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Sequential Injection Analysis for Optimization of Molecular Biology Reactions

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Abstract

In order to automate the optimization of complex biochemical and molecular biology reactions, we developed a Sequential Injection Analysis (SIA) device and combined this with a Design of Experiment (DOE) algorithm. This combination of hardware and software automatically explores the parameter space of the reaction and provides continuous feedback for optimizing reaction conditions. As an example, we optimized the endonuclease digest of a fluorogenic substrate, and showed that the optimized reaction conditions also applied to the digest of the substrate outside of the device, and to the digest of a plasmid. The sequential technique quickly arrived at optimized reaction conditions with less reagent use than a batch process (such as a fluid handling robot exploring multiple reaction conditions in parallel) would have. The device and method should now be amenable to much more complex molecular biology reactions whose variable spaces are correspondingly larger.

Keywords

Sequential injection; endonuclease; design of experiment; fluorogenic; optimization; feedback; automation

Introduction

Many reactions commonly used in biochemistry or molecular biology require specific and often idiosyncratic reaction conditions. As examples, biochemical reactors with precise control of internal conditions are now being used for chiral chemical synthesis¹. Large biomolecule therapeutics can be produced enzymatically. Modified RNA molecules can be produced by *in vitro* transcription (IVT)². *In vitro* transcription and translation (IVTT) can produce active proteins that would otherwise be toxic to host cells³. In each instance, optimizing the yield of product essentially boils down to optimizing the reaction conditions and reactant concentrations, including enzyme concentrations.

In general, there are several strategies to search for optimal reaction conditions (Figure 1A–C). Manual approaches to reaction optimization generally refine one parameter at a time (see Figure 1A, vertical column). A range of conditions are then selected for a second parameter using the optimal value from the first (Figure 1A, horizontal row). While this method finds an improved set of conditions, it may not find the most optimal conditions. Instead, a grid-based search (shown in Figure 1B) does find the local maximum, but at the cost of requiring a much larger number of experiments and consummately large material requirements. While

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Supporting Information Available. MATLAB source code (Simulation_Of_Optimization.m) is available on-line.

grid-based optimization can be automated to improve speed, but the problem rapidly scales with the power of the number of parameters, and is ultimately impractical. Instead, optimization should be carried out using all available data after each iteration of an experiment (Figure 1C). In this instance, an algorithm can choose each subsequent set of reaction conditions according to a rational set of criteria.

As a proof-of-principle, we have combined automation and an algorithm to initially optimize a simple molecular biology reaction, restriction digestion. For automation we rely on a Sequential Injection Analysis (SIA) instrument, while the algorithm is based on the Design of Experiments (DOE) implementation in the MATLAB Statistics Toolbox, which defines a statistically significant pattern of experimental conditions⁴. Iteratively applying DOE followed by model-fitting is analogous to a Newton method for optimization. The values of the model function constitute a 'response surface.' Response surface methodology has been used for optimization in many analytical contexts. For example, Bosque-Sendra et al.¹⁰ use this method to optimize the assay of formaldehyde. In the context of SIA, dos Santos and Masini¹¹ applied a response surface approach to optimize a wastewater treatment reaction. For a review of response surface methodologies for analytical applications see Bezerra et. al¹².

The SIA system performs an experiment and reports the results to the algorithm which in turn makes a decision: either refine within the searched space, expand the search space, or stop. While DOE has been used to efficiently optimize biochemical reactions before⁵, and has been used to optimize a SIA protocol^{6, 7} for a single analysis, an integrated, automated system for the optimization of molecular biology reactions has never before been developed. To the extent that our approach is successful, it should be possible to greatly expand its use. SIA has been widely used to automate analytical reactions with fluorescent or colorimetric detection⁸. Enzymes, fluorogenic substrates, and small sample volumes have all been demonstrated with SIA⁹.

Results and Discussion

Experimental design

In order to establish a protocol for using sequential injection analysis (SIA) to optimize molecular biology reactions, we carried out several experiments in parallel. First, we configured the SIA platform to be able to read a fluorescent signal and provide feedback that would impact the amount of reagents delivered to a given reaction 'plug.' Second, we developed a simple assay for a restriction enzyme that could be easily read by the SIA platform. Finally, we progressively altered the configuration of the device and the variables of the restriction digest in order to perform automated DOE.

Configuring the SIA platform

SIA is an analytical automation technique that typically utilizes a valve, pump, and detector (see Figure 2A–B, schematic), connected to a desktop computer⁸. The flow cell is a machined Delrin block which includes a fluid path and fused silica windows to allow excitation with a fiber-coupled blue LED and detection by a fiber-coupled photodiode. The optimization algorithm and SIA control software were written in MATLAB and PERL, respectively. This platform not only automated dispensing and mixing, but also iteratively and autonomously changed and measured conditions in order to determine ideal conditions for the reaction in question.

SIA functions on the principle of sequentially injecting volumes of reagents into adjacent zones within a narrow tube¹³. The zones are then mixed by dispersion as the fluid is dispensed through the tube toward the detector. Figure 3A shows a schematic of how the

reagents and sample are injected into the system with variable volume. The sharp plugs are mixed by dispersion (see Figure 3B) and variation in injection volume results in tunable concentration in the flow cell (dotted lines indicate flow cell location and change in concentration).

Design of fluorogenic substrate for HaeIII

In order to assess the SIA platform and DOE optimizations for molecular biology reactions, we designed and tested a fluorogenic substrate for the restriction enzyme HaeIII. The substrate is a double-stranded DNA (dsDNA) with a fluorescein and quencher immobilized at one end (see Figure 3C). HaeIII is a particularly good choice for initial optimizations because it can operate on relatively short oligonucleotides, and thus the products of cleavage (a 6 base pair product which has a calculated melting temperature of 18 °C) should readily dissociate and dequench at room temperature.

To test the fluorogenic substrate, we prepared solutions in the manufacturer's recommended buffer (Buffer C) in a 96-well plate. Control samples lacked enzyme. We then measured the fluorescent response upon addition of HaeIII. Figure 3D shows the results, with error bars corresponding to the standard deviation of triplicate measurements. Fluorescence rises dramatically above background upon digestion with HaeIII, confirming that our assay design is functional.

Assessing reagent mixing within the injection plug

Optimization of reaction conditions by SIA can only be accomplished if the injected volumes of reagents mix to create different concentrations in the reactions. Depending on the location and volume of the injection, final fluorescein concentrations ranged from undiluted to a dilution by a factor of $>10\times$ after passing into the flow cell. The mixing of adjacent injection plugs occurred primarily by a phenomenon known in the SIA literature as inter-dispersion due to the parabolic flow profile. Such zone penetration has been discussed and modeled extensively in the literature¹⁴. So long as all of the injection plugs had a total volume of $\sim 100\ \mu\text{l}$, measurable quantities of fluorescein could be detected in the flow cell, within rough agreement with predictions from Taylor dispersion. Only the central region ($16\ \mu\text{l}$) of the total reaction plug ($\sim 100\ \mu\text{l}$) is interrogated within the flow cell. Within this small sub-volume the concentration gradients are relatively small. We have used the average conditions within this small volume to prepare conditions in bulk. The success of our bulk conditions suggest that this approximation is roughly accurate.

In addition to ensuring mixing, each experiment during the optimization must be free from carryover of solutions between runs. In order to assay carryover, we used fluorescently-labeled lysozyme, a protein with a high pI (10.7) that is known to potentially stick to surfaces. Rinsing with at least $300\ \mu\text{l}$ of deionized water between each experiment reduced carryover to below background. To avoid misreading background due to detector drift or the presence of adsorbed product in the flow cell, we performed background subtraction with a new blank between each experiment.

DOE implementation

Our priority was to accomplish the optimization with a limited number of experimental iterations. We therefore based our search algorithm on a Design of Experiments (DOE) approach. We chose to use DOE because it can efficiently and effectively study "black box" problems¹⁵. DOE has been previously used to optimize analytical methods via SIA^{6, 16}, but our approach is distinct in that DOE provides a direct and immediate feedback on the automated design and execution of incremental SIA experiments. The MATLAB Statistics Toolbox has a number of convenient functions for implementing DOE, including functions

for generating several useful factorial and center composite designs. MATLAB also includes methods for efficiently fitting a model function to experimental data (i.e., generating the response surface). Because only discrete integer values of volume could be used experimentally, we simply evaluated the model function at all integer values over the relevant range and chose the maximum as the new center point. This is very similar to the algorithm described by Joshi et. al. in 1998¹⁷. This algorithm offers advantages over steepest ascent algorithms, in that it takes into account any curvature in the response of the system and fits all data available at the time.

In principle, this algorithm is expandable to many (10–16) independent parameters, but the throughput is ultimately limited by the incubation time per experiment. The Box-Behnken design has an advantageous progression in the number of experiments versus the number of parameters. It explores the minimal number of points in parameter-space necessary to fit a quadratic model function¹⁸, and has proven useful in many analytical applications¹⁹. In the three-parameter case, the Box-Behnken design can be visualized as choosing points in parameter-space corresponding the center point and twelve edges of a cube. This contrasts with full-factorial designs which cover all possible combinations of three values from each of the parameters: the center, corners, edges and faces of a cube. Full-factorial designs may give better results in cases where variables are more tightly-interrelated, but require more experimental iterations (and hence more reagents). The Box-Behnken design reduces the growth of the number of experiments (N) as a function of the number of parameters (K) from exponential ($N=3^K$ for full-factorial designs) to approximately quadratic ($N \approx K^2$)²⁰. As a consequence, optimizing six parameters rather than three (as presented here) would have required approximately four times the number of experimental iterations. Our full optimization in terms of three parameters required about 6 hours. Moving to six parameters would have the practical limitation of requiring a full day.

Figures 4A–D show a demonstration of a simulated, automated 2-parameter search, which optimizes an arbitrary reaction rate depending on the magnesium ion and dithiothreitol (DTT) concentration. The initially chosen starting conditions are shown as filled circles (see Figure 4A). The data from experiments at these conditions are fit to a model function. The values of that model function are shown as colored contour plots. New experimental conditions (five additional circles) are chosen based on the first evaluation (Figure 4B). The search pattern shrinks as the algorithm better models the underlying equation (Figure 4C). The color map for the arbitrarily chosen, underlying quadratic function (Figure 4D), is congruent with the final, chosen optimum (open circle). The previous contour plots (Figure 4A–C) can be seen as successive simulations of this color map. Successive reaction rates improve because the reaction conditions better approximate the maximum of the underlying, previously unknown quadratic rate function (Figure 4E). This same series of steps will now be applied to the optimization of a standard restriction digest, as described below.

Optimization of a HaeIII restriction digest

The first proof of principle of our method was a two-parameter optimization of HaeIII activity (as a function of magnesium chloride and DTT concentrations). Preliminary experiments (data not shown) suggested that DTT concentration could dramatically change HaeIII activity, while magnesium concentration would have a more limited effect. The initial question was therefore whether the SIA device and algorithm could reach similar conclusions.

The iterative optimization procedure involves injecting a plug of carrier buffer, then injecting sample and additional buffer components, examining the result, and calculating the injection volumes for the next experiment. The manufacturer suggests running the digest in Promega Buffer C (PBC, 1 mM DTT, 50 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris, pH 7.9).

Therefore, the carrier buffer was initially chosen to be attenuated PBC that contained no DTT. The additional reagents to be injected (termed “spiking buffers” throughout, see **Methods**) were solutions of PBC with a 25× concentration of either DTT or magnesium chloride. In each experiment ~5 µl each of enzyme and substrate and variable volumes of additional reagents were injected into the middle of a ~100 µl plug of attenuated PBC carrier buffer. Mixing occurred as this plug moved into the flow cell. After an incubation period of 4 minutes at room temperature within the flow cell under static conditions the fluorescence was measured.

The amount of fluorescence at 4 minutes represented the relative rate of the reaction. Initial characterization of the kinetics of the reaction (data not shown) indicated that fluorescence increased linearly for ~20 min. The fluorescence output of each sequential experiment over the course of the optimization is shown in Figure 5A. The low initial fluorescence at the beginning of the search was largely due to the lack of DTT in the carrier buffer. As can be seen, the SIA device readily works to optimize reaction conditions. Over the course of 56 iterated conditions, the algorithm methodically improved the fluorescence output from the enzymatic digest (see Figure 5A). As the algorithm found a steep slope in the response surface (primarily due to the strong improvement in cleavage in the presence of higher DTT concentrations), the observed fluorescence rose sharply. Upon reaching a plateau, the algorithm refined the search, determined the optimal conditions for cleavage, and terminated. Upon completing the optimization series, the instrument replicated several reaction conditions in triplicate: attenuated PBC, the first condition explored (0.16 µl each of DTT and magnesium chloride spiking buffers), and the putative optimal condition (19 µl of DTT and 20 µl of magnesium chloride spiking buffers). These results are shown in Figure 5B.

Simultaneous optimization of three parameters

In order to determine the extent to which both the device and the algorithm could navigate a more complex experimental landscape, we then optimized in terms of three parameters (pH, magnesium chloride and DTT) and at a considerably lower signal-to-background ratio. Computationally, this required three times as many iterations as the two-parameter optimization. In addition, we used a lower substrate concentration to conserve reagents and further assess the capabilities of the instrument. These changes led to a lower signal-to-noise ratio (down to 8-fold, as compared to 22-fold in the two-parameter case). However, the instrument and algorithm were still able to readily optimize reaction conditions. The results of each successive iterated experiment are presented in Figure 6A.

The system again started with attenuated PBC, and the measured fluorescence increased as the system systematically improved reaction conditions. The final, optimized conditions were achieved with injections of 6 µl DTT spiking buffer, 2 µl of magnesium chloride spiking buffer and 13 µl of pH spiking buffer. DTT was found to account for much of the signal increase, with pH being next most important and magnesium having the least effect, based on the best fit model coefficients. The relative impact of different components on the reaction rate recapitulated the results from the 2-parameter optimization, though the volumes were not directly comparable due to the SIA system being reconfigured to better accommodate 3 component injections.

Reproducing optimal conditions outside the SIA device

For the optimization routine to be useful outside the context of SIA, it is of course necessary to convert the optimized spiking buffer volumes into spiked component concentrations. To convert the set of injected spiking buffer volumes in the 3-parameter system to concentrations and pH we used “dye injection” per examples in the SIA literature⁹. We

measured the dilution of fluorescein when it was introduced in the same manner as the optimal spiking buffer volumes. From the measured dilution of the analog, the likely buffer concentrations could be inferred.

For the plateau reached by the system, we recreated putatively optimal conditions off-device in a well plate and verified enzymatic activity. For example, fluorescein in the place of the optimal volume of pH spiking buffer was diluted by a factor of 11.6 after transit to the flow cell. Diluting pH 11.1 spiking buffer into pH 7.5 attenuated PBC by the same factor gave a solution with a final pH of 8.0. The optimal DTT and magnesium chloride concentrations were determined in the same manner to be 10 mM and 11 mM respectively. The putative optimal conditions had a slightly higher magnesium chloride concentration than PBC, a lower pH value, and a greatly increased DTT concentration. The change in pH is likely due to the fact that PBC was optimized by the manufacturer for reaction at 37 °C, while our reactions were carried out at room temperature; the Tris buffer has a strong temperature dependence. The DTT concentration in our suggested optimal conditions was ten times higher than that recommended by the manufacturer. That said, it has been noted by Davidson et. al.²¹ that the HaeIII enzyme shows an increased rate at higher DTT concentrations.

Figure 6B shows result of the reaction in our putative optimal conditions compared to the manufacturer's recommended buffer and the attenuated PBC. These reactions took place outside of the SIA device in order to show that the overall improvement is not limited to on-device applications. Reactions were run in triplicate. The reaction rate obtained in our optimal buffer was similar to the rate in the buffer supplied by the manufacturer. This fluorogenic reaction is meant to be a proxy for the generic endonuclease digestion. Optimization by proxy is always limited by the faithfulness of the proxy to the more generalized reaction. To present a more direct assessment of the results of optimization, we also digested the pUC19 plasmid with HaeIII under the same three conditions and separated the products on a 8% denaturing PAGE (Figure 6C). The optimal conditions produced the most thorough digest; fragments less than 100 bp are visible at the far right.

Conclusions

Recent work in the field of robotics has expanded automation into nearly every aspect of the process of science; computers can form a hypothesis at a very high level and then test that hypothesis by robotic experimentation²². However, while these demonstrations are extremely novel, they are far from convenient for the vast majority of researchers who still carry out bench-based experiments and who need automation assistance rather than automated replacements. Our work focuses on applying automation to a narrow set of optimization problems, and needs only a PC and SIA setup costing a few thousand dollars.

The SIA system that we have designed automates both fluid-handling and decision-making. DOE has previously been used to determine the best conditions for repeated SIA measurements⁶, but has not been routinely applied in molecular biology research. Our work leverages DOE to perform optimizations that can also be applied to off-device molecular biology reactions. We chose DOE because it has precedent in the analytical context²³, but other algorithms could also be designed. A whole field of study is devoted to using limited computational resources to solve optimization problems with difficult computational simulations²⁴, and by coupling iterative, automated reactions with computational assessment we stand poised to compare different algorithms for optimization.

Into the future, SIA-based automation of molecular biology reactions should be extensible to many more than three parameters. Healy et. al. reduced 60 parameters in a simulated

semiconductor process to 7 principal components and optimized those 7 components with a DOE approach²⁵. While the necessity for experimental determination of off-device reaction conditions is cumbersome, the reproducibility of the method argues strongly for the development and execution of assays and procedures on the SIA device itself. Within the context of SIA, this might be used to maximize the analytical signal for a given assay by changing the chemical conditions of the assay as demonstrated here. Additionally, it would be quite simple to use the same techniques to optimize the physical parameters of the experiment (e.g., the number of cycles of mixing or the volumes dispensed to the flow cell). The throughput of the HaeIII digest was ~14 experiments per hour, with most of the time being incubation of the enzyme and substrate. Given these results, it is also possible that existing SIA analyses relevant to molecular biology, such as bead injection ELISA^{26, 27}, could be quickly optimized for new analytes or changes in methodology. Finally, using automated feedback control and DOE melds their advantages in a way that could potentially be expanded to process control for microreactors or bioreactors²⁸.

Materials and Methods

Construction of the SIA instrument

We constructed the SIA instrument from a personal computer and several off-the-shelf components. These included the fluidic parts from FIALab instruments (Seattle, WA) such as the syringe pump, 10-port selection valve, and the fluorescence flow cell. It should be noted that we used the low-background Delrin flow cell rather than the PEEK version. We used an Ocean Optics LS-450 LED illuminator connected by fiber optic cables to the flow cell. We attached a Femtowatt Photoreceiver (Thorlabs, Newton, NJ) with a 515 nm long-pass filter (Chroma, Bellows Falls, VT) to the flow cell via a fiber optic cable. Finally, we recorded the data and controlled the illuminator with a National Instruments (Austin, TX) USB-6009 data acquisition module.

Design and assay of the DNA substrate

We designed an 18-mer DNA substrate containing a HaeIII restriction site and a fluorescein modification at the 3' end (sequence 5'-TGAAGCAGCTGGCCGATT-3'-FAM, HaeIII restriction site underlined). A complementary oligonucleotide contained a Black Hole Quencher at its 5' end. These components were ordered from IDT (Coralville, IA) and used without further purification. To prepare the double-stranded substrate for use, we dissolved the DNA to a final concentration of 1 mM, mixed the strands together at 250 μ M in PBS, and heated to 75 °C followed by cooling at 0.1 °C/sec.

The substrate was assayed by diluting it to 100 nM in the manufacturer's recommended buffer (PBC, 1 mM DTT, 50 mM NaCl, 10 mM MgCl₂, 10 mM Tris, pH 7.9 @ 37°C). A 384 well plate was pre-treated with Denhardt's solution and then loaded with 30 μ l of the substrate solution. A Safire microplate reader (Tecan, Männedorf, Switzerland) adjusted to 37°C was used to measure triplicate samples of this mixture every minute for several hours. The reaction was initiated by adding 1 unit of HaeIII (Promega, Madison, WI.). The reaction produced a fluorescent signal that was well above background and ran to completion (no further increase in fluorescence) within approximately one hour.

SIA automated 2-parameter optimization

A biochemical digest with two parameters was iteratively optimized using the SIA instrument. The multi-connector valve was connected to the fluorescence flow cell, and from there to waste. Other ports on the valve were connected to various buffer reservoirs (see Figure 2). The original or default procedure (without spiking buffers and prior to optimization) was as follows: a volume (~100 μ l) of attenuated reaction buffer without DTT

(50 mM NaCl, 10 mM MgCl₂, 10 mM Tris, pH 7.9 @ 25°C) was aspirated into the holding coil, and approximately one-third was then dispensed into the flow cell. Approximately 5 µl each of substrate (10 µM dsDNA in PBC) and enzyme (1 unit/µl in PBC) were introduced into the middle of the carrier buffer plug. This mixture was then dispensed into the flow cell. After 4 minutes, the amount of fluorescent product that had accumulated was measured. To adjust and optimize the concentration of the buffer components within the SIA cell during the reaction, we then added variable volumes of “spiking” buffers just behind the enzyme and substrate (see Figure 3A). As the substrate and enzyme were pushed into the cell, they mixed with the additional material (see Figure 3B). In order to avoid unintentional changes to the reaction conditions, the spiking buffers were prepared with one component concentration increased and all other buffer components held at concentrations equivalent to the carrier buffer. DTT spiking buffer was 100 mM DTT, 50 mM NaCl, 10 mM MgCl₂, 10 mM Tris, pH 7.9 @ 25°C. Magnesium chloride spiking buffer was 50 mM NaCl, 970 mM MgCl₂, 10 mM Tris, pH 7.9 @ 25°C. The injected volumes of the spiking buffers change DTT or magnesium chloride concentrations respectively, without changing Na⁺ concentration or pH. Different volumes of spiking buffers determine the reaction conditions for any given experiment. The entire optimization took 5–6 hours (80 experiments at ~4 minutes each). The reagents were drawn from vials stored in a cooler at 4–8 °C.

The program for injection and optimization, written in MATLAB, iterates the following routine:

1. Design a set of experiments based on current search size
2. Run the set of experiments; add fluorescence measurements to a log
3. Fit all logged data to quadratic response surface model (see Supporting Information)
4. Determine the maximum of the model over the current search size
5. If the maximum is within the bounds of the search size, shrink the search size
6. Repeat until search size has shrunk twice

See supporting on-line information for the MATLAB source code.

SIA automated 3-parameter optimization

We performed a 3-parameter optimization in the same manner as the 2-parameter optimization described above with the following adjustments: Substrate was adjusted to 3 µM and enzyme was reduced to 0.5 u/µl. The attenuated PBC carrier buffer was at pH 7.5. DTT spiking buffer was 500 mM DTT, 50 mM NaCl, 10 mM MgCl₂, 10 mM Tris, pH 7.5 @ 25°C. A third spiking buffer for pH was added as the third parameter, and this spiking buffer was PBC at pH 11.1. The large DTT concentration in the DTT spiking buffer and the high pH in the pH spiking buffer again minimized the required injection volumes, which in turn ensured adequate mixing of all three spiked components within the reaction volume. The DOE analytical method picked successive experimental conditions based on a Box-Behnken design²³. See Supporting Information for source code.

Determining the concentrations of spiked buffer components

Optimization experiments return on-device optimal conditions for spiking buffer volumes. The actual final concentrations of the spiked components can be estimated in several ways (e.g., based on Taylor dispersion), but we also wished to measure the final concentrations as directly as possible. To do this, we used a technique known in the literature as dye injection⁹. A “blank” experimental procedure (with all fluids replaced by buffer) that had all the same fluidic steps and volumes as the optimal conditions was carried out. One spiking

buffer was replaced with a known concentration (10 μ M) of fluorescein (Invitrogen, Carlsbad, CA) in PBC. The fluidic steps that had been determined for the optimized conditions were then repeated. Calibrated fluorescence measurements were then used to determine the degree of dilution of the fluorescein plug, and this dilution factor was in turn used to estimate the value of the spiked component. For example, DTT spiking buffer was replaced with 10 μ M fluorescein, the fluidic steps for the procedure that arose from the 3-parameter optimization were carried out, and we determined that the final concentration of fluorescein in the flow cell was 203 nM (from the original 10 μ M). This gives us a dilution factor of 49. The concentration of DTT in the original spiking buffer was 500 mM, and assuming the same dilution factor this implies that the concentration of DTT in the flow cell during the optimum reaction condition found during the 3-parameter optimization was 10 mM DTT.

This dilutions observed are caused both by inter-dispersion during transit from valve to flow-cell and by diffusion during reaction incubation. We estimated the degree of mixing due to dispersion during transit using the Taylor dispersion equations²⁹. Taylor Dispersion mixing during transit should be approximately 10^4 times greater than mixing due to diffusion during incubation. Thus we can safely neglect diffusional mixing and dilution during the reaction.

Assessment of optimized reaction conditions

The final reaction conditions were separately assayed in bulk samples. The final concentrations were as follows: the 'attenuated' sample contained 50 mM sodium chloride, 10 mM magnesium chloride and 10 mM HEPES at pH 7.5 at room temperature plus 1 unit per 10 μ l of HaeIII and 100 nM substrate. The manufacturer's recommended buffer sample contained 50 mM sodium chloride, 10 mM magnesium chloride, 1 mM DTT, and 10 mM HEPES at pH 8.2 at room temperature plus 1 unit per 10 μ l of HaeIII and 100 nM substrate. Our putative optimal buffer conditions were 50 mM sodium chloride, 11 mM magnesium chloride, 10 mM DTT, and 10 mM HEPES at pH 8.0 at room temperature plus 1 unit per 10 μ l of HaeIII and 100 nM substrate. Bulk reactions (100 μ l volume including 100 nM substrate and 10 units of HaeIII in the above buffers) were assayed on a Safire well-plate fluorimeter (Tecan, Männedorf, Switzerland). We assessed fluorescence intensity once per minute for 15 minutes and took the slope component of the linear regression to be the rate shown in Figure 6B.

To ensure the generality of our results, the HaeIII digestions were carried out with another substrate, PUC.19 plasmid DNA. The buffers described above were mixed with 0.5 μ g of plasmid and 0.01 units of HaeIII (Promega, Madison, WI) in 10 μ l final volume. The samples were incubated at room temperature for 6 hours. We then separated the products on a 8% denaturing PAGE, stained with SYBR gold (Invitrogen, Carlsbad, CA), and scanned the gel with a Storm fluorescence imager (GE Healthcare, Piscataway, NJ).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

A comparison of different approaches (A–C) to optimization: (A) shows a one-at-a-time approach that misses the local maximum. (B) shows a grid-based approach that requires excessive resources. (C) shows an iterative approach saves sample while finding the local maximum.

Figure 2. SIA device. (A) A photograph of the experimental arrangement shows the pump and valve. (B) Schematic showing the pump and valve connected by tubes (solid lines) to the flow cell (FC). The flow cell is connected by fiber optics (blue and green colored lines) to the light source (LED) and photodiode (PD). Finally, the pump, valve and photodiode are connected to a controller (PC, via dotted lines). (C) The flow cell is diagrammed to show the light (blue input and green output) and fluid connections.

Figure 3. Sequential injection analysis of restriction digests. Variable volumes of reagents injected (A) become variable concentrations (B) in the detector. (C) Fluorogenic endonuclease substrate. (D) Cleavage over 15 minutes yields a strong increase in fluorescence. Fluorescence is measured in arbitrary units (AU).

Figure 4. Simulated 2-parameter search with DOE. An underlying quadratic function is used for data generation. (A–C) Each search (five closed circles) of the space yields a new contour plot. The search patterns and contours are refined algorithmically using a DOE approach. (D) The color map corresponding to the underlying quadratic function, and the final search optimum (open circle). (E) Reaction rate values improve following each successive search.

Figure 5.

Experimental 2-parameter optimization of a HaeIII restriction digest. (A) Fluorescence yield increases in successive experiments within the flow cell over the course of the optimization. (B) The final, Best Fit reaction conditions produce much more signal over 4 minutes than Attenuated buffer or Starting reaction conditions. Fluorescence is measured in arbitrary units (AU).

Figure 6.

Experimental 3-parameter optimization of a HaeIII restriction digest. (A) In the 3-parameter optimization, reaction yields increase within the flow cell over the course of the optimization. (B) The rate of the reaction under Best Fit conditions is comparable to the Manufacturer's recommended buffer. Reaction rates are measured in Arbitrary Units per min (AU/min). (C) PAGE separation and fluorescent staining of DNA fragments resulting from a plasmid digest with HaeIII under Attenuated, Manufacturer recommended and Best Fit conditions. The fourth (bottom) lane is a 100 bp DNA ladder.