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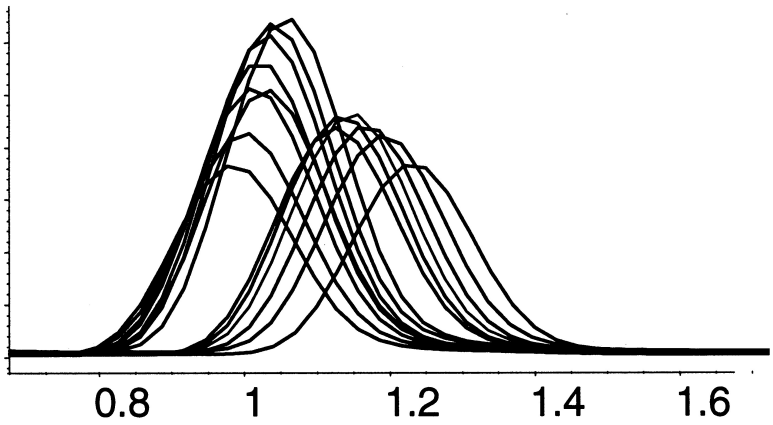

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Articles

Improved Methods for Encoding and Decoding Dialkylamine-Encoded Combinatorial Libraries

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Optimized methods for utilizing and analyzing dialkylamine tags in encoded combinatorial chemistry are reported. A previously described LC/fluorescence method has been shortened from over 1 h to 6 min. A novel ion exchange LC/MS method for decoding has been developed which is both fast and rugged. The use of quantitative encoding schemes is described. These new techniques have allowed a simplified, automated sample preparation scheme, and over 300 encoded bead samples can be read in a day.

Introduction

Encoded combinatorial libraries are an important new technology for modern drug discovery.[1−5] They combine the productivity of split-pool synthesis with many of the screening advantages offered by collections of discrete compounds. One of the technologies required to realize such libraries was the invention of polyamide "hard tags" which were shown to be compatible with a wide range of synthetic chemistries and of demonstrable utility in drug discovery.[6,7] The initial description of the Affymax encoding technology introduced the basic synthetic scheme, an analytical method for reading the codes, and examples of encoded library synthesis. Here are presented new developments in encoding technology, especially improved decoding methods, which have made encoded libraries much more practical.

Figure 1 is adapted from the earlier article.[6] It shows the Affymax process for a three-step synthesis where the first two steps are encoded: the differentiated bead (a), the tagging monomer units (b and c), and the generic, final encoded bead (d). The cleavable linker contained a photo- or acid-cleavable group optimized for screening; the tag will stay with the bead until mineral acid hydrolysis frees it for structure determination. The protecting groups PG^L and PG^T are normally Fmoc and Boc, respectively. The Alloc protecting group of Figure 1b masks the branch point and was readily removed for subsequent encoding steps. The tags were released for analysis with strong acid, dansylated, and determined by HPLC with fluorescent detection. The beads used in this process typically have a few hundred picomoles of synthesis sites. They are differentiated so that approximately 90% is

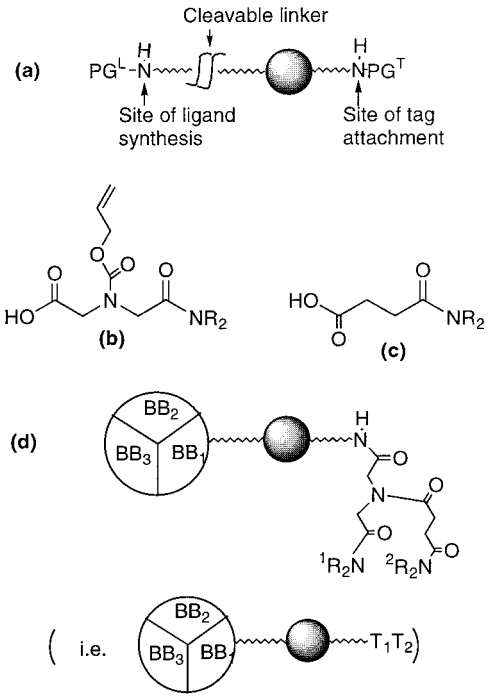

Figure 1. Encoded synthesis using secondary amine tags on a differentially functionalized polymer support: (a) functionalized resin, (b) structure of Alloc tagging monomer unit, (c) structure of succinoyl monomer tagging unit, (d) schematic representation of the product of a two-step encoded synthesis.

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Table 1. Number of Compounds Encodable with a Given Number of Tags Using Different Encoding Strategies

	no. of tags	2	3	4	5	6	7
a	binary	3	7	15	31	63	127
b	binary adjusted	3	7	14	25	41	63
c	ternary	5	19	65	211	665	2079
d	ternary adjusted	7	22	51	100	176	287

ligand (B1−B2−B3) and 10% is tag. Smaller beads, with fewer synthesis sites, require a higher tagging percentage. For reasons of analytical sensitivity, a maximum of three amines is used to encode each step. The individual amines will be present at levels from a few picomoles to a few tens of picomoles per analysis.

Prior to biological screening of the encoded library, it is desirable to determine the quality of the library by analyzing a population of single beads representative of the whole.[8] This quality control consists of comparing the structure of the ligand on each bead as analyzed by mass spectrometry to the structure indicated by the codes on that bead. This process makes great demands on the throughput of the analytical techniques. In the original Affymax method, the HPLC cycle time, on a C_{18} column, was over 80 min. This report details better, faster, and more readily automated methods for decoding combinatorial libraries.

Results and Discussion

Changes to Encoded Synthesis. In reducing encoded synthesis to practice it has been found that it is often not necessary to encode more than two positions. Current best practice is to use an Alloc protected tag (Figure 1b) for the first step of encoding and a succinoyl tag (Figure 1c) for the second step of synthesis. The construct at this point will then not have an Alloc group, and slightly more freedom is obtained for the chemistry of a third chemical synthesis step. In cases where a third encoding step is required, the Alloc tag would be used for the second encoding step and the succinoyl tag for the third step.

Table 1, row a, shows the number of building blocks which can be encoded in a simple binary encoding using all combinations of tags (excluding the null set). In binary encoding, the two states of "detected/undetected" or "1/0" carry the information. The number of "bits" then decides how many items can be encoded. In the original work, 18 tags were chosen to allow for about 50 building blocks and three synthesis steps. However, experience has shown that analytical sensitivity becomes an issue when more than three tags are used per step. Table 1, row b, shows the number of building blocks encodable by a binary system with a maximum of three tags per step. For these reasons, a set of 14 tags has been selected for routine use. This set will encode anything up to a 63×63, two-position library or equivalent.

A New Set of Tags. The application of mass spectrometric detection methods to decoding required a new tag set. It was desirable to have tags, which were distinguishable both chromatographically and by mass. In addition, a persistent problem of contamination among the lower homologues due to the presence of hydrolyzable dialkylamines in ligand molecules had been observed. It was hoped that switching to a higher molecular weight, highly hydrophobic amine set would ameliorate this problem. Finally, the methyl series of tags was excluded because, in certain encoding chemistries, their higher reactivity can lead to poor coding ratios.

Table 2. Optimized Tag Set

no.	name	mol wt
1	2,2,2-trideuteroethylpentylamine	118.2
2	2,2,2-trideuteroethylhexylamine	132.3
3	2,2,2-trideuteroethylheptylamine	146.3
4	2,2,2-trideuteroethyloctylamine	160.3
5	2,2,2-trideuteroethylnonylamine	174.3
6	2,2,2-trideuteroethyldecylamine	188.4
7	pentyloctylamine	199.4
8	diheptylamine	213.4
9	heptyloctylamine	227.4
10	dioctylamine	241.4
11	pentyldodecylamine	255.5
12	hexyldodecylamine	269.5
13	heptyldodecylamine	283.5
14	didecylamine	297.6

Excluding the methylamines, the HPLC retention of the dansyl derivatives was found to be well correlated to carbon number. A homologous set from C_7-C_{20} was chosen. This would also ensure the unique masses required for the MS method. The C_6 amines were specifically excluded because triethylamine is a common laboratory contaminant; the other low-carbon tags were converted to trideutero analogues. This last change does not eliminate contamination problems when the fluorescence method is used but is very helpful in conjunction with the LC/MS method; the 3 amu offset puts them at masses very unlikely in basic contaminants.

The selected set is shown in Table 2. Unfortunately, only the dioctyl- and didecylamines are commercially available. The other nondeuterated amines are readily prepared by reductive amination from the appropriate primary amines and aldehydes. The trideuteroethyl derivatives were chosen because they can be prepared in reasonable time and cost by reaction of trideuteroacetyl chloride and the appropriate primary amines followed by reduction. The Alloc and succinoyl derivatives are synthesized with standard anhydride chemistry.[6]

Accelerating the Dansylation Decoding Method. The original Affymax method (Figure 2 of ref 6) for separating the 18 tags by LC/fluorescence of their dansyl derivatives, using a gradient of acetonitrile and water on a reverse-phase column, required over 80 min. Two of the main obstacles to shortening the run were that the tags were unevenly spaced (spacings varied from 1 to 7 min) and that several tags had very long retention times. These required lengthy isocratic elution with pure nonpolar solvent; thus their retention cannot be accelerated using these chromatographic conditions. The following three approaches were used to overcome these problems.

(a) Rational Choice of Amines. A systematic study of the HPLC separation of available amines with a cyano column was carried out in order to select amine tags that could be adequately resolved within a minimum run time. This investigation was facilitated by the use of computer simulation using DryLab for Windows software, version 2.0.[9]

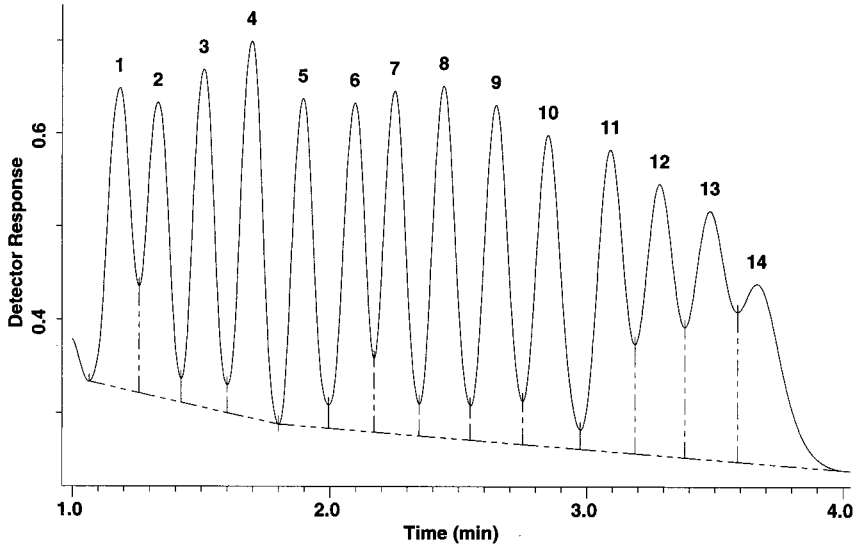

Figure 2. Gradient reverse-phase HPLC/fluorescence analysis of dansylated tags from a single bead encoded with an equimolar mixture of 14 tags. Tags identified as per Table 2.

As described above, further simplification was achieved by reducing the number of tags from 18 to 14. These choices are reflected in the new tag set.

(b) Use of a Less-Retentive Stationary Phase. The original separation of the amine tags used a C_{18} reversed-phase column, which resulted in late retention of higher molecular weight tags and their slow elution after the gradient. Substitution of the present cyano column for a C_{18} column allowed much faster separation of these heavier tags.

(c) Chromatographic Optimization. After combining the optimized tag set and a more appropriate cyano column, standard HPLC optimization could be performed to speed up the analysis. A wider diameter (4.6 vs 2.1 mm), shorter (50 vs 250 mm) column combined with a higher flow rate (2.0 vs 0.25 mL/min) reduced wasted time due to equipment hold-up volume. All tags now eluted within the gradient. Fine-tuning of the gradient conditions yielded the chromatogram shown in Figure 2. In this example, an undifferentiated bead, which has been fully encoded with an equimolar mixture of all 14 tags, has been hydrolyzed, derivatized, and analyzed. The reduction of analysis time from 80 to 4 min allows many more samples to be completed in a day.

Quantitative Encoding Schemes. Quantitative coding[5] uses the *amount* of a given amine as well as simply its presence or absence to increase the information encodable by a fixed number of tags. For binary tagging, where two states of each amine are determined, the number of codes doubles as the number of amines used is increased by one. If three states are distinguishable (i.e., 0, 1, and 2; ternary tagging), then the number of available codes triples with each additional tag. Thus, as shown in Table 1, row c, a real increase in encoding power is available simply by moving to base three.

The number of codes in ternary schemes is less than $3^n - 1$ because simple multiples are not distinguishable from one another, i.e., the codes 102 and 204, or 01 and 02, cannot be used together because the ratio of each tag is the same in each case. Table 1 also indicates that the relative efficiency of ternary encoding increases with library size. To fully encode a 100^3 library by the binary method requires at least 21 tags, but this could be readily encoded with 15 amines by a ternary strategy. Quantitative coding makes possible experiments, which are not achievable using binary technology. Figure 3 shows examples of relatively simple quantitative codes, which have been utilized in library synthesis. In practice, codes with any analyte present in less than 25% of the total are difficult to reliably read. Limiting the ternary codes to those shown in Table 1, row d, solves this detection problem. The adjusted set still offers a large increase in the

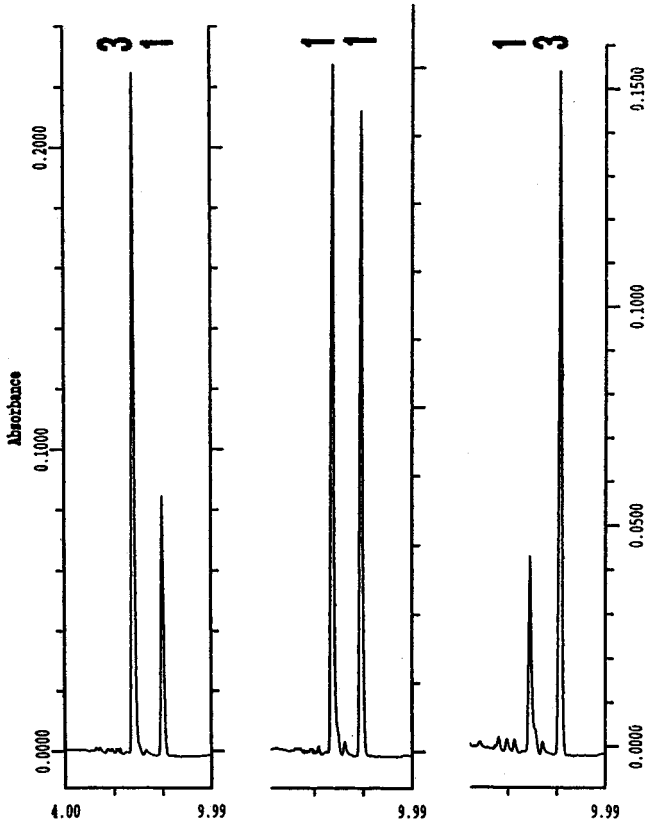

Figure 3. HPLC decodes of three beads with three different ratios of the same two amines.

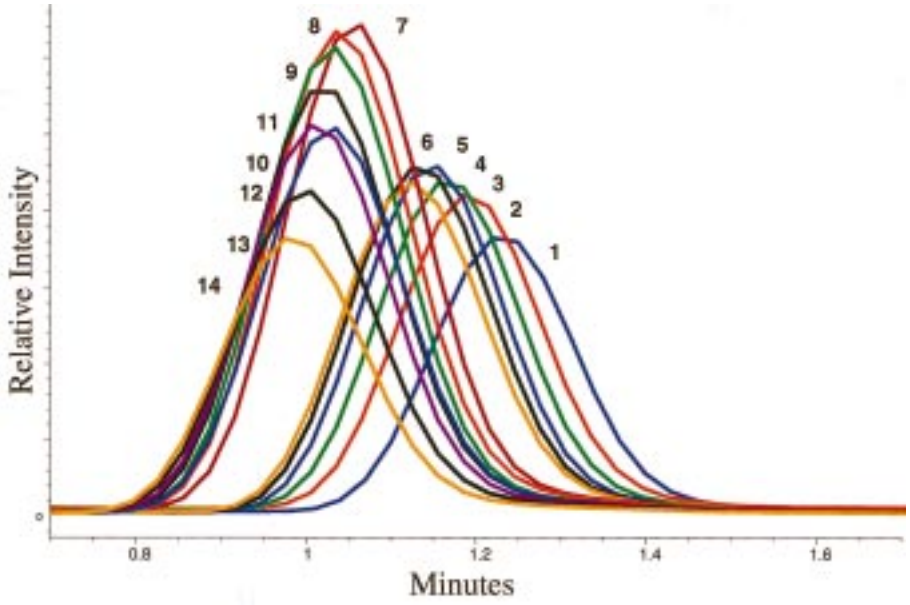

Figure 4. Isocratic cation exchange LC/MS analysis of underivatized tags from a single bead encoded with an equimolar mixture of 14 tags. Tags identified as per Table 2. Each tagging molecule generates a single ion, which is detected by a selected ion monitoring chromatogram. The 14 such chromatograms are colored and overlaid in the figure.

number of available codes over the useable, binary set employing the same tags.

Mass Spectrometric Analysis Methods. All the above studies have been based on HPLC of dansylated amines. While this has proved to be a reasonably versatile and efficient method, there are some inherent limitations, which may be overcome by other analytical methods. Mass spectroscopy offers the obvious advantage of providing mass information on the molecular species present. If used in conjunction with chromatography (e.g., LC/MS) an additional dimension of data is provided which can assist in decoding. As an example, amines are often used as building blocks in library assembly. If, for some reason, they remain associated with beads during the tag analysis process, they will be dansylated and may elute near one of the tags, interfering with tag assignment. Mass information would eliminate such interferences and thus increase the reliability of decoding.

The dialkylamine tags were originally chosen in the hope that they could be readily analyzed as the underivatized protonated amines by high throughput, flow injection, electrospray MS. Despite considerable effort, adequate detection limits were never achieved, due to ion suppression effects of the residual HCl and other impurities in the samples. Also investigated were GC/electron impact and GC/negative chemical ionization MS of pentafluorobenzoyl derivatives as well as flow injection/MS/MS, LC/MS, or LC/MS/MS of the dansyl derivatives. Lane and Pipe have recently described the CEC/MS/MS of dansylated tags.[10] However, none of these methods satisfied the demand for sensitivity and speed until ion exchange LC/MS was explored.

Strong cation exchange chromatography[11] in a highly organic solvent mixture is ideally suited to the task of determining strongly basic amines. Only cations are retained on the column. Since most of the impurities in the samples flush right through, isocratic elution with dilute trifluoroacetic acid (TFA) is possible. Several variables were investigated in optimizing this chromatography. Acetonitrile was chosen as the organic cosolvent. For both acetonitrile and methanol, increasing the percent of organic solvent led to an increase in retention, and this effect was more pronounced in methanol. As H^+ is the cationic displacer, increasing TFA concentration caused decreased retention. In this system, the amines with most hydrophobic character (and consequently least cationic character) elute first, followed by the increasingly polar amines. The sample dissolving solvent is 100% acetonitrile, the weak solvent for this separation. On-column concentration is observed, so a large volume injection gives acceptable results.

After optimization of the isocratic chromatography, the amine tags could all be eluted in a close packet separated from the solvent front but with overall a remarkably short run time of 3 min. By having unique masses for each tag, it was possible to set up selective ion monitoring channels so, although the tags are not well-separated in time, they are separately analyzable by mass. This method quickly pointed out problems with several of the lower mass tags. The combinatorial chemistry laboratory environment is likely to be contaminated with reagents such as triethylamine or diisopropylethylamine, which will be poorly separated from isomeric dialkylamines. However, the optimized tag set solved this problem and led to the chromatogram shown in Figure 4, with the 14 separate selected ion monitoring channels overlaid. In this example, an undifferentiated bead, comparable to that used for Figure 2, has been hydrolyzed and analyzed. This bead contains a nominal 300/14 = 20 picomoles per tag. In normal encoding practice a 10/1 differentiated bead sample will never contain more than three tags per synthesis step, for a nominal 300/10/3 = 10 picomoles for the smallest analyte. The sensitivity of the method is adequate even if tag incorporation has been poor. Tags present at 5% of the expected level have been successfully read. At the subpicomole level the method fails,

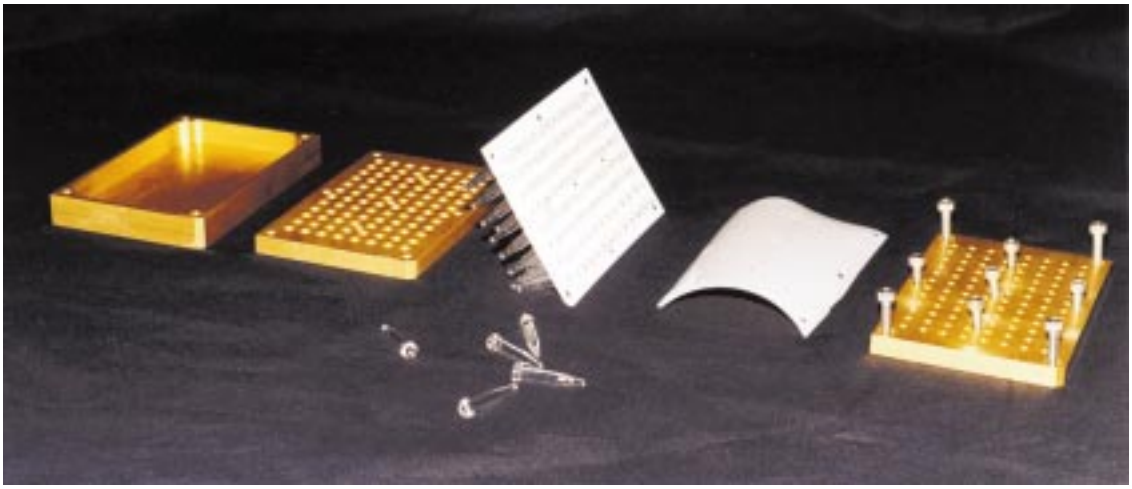

Figure 5. Tag hydrolysis automation device shown disassembled.

not from raw sensitivity but rather from contamination in the laboratory and electrospray background. The precision and accuracy of the method are adequate to the decoding purpose.

Over time, the cation exchange capacity of the column decreases and retention of the amines drift downward. It was found advantageous to regenerate the column with a high aqueous wash every 48 samples. Even with this treatment, after a few thousand injections, the overall retention has slowly decreased to where the tags all elute within 1.8 min. However, they are still adequately detectable as evaluated with a standard reference mixture. These strong cation exchange columns have been shown to be remarkably stable for long-term use.

It was critical to remove methanol from the system; even small amounts led to a high background at *m*/*z* 119, attributed to an ion of composition $(MeOH)_3 + Na$. This background at *m*/*z* 119 led to high constant noise in the M + H channel for tag 1 with concomitantly poorer detectibility. It was also discovered that dilute anhydrous TFA in acetonitrile led, over time, to increasing background at *m*/*z* 228 with corresponding harm to detection of tag 9. A simple solution was to use aqueous TFA as solvent A and pure acetonitrile as solvent B. The high-throughput (4 min per sample), specificity, and simplified sample preparation (relative to derivatization) make this a highly promising alternative to HPLC methods.

Automation of Sample Preparation and Sampling. With a new detection method in hand, the decoding bottleneck became the slow, manual, sample preparation steps. This was especially true in the library quality control process,[8] where specific beads are explored both for ligand and for tags. The earlier method[6] required manual (using a glass capillary and a microscope) transfer of the bead after ligand cleavage to a screw cap vial, addition of HCl, heating, lyophilization, derivatization, and filtration. A new apparatus for automating this whole process in a 96-well format has now been constructed. Figure 5 shows a photograph of the disassembled apparatus. The three metal pieces are made from anodized aluminum. The bottom plate sets the correct microtiter plate footprint. The middle piece holds the glass vials in place. The top piece has holes appropriate for a 96-well autosampler. Tapered glass vials are used for their cleanliness, acid resistance, and volume efficiency. A Teflon insert between the middle metal piece and the lips of the vials prevents vial breakage when the device is sealed with a Teflon/silicone membrane and screw clamps. Assembled (Figure 6), the apparatus has a standard 96-well plate footprint.

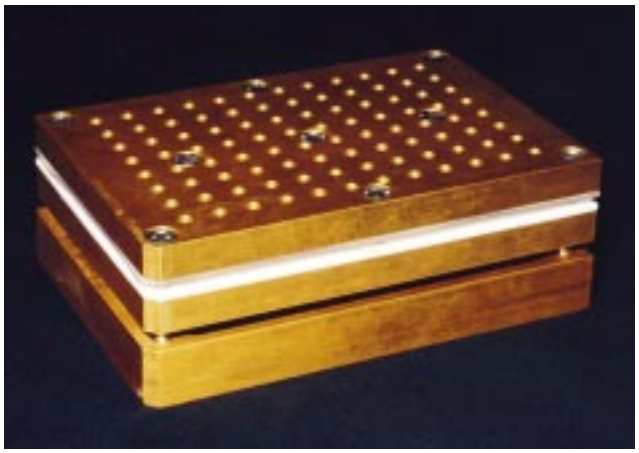

Figure 6. Tag hydrolysis device after assembly.

This apparatus has allowed us to adopt many features of automation such as multichannel pipetting and direct autosampling. Appropriate negative controls for ensuring contamination control, positive controls for ensuring hydrolysis, and reference standards for optimizing peak identifications are readily included in the sample queue of the hydrolysis plate. Once the beads are placed in the vials, the steps of ligand cleavage, ligand LC/MS, code hydrolysis, acid lyophilization, and code LC/MS can be automated with no further bead manipulation during the library quality control process. The fast MS run times allow completion of several 96-well plates in a day.

Conclusions

Hard tag encoding technology at Affymax has become more robust. The newest techniques are faster and more rugged. The dansylation HPLC decoding method has been optimized through the application of chromatography software, a new tag set, and a more appropriate column chemistry. A novel cation exchange LC/MS method has been

developed. This LC/MS method has been automated and resulted in a truly high-throughput, library quality control and decoding operation. Applying these new approaches to the next generation of encoded libraries will further help realize the potential of combinatorial drug discovery, synthesizing and screening libraries of higher quality and greater diversity.

Experimental Section

Chemicals. Solvents were from Burdick and Jackson. Hydrochloric acid was constant boiling from Pierce. Trifluoroacetic acid was from Baker. Dioctyl- and didecylamines are from Aldrich Chemical Co. The other undeuterated dialkylamines were prepared by reductive amination from the appropriate aldehyde and primary amine. Each amine was characterized by ^{1}H NMR and GC/MS.

Chromatography Instrumentation. The HPLC system used was either a Beckman System Gold or a Hewlett-Packard 1050, modified with an Eldex CH-150 column heater. The fluorescence detector was a Shimadzu model RT-551. LC/MS was conducted with a Hewlett-Packard 1100 Series LC/MSD system. LC autosampling from microtiter plates was done with a CTC PAL autosampler. Proton NMR spectra were collected with a Varian Mercury 400 model.

Code Release by Acid Hydrolysis. Single beads, preferably relieved of ligand by prior cleavage, were placed in individual tapered glass microvials (National Scientific, part no. C-4008-632C, decontaminated before use by heating at 400 °C for 2 h). HCl (Pierce 6 N constant boiling sequenal grade, part no. 24309, 25 μL) was added, and the sealed vials were heated at 135 °C for 12−16 h. The HCl was removed from the cooled vial in vacuo.

Derivatization for LC/Fluorescence. Samples were treated with aqueous lithium carbonate (54 mM, 25 μL) followed by dansyl chloride solution (0.74 mM in acetonitrile, 25 μL). After 0.5 h at room temperature, the sample was diluted with acetonitrile/H_2O (2/1; 200μL). Off-line sample filtration was found to introduce contaminants and was no longer performed.

LC/Fluorescence Method for Dansylated Tags. The column used was a Zorbax SB-CN column, 4.6 m × 50 mm, in a column heater at 40 °C. Injection volume was 20 μL. Flow rate was 2.0 mL/min with a gradient of 50/70/75/50/50% acetonitrile/water in 0/3/4.5/4.5/6 min. Detection was by excitation at 352 nm and emission at 510 nm. A precolumn, in-line, 0.45 μm, filter protected the column from plugging.

LC/MS Method for Underivatized Tags. The sample was dissolved in 200 μL of acetonitrile. The column used was a Supelco LC-SCX column, 1 m × 50 mm, in a column heater at 40 °C. Injection volume was 20 μL. Flow rate was 0.2 mL/min isocratic with 85% acetonitrile/15% 0.4% aqueous TFA with a total run time of 3.5 min. Electrospray drying gas was set at 7 L/min and 350 °C. Capillary voltage was held at 2000 V. Fragmentor was ramped from 70 to 120 V, based on the masses sampled. Detection was by selected ion monitoring of the appropriate M + H values. A precolumn in-line 0.45 μm filter protected the column from plugging. The ion exchange capacity of the column slowly changed under isocratic conditions, causing a slow reduction in retention times. The drifting retention times were readily recalibrated by running standards every 24 samples. After every 48 samples the column was regenerated by switching to 50% acetonitrile/50% 0.4% TFA for 15 min, followed by a 15 min reequilibration.

Synthesis of Trideuteroethylamines. To a stirred solution of acetyl-d_3 chloride (43.28 g, 531 mmol) in THF (430 mL) was added heptylamine (79 mL, 531 mmol) dropwise at 5−10 °C. Dimethylethylamine (58 mL, 531 mmol) was then added dropwise at 5−15 °C. The reaction was stirred at room temperature for 4 h. After the reaction mixture cooled to −10 °C, $LiAlH_4$ powder (31 g, 811 mmol) was slowly added in portions, keeping the temperature below −10 °C. After the mixture was stirred overnight at room temperature, the reaction mixture was refluxed for 1 h. The reaction mixture was then quenched by cooling to −10 °C and carefully adding water (32 mL). Aqueous NaOH (22.0 g, 550 mmol, dissolved in 25 mL of water) was then added dropwise, allowing the temperature to warm to 20 °C. After being stirred for 1.5 h, the reaction mixture was filtered, dried over Na_2SO_4, and concentrated in vacuo. The resulting cloudy liquid was redissolved in ether (200 mL), redried with Na_2SO_4, and concentrated again. The pale yellow liquid was distilled to yield 61.0 g (78%) of colorless liquid, bp 178−184 °C/760 mm. In a similar way, the pentyl, hexyl, octyl, nonyl, and decyl derivatives were prepared. Electrospray MS of the free amines showed the correct M + H. ^{1}H NMR of each free amine confirmed the structure and showed the purity of each to be >95%.

Synthesis of Alloc Protected Amines. This procedure was described previously.[6] Each of the 14 Alloc amines gave appropriate M + H by electrospray MS and appropriate ^{1}H NMR.

Synthesis of Selected Succinoyl Protected Amines. The appropriate dialkylamine was reacted with 1 equiv of succinic anhydride in dichloromethane at 0 °C. After evaporation, the compounds were sufficiently pure for use. In this way the succinoyl derivatives were prepared for amines 9−14 (Table 2). Electrospray MS and ^{1}H NMR of the compounds confirmed the structures.

Synthesis of Fully Encoded Beads. Three equivalents of an equimolar mixture of the 14 tags as their Alloc derivatives in DMF at individual concentrations of 36 mM (total tag concentration, 0.5 M) were activated with DIC/HOBT and allowed to react with undifferentiated TentaGel S−NH_2 (100 mg) for 16 h. After extensive washing (DMF, MeOH, DCM), the resin was dried under high vacuum.

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