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HIGH THROUGHPUT PURIFICATION OF COMBINATORIAL LIBRARIES

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Abstract: This article summarizes recent advances at MDS Panlabs which provide for the high-throughput preparative HPLC purification of our Optiverse™ screening library. Topics covered include unique methods for the preparation, purification, characterization, and plating of purified screening compounds. Also discussed are procedures for data tracking as samples proceed through the purification process.

Introduction: Approximately 10 years ago the field of combinatorial chemistry was born.[1] High throughput screening had advanced such that compounds prepared by traditional means could no longer be supplied quickly enough to meet the demands of high throughput screening. With reaction products prepared *en masse*, combinatorial libraries readily filled this void. As the number of samples assayed increased so did the time and effort required to isolate and characterize active compounds. This situation was further aggravated by the fact that mixtures of compounds can sometimes give false positives or negatives as well as less reliable SAR data. To provide compounds of high purity, MDS Panlabs became the leading co-developer of the Parallex™ high throughput preparative HPLC system in collaboration with Biotage, Inc. (Charlottesville, VA). In addition, MDS Panlabs developed new protocols and methodologies to support preparation, characterization and plating of the purified libraries as well as data tracking of samples moving through the purification process.

Overview (See Figure 1): The entire process of library production, purification, analysis and plating is based on modularity. This has the advantage of allowing flexibility in the workflow. Additional equipment may be brought on line to address bottlenecks or the process may be adjusted to allow for greater efficiency. The focus of these efforts is high throughput, not fast turnaround. The goal is to make each phase of the purification process as efficient as possible to maximize the number of samples purified. For example, mass spectral data is acquired in a separate step from HPLC purification. Performance of one is not dependent on the other. Further, by keeping these two steps separate, idle time is reduced on the mass spectrometers, which reduces the number of instruments required and increases overall efficiency.

The preparation of the libraries has been covered in detail elsewhere[2] and will be repeated only briefly here. Reactions and scaffolds are selected from current literature or more traditional organic chemistry. After reaction conditions have been validated, parameters for starting materials are determined for the design of each library. This information is then provided to Tripos, Inc. (St. Louis, MO) who, in collaboration with MDS Panlabs, currently designs the libraries[3,4] for maximum diversity while imposing a variety of filters. As an example, filters are applied to exclude compounds with chemically or biologically interfering functionalities,

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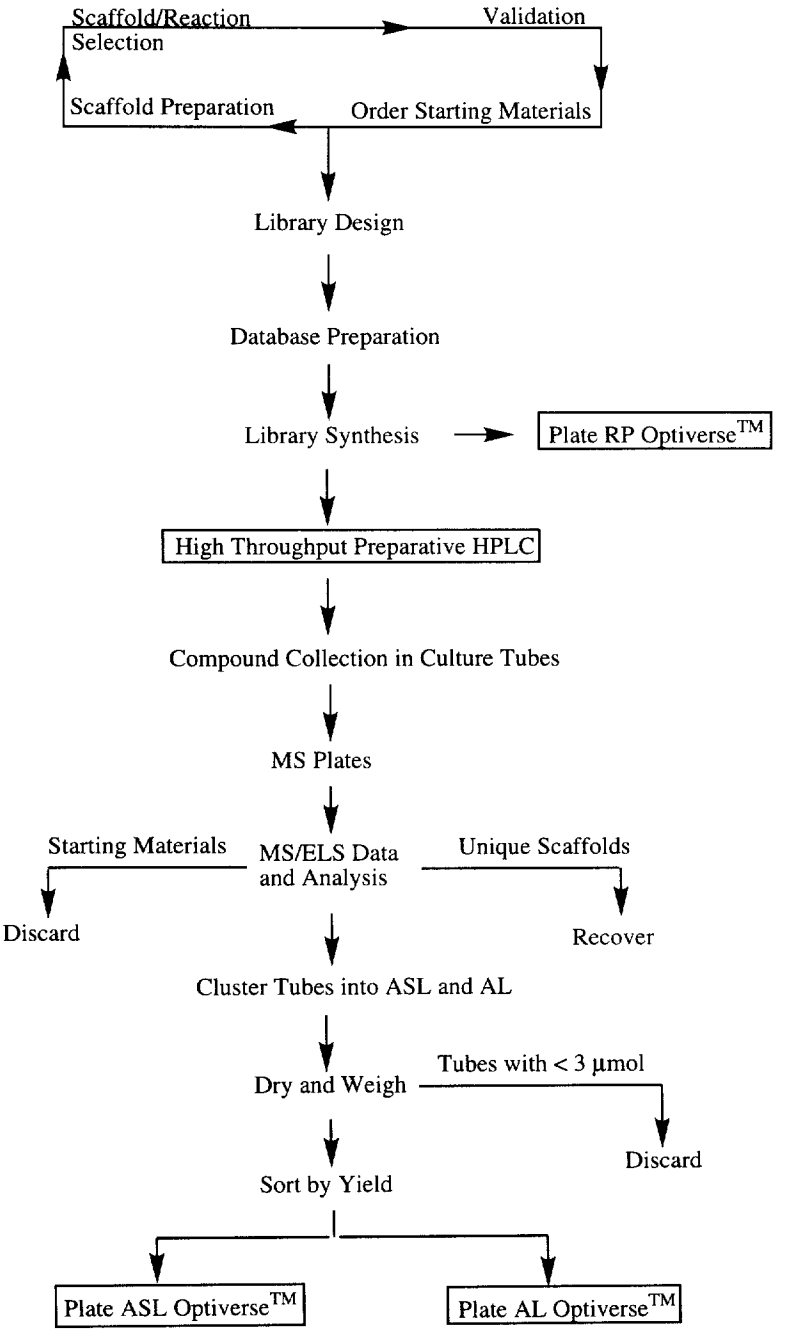
Figure 1


Scaffold/Reaction Selection
Validation
Scaffold Preparation
Order Starting Materials
Library Design
Database Preparation
Library Synthesis
Plate RP Optiverse™
High Throughput Preparative HPLC
Compound Collection in Culture Tubes
MS Plates
Starting Materials
MS/ELS Data and Analysis
Unique Scaffolds
Discard
Recover
Cluster Tubes into ASL and AL
Dry and Weigh
Tubes with < 3 µmol
Discard
Sort by Yield
Plate ASL Optiverse™
Plate AL Optiverse™

toxicity, or unacceptable calculated octanol/water coefficients (cLogP). Starting materials are ordered and unique scaffolds, or intermediates, are prepared in house. All initial steps are interrelated. For example, library designs are affected by validation parameters as well as the availability of starting materials. After preparation of the ISIS database (MDL Information Systems, Inc., San Leandro, CA) all reaction products are prepared in parallel from solution-phase chemistry on a one-millimolar scale. Preparation of the libraries has been streamlined through the use of custom automated workstations for quenching and extraction as well as plating, capping and labeling (built by Bohdan Automation, Inc., Mundelein, IL).

After preparation, each reaction mixture is evenly divided into 4 vials of ca. 250 μmol (theoretical). Two vials are placed in archival storage. One vial is dispensed into multiple microtiter plates for assay and entered directly into the unpurified reaction products library or RP Optiverse™. The remaining vial is placed into an individual well of a microtiter plate for purification. The purification plates are subjected to high throughput preparative HPLC and collected into tared culture tubes. Collection occurs only when a peak is detected (intelligent fraction collection). Microtiter (MS) plates are created in which a small aliquot is taken from the first tube of each detected peak in the HPLC trace. Volumes greater than 18 mL (one culture tube) are not collected. The compound in each well of the MS plate is subjected to mass spec analysis and ELS (evaporative light scattering) response to assess the compound's identity and approximate mass. After data analysis, culture tubes which contain the designed reaction products are clustered and entered into the advanced screening library or ASL Optiverse™. Culture tubes which contain products other than the designed product are reserved for the alternate screening library or AL Optiverse™. Each tube is evaporated to dryness, weighed, and the micromoles of compound calculated. Tube are sorted according to the number of micromoles they contain to allow maximum efficiency in plating.

High Throughput Preparative HPLC System, Hardware: The primary system requirement is high throughput-up to 600 fractionations per day. The Parallex™ HPLC from Biotage was chosen because of its close fit to these needs. Injector and collectors use a footprint which accepts eight microtiter plates with 24-, 48-, or 96-well formats. Collectors have been modified to accept 16 × 125 mm culture tubes in a 24-rack format. All plate numbers can be entered manually or by bar-code reader. Samples are loaded sequentially in multiples of 4 into a 6 way injector while HPLC runs are in progress. As soon as an HPLC run is completed, the next run begins immediately. Four HPLC injections are run in parallel, using the same gradient. Each injection loop has its own dedicated HPLC column and fraction collector. The UV detector is unique; it is a single UV cell with 4 flow paths. Output can be monitored at 219 nm, 254 nm, 280 nm, or 307 nm through the use of interchangeable filters. Any two wavelengths can be used to monitor and trigger fraction collection simultaneously. The fraction collectors are triggered only when eluted peaks exceed entered threshold values (when peak height exceeds preset absorbance units or AU) or slope enable/slope values (when peak height exceeds preset AU and the slope rises faster than preset AU per second).

High Throughput Preparative HPLC System, Software: The Parallex™ software[5] is designed to operate in a Microsoft® Windows NT™ environment. Through Parallex Explorer™, data is entered and extracted: spread sheets are imported and exported using Microsoft® Excel or Access, HPLC conditions and collection parameters are entered, and bar-code numbers are accepted. Parallex Explorer™ also allows one to view data after it has been collected and processed. Parallex ControlTM is used to produce the movements of the Gilson injector and collectors, loading of injection loops, and detector sensitivity. ControlTM also operates the programmable logic controller (PLC) and thereby the HPLC pumps, gradient valves, injection valves and pressure alarms. Microsoft® Access communicates with Parallex ExplorerTM and Parallex ControlTM and saves all data. This includes all samples imported for fractionation, processed data and their relationship. Data fields include sample name, plate ID and well location, peak and peak cut number, volume collected for each peak and retention time as well as HPLC conditions and collection parameters. All HPLC traces are saved in ANDI format (a standardized format for chromatographic data supported by the analytical instrument association).

HPLC Protocol: The goal is high throughput preparative HPLC separations. To this end what is required is a "rapid universal" gradient which can be applied to all samples as they are purified.[6] Our standard method uses reverse phase chromatography employing a 10 μ C_{18} column which is endcapped for the purification of combinatorial libraries containing small organic molecules with amines. A 20 × 100 mm column allows sample loads of 75 mg to 100 mg while balancing resolution and throughput. A standard reverse-phase binary gradient of acetonitrile (or methanol) and water is employed. Typically a trifluoracetic acid (0.1%) or ammonium acetate (10 mM) buffer is added to the aqueous phase. The method chosen allows for a gradient from 80% aqueous/20% organic to 100% organic over 5.5 min at 30 mL per minute followed by a wash of 100% organic for 1.5 min at 35 mL per minute then equilibrating at starting conditions and holding for 2 min (35 mL per minute). Peaks are collected during the gradient, wash, and equilibration steps. Typical output on a daily basis is 288 samples per instrument.

Sample Preparation: All samples are plated in 5 mL 48-well microtiter plates. They are dissolved by first adding 1 mL 50/50 DMSO/MeOH and then sonicating for 30 min. At this point any compounds that do not dissolve are removed from the plate and treated separately.

Post Purification Processing: At the end of each day all collected samples and data are transferred to the mass spectrometry lab. An aliquot of 220 μL is sampled from each peak and transferred to a 96-well MS plate using a 204 Packard MultiPROBE. (Four culture tube racks are reduced to one 96-well plate to allow archival storage of the MS plates.) Flow inject electrospray mass spectral analysis in positive and negative mode is obtained on each peak (Sciex, API150 MCA mass spectrometer) to verify each compound's identity. In addition an ELS response (S.E.D.E.R.E. Sedex 55) is measured to determine those products which do not have

sufficient mass to be of interest. Samples are introduced by a Gilson 215 liquid handler using a HP1100 series binary pump for solvent delivery with the eluant (80% acetonitrile/20% water) being split between the mass spectrometer and ELS detector. Throughput is 50 seconds per sample to allow the analysis of 72 peaks per hour per instrument. Apple scripting autoanalysis programs (PE Sciex, Concord, Ontario) reduce the raw data files and summarize the molecular weight of the base peak as well as the presence of the designed product, scaffolds and starting materials. The resulting text file is converted to Excel format. Based on the results of the analysis of the MS plate, culture tubes with starting materials only are tagged for disposal. Culture tubes with unique scaffolds are combined into appropriate vessels for recovery. Culture tubes with ASL and AL compounds which have mass spectral counts and ELS responses above preset thresholds are tagged. Each selected tube is transferred to a secondary rack for further processing. The solvent from each tube is removed by lyophilization or speed-vac (vacuum-assisted centrifugal evaporation) and each tube is then shuttled to a weigh station.

Weigh Station: Before the initiation of each Parallex HPLC run, culture tubes (16 × 125 mm, 19 mL volume) are bar-coded and tared. A spreadsheet is prepared with bar-code number, tare weight and rack ID/position. Upon completion of each HPLC run, information on sample ID and rack ID/position is uploaded to the CTS (compound tracking system) to allow sample name and bar-code number to be correlated. After each culture tube has been dried, the weight of the tube is taken. The tare weight is subtracted from this number to obtain the mass of compound contained to the nearest tenth of a milligram. Using the molecular weight of the compound in each well, the micromoles of compound in each tube are calculated. After an intermediate step where tubes are sorted according to micromolar quantity, the tubes are transferred to a liquid handling station where the contents are brought to a known concentration for dispensing into assay plates.

Data Tracking: A key component of the modular system is data management and tracking. A SQL server houses the compound tracking system or CTS (custom designed by AXC Interactive Solutions) dedicated to tracking samples as they move through the purification process. As the design of each library is completed, this information is downloaded to the ISIS database. Compound ID, plate ID, well location, molecular formula, molecular weight, and starting material IDs are downloaded to the CTS, which serves as a hub—sending and receiving data to each module as it is needed.

Parallex Explorer™ receives sample ID and well location in an MS Excel spread sheet. As each sample is purified, sample ID, collection rack and position, peak number, and retention time are saved in the Microsoft® Access database while HPLC traces are saved in ANDI format. Upon completion of a run, the information is uploaded, through Parallex Explorer™, to the CTS. Based on this information, a MS plate, which contains an aliquot from each peak, can be prepared for mass spectral characterization and ELS response. The relationship between the collections racks and MS plates is tracked by the CTS. As each well of the MS plate is subjected to characterization, the mass spectral and ELS data are analyzed. The data are uploaded to the CTS along with the results of the analyses. Review of this data allows the first keep/discard decision to be performed. These results

are again tracked by the CTS. Instructions are sent to a robot which pulls culture tubes for both the ASL and AL and clusters these tubes in secondary racks. After the tubes are evaporated to dryness, they are taken to the weigh station. Each tube is tracked by bar-code number and its weight entered into the CTS where the mass of the contents is determined. Further, by accessing the molecular weight of the compound, the millimoles of each compound can be calculated along with the volume of solvent required to bring the sample to a standard concentration. A second keep/discard decision is performed. All tubes which contain less than 3 μmol are discarded. Under the direction of the CTS, the culture tubes which are to be included in the ASL or AL are clustered according to amount of material contained by a sorting automated work station. A layout of the purified material in assay plates is written. A liquid handling station then receives this information, dilutes the samples to a standard concentration and plates the samples into designated wells. The information for the purified libraries is uploaded to the ISIS database.

Summary: In order to provide reaction products of high purity, MDS Panlabs became the leading codeveloper of the Parallex™ high throughput preparative HPLC, put together a modular system for the preparation, analysis and plating of purified screening compounds, and developed a data tracking system for samples as they move through this unique process. The purified compounds provide screening libraries of increased value. High throughput screening groups can more rapidly identify lead compounds through more reliable assay and SAR data.

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6. Special thanks to Dr. Peter Rahn of Biotage for may helpful discussions.