions to the synthetic compartments. The capacity of these synthesizers ranges from 12 to 384 compounds that can be synthesized in one run. However, none of these instruments would be adaptable to oligonucleotide synthesis. Besides instruments capable of parallel synthesis of oligonucleotides mentioned above using 96-well multititerplates equipped with filters at the bottom of each well[1,3,4], we should mention the alternative machine developed by ProtoGene Laboratories[8], based on the synthesis on planar surface driven by the surface tension.

One of the key processes in multiple solid-phase synthesis is the parallel removal of excess reagent and wash solutions from the solid support in all synthetic compartments. In most of currently available synthesizers, this is achieved through the porous bottoms of the synthetic compartments, either by vacuum filtration (*e.g.* in instruments from Argonaut Technologies, Bohdan Automation), or application of pressure from the top of the compartments (*e.g.* in instruments from Advanced ChemTech). These methods bear the inherent risk of clogging of one or more compartments, resulting in insufficient liquid removal from the clogged compartments, overflow, and, consequently, contamination of neighboring compartments. An alternative method, which employs aspiration of the liquid from the surface[9,10] requires dedicated equipment and is not commercially available.

Synthesis Automation Based on Tilted Plate Centrifugation

We have devised a new technological concept for the automation of the solid-phase synthesis of large compound arrays[11,12]. The key feature of this technology is a new method for separation of the solid support from reagent solutions, termed "tilted plate centrifugation", which uses centrifugation as a means of liquid removal in conjunction with the use of tilted microtiter plates as reaction vessels. This technology has its roots in an earlier multiple peptide synthesizer developed in the Institute of Organic Chemistry and Biochemistry in Prague, which utilizes centrifugation for liquid removal from the solid support resin contained in polypropylene mesh bags or cotton pieces[13,14]. The tilted plate centrifugation technology improves the earlier centrifugation method by using the wells of microtiter plates as synthetic compartments, thus enabling the parallel synthesis of much larger compound arrays. The plates are mounted on a centrifugal plate and slightly tilted down towards the center of centrifugation, thus generating a pocket in each well, in which the solid support is collected during centrifugation, while the supernatant solutions are expelled from the wells (Fig. 1).

In order to ensure efficient liquid removal (*i.e.*, no solution remaining in the wells after centrifugation), and at the same time avoiding any loss of solid support during centrifugation, the volume of the well-pockets should be equal to the volume of solid support in each well. This can be achieved by adjusting the pocket size by using plates with varying well volumes and/or modifying the tilt angle, as well as the speed of rotation.

An essential feature of this approach is that well-to-well cross-contamination with reagent solution or resin is avoided by the fact that the

plates are tilted, while the direction of centrifugation is horizontal. Consequently, any liquid or resin expelled from the wells is either captured in the inter-well space of the plate, or collected on the wall of the centrifugal drum. The fact that the cross contamination is not an issue we have proven by analyzing all products prepared on the microtiterplate by HPLC/MS.

We have built high-throughput synthesizer "Oligator™ 768" (Fig. 2) which features the following capabilities: Parallel solid-phase synthesis of oligonucleotides in the wells of polypropylene microtiter plates, which are slightly tilted down towards the center of rotation, thus generating a pocket in each well, in which the solid support is collected during centrifugation, while the liquid is expelled from the wells. Wash solutions and reagents common to all synthesized species are delivered automatically through a multichannel distributor connected to a serially linked four-port selector valves. Building blocks and other specific reagents are delivered individually to the respective wells by distribution through the banks of solenoids.

The oligonucleotide synthesizer requires extremely fast building block and reagent delivery since prolonged times may destroy the prepared intermediate. Furthermore, the synthesis has to be performed under inert atmosphere. On the other hand, it is not necessary to further develop synthetic protocols since all the reagents (building blocks, activators, solid carriers) have been tested and optimized by others and are commercially available. However, there is still not an instrument on the market, which would be

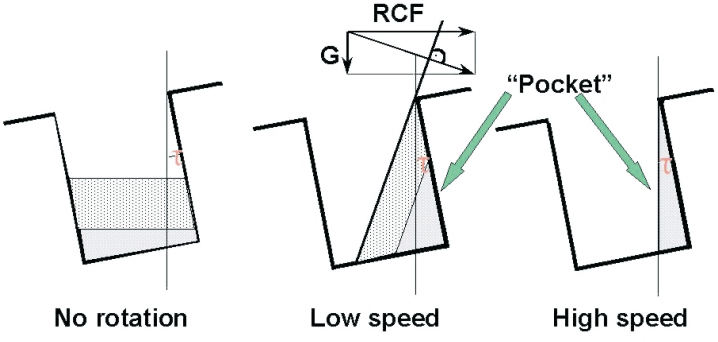

FIG. 1
Formation of the pocket in the well of a tilted plate during centrifugation (direction: left to right). The solid support (lower layer) is collected in the pocket, while the liquid (upper layer) is expelled from the well. The liquid surface angle is perpendicular to the resulting force vector of the relative centrifugal force (RCF) and gravity (G)

comparable to the Oligator™ 768 in its synthesis productivity (the number of compounds synthesized in one batch).

Synthesis in Oligator™ 768 is performed in the 96 well polypropylene microtiterplates. The liquid delivery system is based on moving banks of nozzles. The basic idea of this system is illustrated in Fig. 3. An array of nozzles is placed on the actuator (single axis robotic arm). Each nozzle in the array is connected through the solenoid valve to a pressurized bottle (argon, 0.2 atm) and can be positioned (as a part of the array) above the particular well of a given microtiterplate. We are using four arrays of eight nozzles for the delivery of four phosphoramidites and one array of eight nozzles for the delivery of the activator solution. Each array serves in parallel the wells of one column of microtiterplate. The arrays are placed in the grid, which covers five columns of the microtiterplate.

To illustrate the process of the delivery of building blocks, let us describe one cycle of the delivery.

The arrays are parked outside the area for delivery (none of the arrays are above the microtiterplate).

All solenoids are opened, nozzles are primed, solenoids are closed, and array of nozzles moves into first positions for delivery. In this position, the

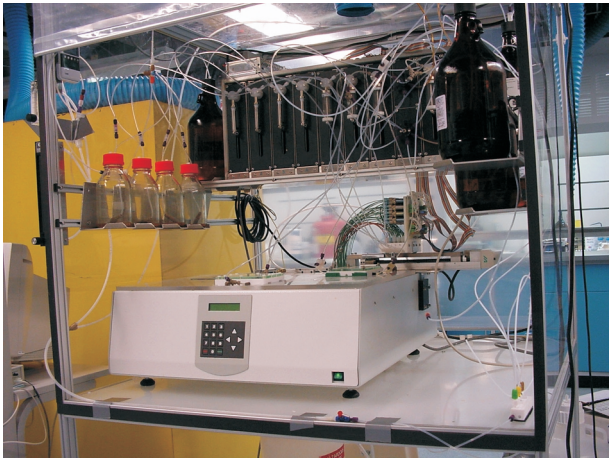

FIG. 2
First-generation Oligator™ 768. All reagents delivery is achieved by Cavro syringe pumps. Note the bulky array of solenoid valves attached to the nozzle array by a bundle of PEEK™ tubing

first array of eight nozzles is above the first column of wells of the microtiterplate. Other arrays are not yet above the plate.

The computer program calculates the volume of the first reagent (dA phosphoramidite) to be delivered into all wells of this column, opens the appropriate solenoid valves for the calculated time period, and the calculated volume is delivered to appropriate wells.

After the delivery, the array is positioned above the second column and the process is repeated. Now the first array of nozzles is delivering (dA phosphoramidite) into the second column of wells and the second array of nozzles is delivering (dC phosphoramidite) into the first column of wells. The arrays for delivery of dG and dT phosphoramidites and activator are not yet above the microtiterplate.

When the first array is placed above the fifth column of the microtiterplate, the array of nozzles delivering the activator solution is placed above the first column and activator is added to all wells (all wells should now contain a solution of one of the phosphoramidites).

After servicing all of the wells of the plate, the nozzle array returns into the parking position and the rotor is actuated to place the second microtiterplate under the delivery system.

We have designed the nozzle array holder allowing quick assembly of the multiarray and which is easy to maintain and troubleshoot (Fig. 4). This array is capable of reproducible delivery of the amounts of liquid in the range

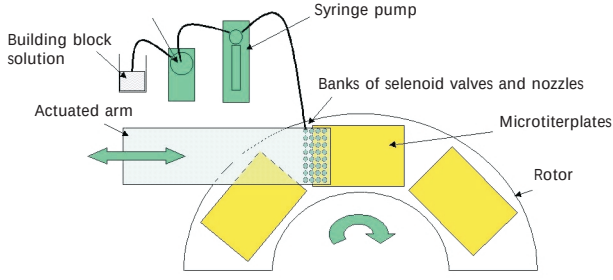

FIG. 3

Four arrays of nozzles are attached to linear actuator placed above a location of plate in the rotor. Building blocks are sucked into the syringe pump (only one shown) and individual solenoids are opened when the delivery of the building block into the particular well is required. Delivery is achieved by actuating of a syringe pump. Linear actuator moves the arrays above the next set of columns and process is repeated until all wells had a chance to be served by all nozzle banks. Delivery from the pressurized vessels follows the same principle

from 5 to 500 μl. We have tested this assembly in production of tens of thousands of oligonucleotides (see below).

Due to the fact that all five arrays of nozzles can be operated at the same time, the delivery of the reagents is fast – one plate can be serviced in 8 s. The fact that the solutions of reagents are added to the system still containing small amount of the solvent is corrected by using repeated addition of activated building blocks. Instead of tetrazole, which has limited solubility, we are using 4,5-dicyanoimidazole (DCI) as an activator. This activator does not cause any problems with crystallization in the system and, as the additional benefit, increases coupling rates of phosphoramidites above the level achievable with tetrazole. The longest time delay after the phosphoramidite is added before the activator solution is delivered into the well is about 2 s. From the practical point of view, the addition can be considered simultaneous, thus simulating the situation in automated synthesizers where phosphoramidite and activator are pumped through the cartridge of solid support as segments of liquid.

Critical factor for successful performance of the oligonucleotide synthesis is the guaranteed retention of all solid support in washing cycles using the centrifugation process. The synthesis of twenty-mer oligonucleotide requires up to 700 centrifugation cycles – therefore loss of even a small fraction of the support in every step would result in complete loss of the product. Fortunately, the sedimentation characteristics of the controlled-pore glass (CPG), which is the carrier of choice for DNA synthesis, are favor-

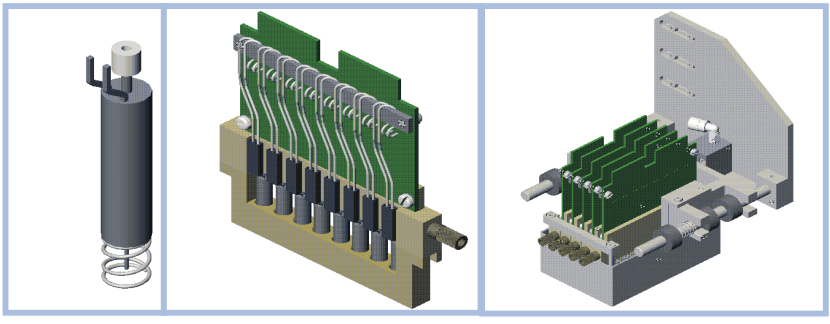

FIG. 4
Solenoid array for delivery of phosphoramidite and activator solutions. From left to right: Individual INKA valve with supporting spring and Teflon connecting insert, one bank of eight solenoids with electrical connections attached to the PC board and liquid connection attached to the central channel, five banks assembled in an array mounted in a holder

able. We have shown that even after several thousands of centrifugations of DNA synthesis, the solid support was retained.

Since the DNA synthesis uses five different solvents and reagents, their separate delivery must be arranged appropriately. One option was to use separate delivery head for each solvent/reagent. This would require dedicated pumps for delivery of each reagent (Fig. 5A). Another option was one delivery head connected to the alternative reagent syringes *via* motorized selector valve (Fig. 5B). The simplest solution, requiring, however, the most thorough washing of lines between operations is illustrated in Fig. 5C. In this case only one motorized syringe and selector valve are needed. This option was simpler in construction of hardware parts and we have implemented it in the first model of Oligator™ 768 (see Fig. 2). The reagents were delivered by two coupled pairs of motorized syringe pumps addressing simultaneously two 96-channel delivery heads to speed up the delivery cycle. Later we replaced syringe pumps by a single gear pump. This modification resulted in significant time reduction due to the fact that the gear pump is delivering the liquid directly without the need for charging before each delivery, which is necessary in the syringe pump. The second generation Oligator™ 768 is illustrated in Fig. 6.

The Oligator™ 786 synthesizer control system (see Fig. 7) utilizes a layered, distributed architecture. The top level of control employs a standard PC architecture that incorporates a flat panel touch screen to facilitate user interaction, as a keyboard and mouse would not work well in a laboratory environment. The PC runs the Linux operating system. The control software is written in the C programming language and uses the GNOME graphical environment to generate the graphical user interface objects required by the operating software. The top level software is composed of two

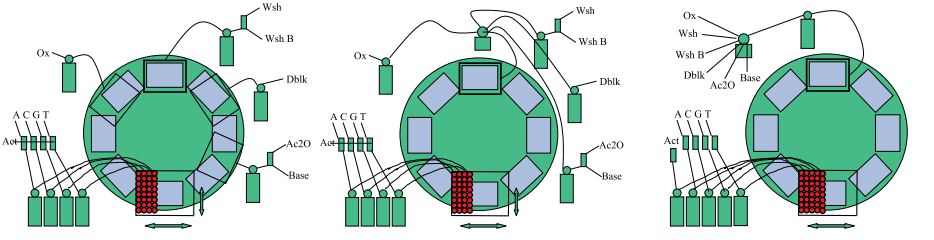

FIG. 5

Alternative arrangements of reagent delivery (Ox – oxidizer; Wsh – washing; Dblk – deblock; Ac2O – cap mix A; Base – cap mix B; A, C, G, T – phosphoramidites; Act – activator)

main components, the run-time component and the direct control component. The run-time component allows the creation and editing of synthesis protocols, *i.e.* the step-by-step instructions for the machine's hardware components. This is accomplished by a user interface that incorporates buttons and dialog boxes that allow facile generation of English text statements representing the instruction and parameters associated with that instruction. The interface also allows the loading, editing, saving and running of protocols once they have been created, as well as the loading of text files that contain the information representing the sequences that are to be synthesized. The second component of the interface allows for direct control over discrete hardware on the system. This allows for set-up, adjustment and testing of the system by service personnel.

The PC communicates with two subcontroller systems to control the entire machine. The first system is a Compumotor 6K2 real-time motion controller. Instructions are sent *via* Ethernet from the PC to the 6K2. The 6K2 contains software that operates as subroutines, and can execute simple or complex operations without continuous supervision from the top-level PC. The 6K2 performs operations, *i.e.*, moving the rotor, operating the dispensing head, *etc.*, and then simply reports back the results, success or error, to the PC. This removes the load from the PC to the 6K2 which is much more capable of real-time, multitasking operations. The 6K2 also provides for interfacing discrete components, such as solenoid valves and sensors, and controlling the various mechanical systems within the machine.

The second control subsystem is a valve controller board (VCB), used to control the fast solenoid valves during the base dispensing process. This dedicated eight-bit controller downloads the sequence information and dis-

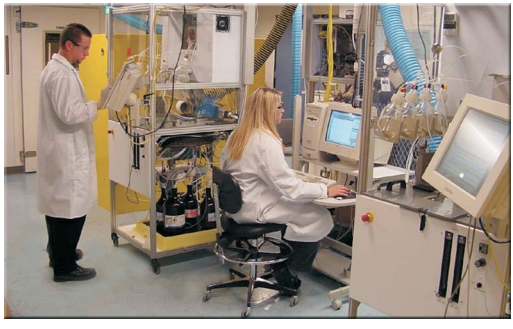

FIG. 6
Two prototypes of second-generation Oligator™ 768 being tested at Illumina, Inc.

penses volumes from the PC *via* an RS-232 link. The controller calculates the actual dispense time based upon an empirically determined calibration curve that correlates pressure and delivery volume to time (the amount of time the valve must be kept open to deliver the desired volume). The dispense cycle is composed of moving the dispensing head across the microtiter plate while the 6K2 signals the VCB when the head is correctly positioned over sets of wells. At each trigger interval, the VCB opens the valves that correlate the desired base in the desired well for the pre calculated time interval, all without stopping the dispensing head motion over

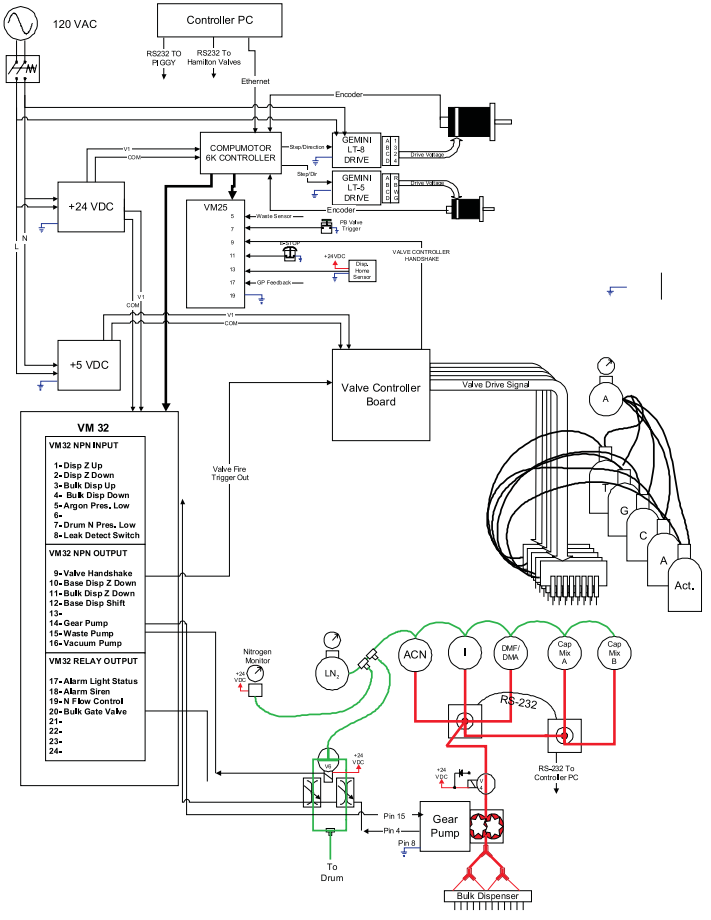

FIG. 7
Control architecture of Oligator™ 768

the plate. This allows rapid dispensing of bases and activator into the plate within 8 s or less for a 96 well plate.

Wash solutions and reagents common to all synthesized species are delivered automatically through a 96-channel distributor connected to a gear pump through two four-port selector valves. We have tested various nozzle materials and diameters to achieve uniform distribution of liquid throughout the plate locations. So far we have tested stainless steel, titanium, and PEEK™. The material of choice for the delivery into 96-well plates seems to be PEEK™ nozzles of 0.25 mm diameter ID.

The delivery into smaller openings of microtiterplates requires bringing the nozzles of the delivery manifolds very close to the plate. However, achieving the tilt needed for the centrifugation step, and placement the nozzles close to the plates are two contradictory requirements. We decided to solve this problem by the application of "dynamic tilt". Plates are placed in the holder supported by the spring, which retains them in horizontal position when centrifugal force is not applied. However, as soon as the centrifugation starts, the weight located under the axes of plate holder hinge rotation pulls the plate into tilted position, the limit of which is defined by locating nuts placed on the screw supporting the springs. Plates can thus be centrifuged in the tilted position and serviced in horizontal position. The carbon-fiber rotor capable of "dynamic tilt" is shown in Fig. 8.

Oligator™ 768 was tested in the synthesis of about 100,000 individual oligonucleotides of the lengths spanning from 20 bases up to 85 bases. Oligonucleotides were analysed by ion exchange and reversed phase HPLC, gel electrophoresis and mass spectroscopy. HPLC analysis of one plate of in-

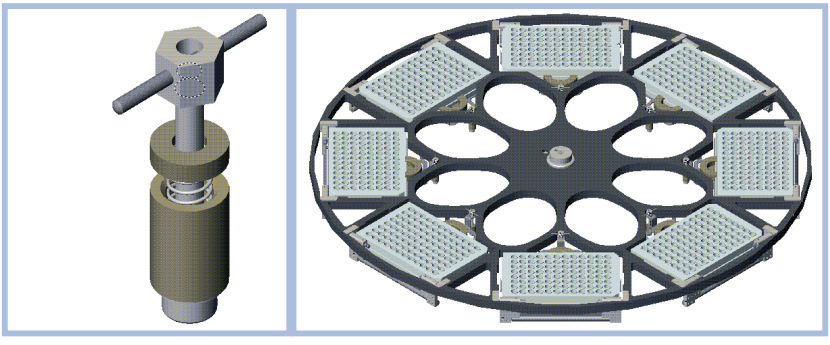

FIG. 8
Carbon-fiber rotor with holders for eight microtiterplates capable of "dynamic tilt". On the left – Adjusting pin defining the level of the tilt by limiting the travel of the spring

dividual 24-mers is given in Fig. 9. As can be seen, some of the sequences did not produce completely homogeneous products (see HPLC trace in row 3, columns 5 and 6, row 7, columns 2 and 5, row 9, column 5, and especially row 11, column 2). We have shown in a separate experiment that these results are reproducible and sequence dependent (*e.g.* sequences with multiple G in the consecutive positions provide disappointing results). As the ion exchange HPLC was shown to separate full-length oligonucleotide from the $n - 1$ product[15,16], we were able to calculate step coupling yield of most of the products. Analysis of average step coupling yield showed 98.9%, which is above the industry-wide accepted value of 98%. Multi-facility survey of 71 DNA core facilities have shown that only 85% of tested laboratories provided products with average coupling efficiency higher than 98% (ref.[17]). In a separate experiment we have evaluated the potential cross contamination between wells of the individual micro-

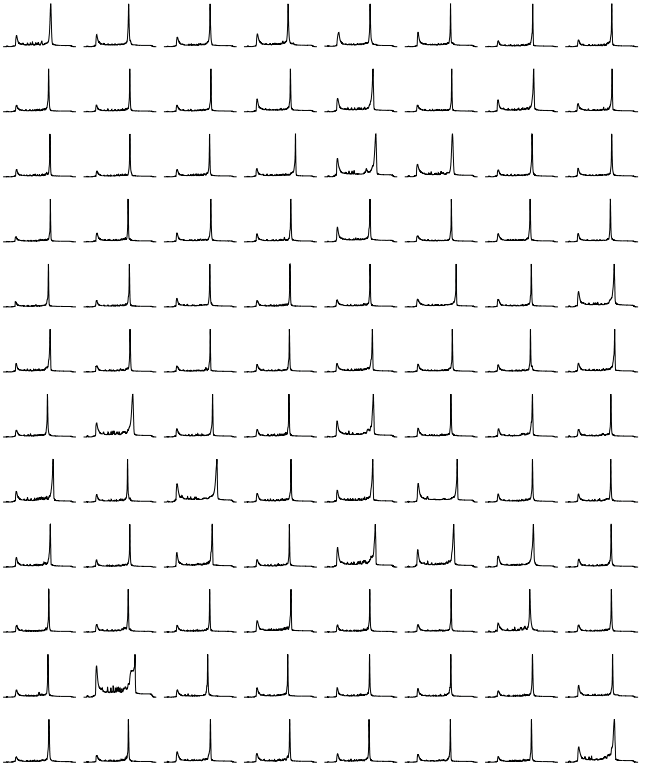

FIG. 9
Ion-exchange HPLC traces of 25-mers synthesized in one microtiterplate

titerplate. Figure 10 shows the HPLC traces of products from adjacent wells of the microtiterplate. Besides the absence of cross-contamination, the high coupling efficiency is further demonstrated (99.2%).

EXPERIMENTAL

Synthesis of 768 25-mer Oligonucleotides

"Universal support" (3.2 g, 40 μmol/g, Glen Research, Sterling, VA, U.S.A.) was suspended in a mixture of dibromomethane and acetonitrile (7 : 3) and aliquot containing 4 mg of solid support was delivered to each well of microtiterplate by manual multichannel pipetting. Microtiterplates were placed on the perimeter of the carbon-fibre rotor and the protocol given in Table I was applied. Plates were dried by 120 s centrifugation, removed from the rotor and a mixture of concentrated aqueous ammonia and methylamine (1 : 1) was pipetted into all wells. Plates were covered by polypropylene foam sheet and placed in the aluminum clamp pressing the foam on top of the plate, preventing thus evaporation of the cleavage reagent. The assembly was placed in the oven heated to 80 °C for 4 h. After cooling, the assembly was opened and the product was extracted by repeated delivery and removal of water (96-channel pipetting by TomTec (Hamden, CT, U.S.A.)). Aqueous extracts were consolidated in the deep-well polypropylene plates and transferred to GeneVac evacuated centrifugal evaporator (Genevac, Ipswich, U.K.). Samples taken from the consolidated extracts were subjected to anion-exchange HPLC (Gen-Pak FAX column 100 × 4.6 mm (Waters, Milford, MA, U.S.A., gradient from 25 mM Tris-HCl, pH 8.0 to 60% 1 M NaCl in 12 min) to evaluate purity. Randomly selected samples (12%) were analyzed by electrospray mass spectroscopy to confirm the identity. Results of HPLC analysis of all products from one microtiterplate are given in Fig. 9 (scales omitted in the figure – *x* axis 0 to 16 min, *y* scale OD 260 0 to 0.5 AU). HPLC traces from eight adjacent wells synthesized in an experiment designed for evaluation of cross contamination are shown in greater detail in Fig. 10.

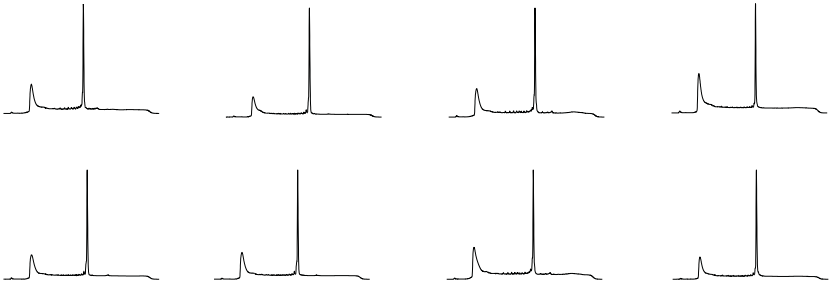

FIG. 10
Ion-exchange HPLC traces of eight 25-mers synthesized in adjacent wells of Oligator 768

TABLE I
Synthetic protocol for Oligator™ 768

Reagent	Number of repetitions	Total time min
Acetonitrile	5	3
2% Dichloroacetic acid in dichloromethane	4	6
Acetonitrile	7	4.2
0.1 M Phosphoramidite and 0.25 M dicyanoimidazole (3 : 2)	3	6.5
Tetrahydrofuran/pyridine/acetic anhydride (8 : 1 : 1) and 10% *N*-methylimidazole in tetrahydrofuran (1 : 1)	4	2.4
Acetonitrile	1	0.6
0.02 M Iodine in tetrahydrofuran/pyridine/water (89.6 : 0.4 : 10)	3	1.8
Acetonitrile	3	1.8
Tetrahydrofuran/pyridine/acetic anhydride (8 : 1 : 1) and 10% *N*-methylimidazole in tetrahydrofuran (1 : 1)	4	2.4

CONCLUSION

Tilted centrifugation was found a suitable principle for construction of parallel oligonucleotide synthesizers capable of synthesis of millions of discrete oligonucleotides in a year. Polypropylene microtiterplates were found to be the ideal reaction vessels for tilted centrifugation-based oligonucleotide synthesis.

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