Strategies for Rapid Deconvolution of Combinatorial Libraries: Comparative Evaluation Using a Model System

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Synthesis and testing of complex mixtures maximize the number of compounds that can be prepared and tested in a combinatorial library. When mixtures of compounds are screened, however, the identity of the compound(s) selected may depend on the deconvolution procedure employed. Previously, we developed a model system for evaluation of deconvolution procedures and used it to compare pooling strategies for iterative and noniterative deconvolution [Freier et al. *J. Med. Chem.* **1995**, *38*, 344−352]. We have now extended the model studies to include simulations of procedures with overlapping subsets such as subtractive pooling [Carell et al. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 2061−2064], bogus coin pooling [Blake and Litzi-Davis. *Bioconjugate Chem.* **1992**, *3*, 510−513], and orthogonal pooling [D'Prez et al. *J. Am. Chem. Soc.* **1995**, *117*, 5405−5406]. These strategies required synthesis and testing of fewer subsets than did the more traditional nonoverlapping iterative strategies. The compounds identified using simulations of these strategies, however, were not the most active compounds in the library and were substantially less active than those identified by simulations of more traditional strategies.

Introduction

Before the advent of combinatorial chemistry, the rate-limiting step in drug discovery was often compound synthesis. With current methods for chemical synthesis of combinatorial libraries, the rate-limiting step in identification of leads has become compound screening. High-throughput screening groups[1−5] are pressed to keep up with the millions to billions of compounds that can be prepared using combinatorial procedures.[6−20] Screening rates can be increased if mixtures of compounds are tested, but mixture screening increases the risk of missing active compounds. In addition, if compounds are synthesized as mixtures,[21,22] a "deconvolution" procedure must be employed to identify the most active compound in the mix. Probably the most straightforward deconvolution strategy is iterative deconvolution first described by Geysen for peptide libraries.[23] Although this strategy has been used successfully to identify several active compounds,[24−45] the rate of compound discovery is limited by the requirement for several rounds of synthesis and testing.

Several ingenious strategies have been devised to eliminate the multiple rounds of synthesis and testing associated with iterative deconvolution. These alternate strategies usually deduce the structure of the most active molecule after synthesis and testing of a relatively small number of compound mixtures and thus offer an advantage over iterative deconvolution that requires multiples rounds of synthesis and testing. These streamlined strategies can successfully identify the most active compound from a library when the library contains one very active compound in a large population of inactive compounds. Many real libraries, however, contain many compounds with a spectrum of activities. This spectrum of activities represents a "molecular landscape",[46] and the question arises how the compounds with suboptimal activity affect the deconvolution.

Previously we used computer simulations to evaluate the effects of compounds with suboptimal activity on the results of iterative deconvolution and position scanning. We compared pooling strategies for iterative deconvolution and position scanning to determine the effect of experimental error on the outcome.[47,48] We also used the computer model to evaluate "mutational SURF" in which series of single compounds are iteratively synthesized and tested to identify leads using an evolutionary process.[49] A model of RNA hybridization was used to create two molecular landscapes. Each molecular landscape is simply a list of activities corresponding to each compound in the library. Activity distributions in these libraries bracketed many real situations and suggested they were applicable to non-nucleotide libraries that typically contain small molecules that bind to targets such as enzymes or membrane receptors.[38,39,46,47,49−51] In this paper, we extend these computer simulations to include four strategies that require much less synthesis and testing than standard iterative deconvolution. They are subtractive pooling,[52,53] bogus coin pooling,[26] orthogonal pooling,[54,55] and position scanning.[29,56,57] We will demonstrate how the likelihood of success can be reduced by the presence of more than one active compound in the library.

Results

Activity Profile of the Library. Simulations were performed using activity profiles described previously.[47−49] Briefly, we calculated molecular landscapes (activity profiles) for a library of asymmetric compounds prepared from 64 building blocks. Each building block was an RNA trinucleotide, and each compound was composed of three building blocks for a total of 262 144

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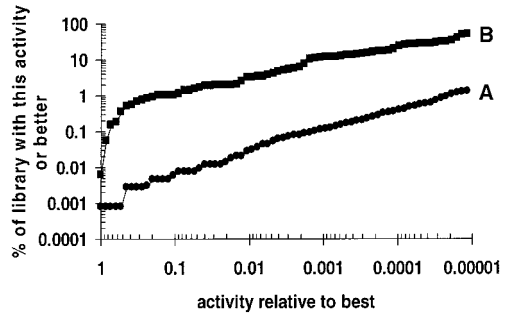

Figure 1. Cumulative frequency distribution of activities for landscapes A (●) and B (■). Activity is plotted relative to the most active molecule in the library. The appearance of "steps" in the profile for landscape A is due to the low number of compounds with high activity. For example, the curve is flat between 1 and 0.5 because there were no compounds in the library with activity between that of the best and 0.5 times that of the best.

(64^3) compounds. Two RNA targets, 5′-GUGUGGGCA-3′ and 5′-UGGGCA-3′, and RNA folding parameters[58] were used to create two different molecular landscapes as described previously.[47] Cumulative frequency distributions of activities in these two molecular landscapes are plotted in Figure 1. Activities are reported relative to the most active compound in the library. Thus the most active compound had an activity of 1.0. We arbitrarily classified compounds with activity within 5-fold of the best (activity >0.2) as compounds with "good" activity. The most active compounds had IC_{50} values of 1 pM and 40 nM for landscapes A and B, respectively. The two landscapes had distinctly different profiles that bracket those of many known drug−receptor complexes. Landscape A was typical of libraries with few active compounds; only 12 (0.005%) had good activity. Landscape B, in contrast, contained many active compounds; 2414 (0.9%) had good activity. These two distinct molecular landscapes provided us with two models for testing deconvolution strategies. We asked how the presence of more than one active compound in the library affected the likelihood that each strategy would identify the most active molecule or one with good activity.

Subtractive Deconvolution. Subtractive pooling is a variation of a strategy reported by Carell et al.[50,52,53] and is particularly advantageous when applied to libraries prepared by simultaneous addition of functional groups to multiple sites on a core scaffold. As diagrammed in Table 1, subtractive deconvolution begins with synthesis and screening of the entire library as a single mixture. If activity is detected in the library, then a set of subtractive subsets, each missing one building block, is prepared. Activities of the subtractive pools are compared to determine which of the building blocks are responsible for library activity. Subtractive subsets that are missing a functional group from the active compound(s) will lose activity relative to the parent library. Thus the least active subtractive subsets identify the most "important" functional groups. After these few building blocks have been identified, a small subset of compounds containing only these functional groups is prepared, and the most active compound in this subset is selected using one-at-a-time synthesis and testing or a small iterative deconvolution. The method of deconvolution preferred for this small subset will depend on the exact number of compounds in the subset

Table 1. Examples of Five Deconvolution Strategies for a Library with 64 Functional Groups and Three Positions[a]

Iterative Deconvolution

Round 1:	X-N-N	Round 2:	*1*-X-N	Round 3:	*1-3*-X
1-N-N	+++	***1-1***-N	++	***1-3-1***	+++
2-N-N	-	***1-2***-N	+	***1-3-2***	++
3-N-N	-	***1-3***-N	++++	*etc.*	
4-N-N	+	***1-4***-N	+++	***1-3-8***	+++++
5-N-N	-	***1-5***-N	-	***1-3-9***	++
etc.		*etc.*		*etc.*	
Winner:	***1***-N-N	Winner:	*1-3*-N	Winner:	***1-3-8***

Subtractive Deconvolution

Round1:			Round 2:
Full library	64^3	++	prepare 27 unique compounds composed of ***3***, ***5***, ***8***
minus ***1***	63^3	+++	
minus ***2***	63^3	+++	
minus ***3***	63^3	+	
minus ***4***	63^3	+++	
minus ***5***	63^3	+	
minus ***6***	63^3	+++	
minus ***7***	63^3	+++	
minus ***8***	63^3	+	
etc.			
important groups:		***3 5 8***	

Bogus Coin Deconvolution

Round 1:	
– Equal representation of all groups at all positions	+
– No α at position 1, double β at position 1, leave γ unchanged	++
– No α at position 2, double β at position 2, leave γ unchanged	-
– No α at position 3, double β at position 3, leave γ unchanged	+
Round 1 winner	βαγ
Round 2:	
Divide each winning group into three subgroups (eg. β_1, β_2, β_3, *etc.*)	
– Equal representation of all β subgroups at position 1, all α subgroups at position 2 and all γ subgroups at position 3	++
– No β_1 at position 1, double β_2 at position 1, leave β_3 unchanged at position 1	+
– No α_1 at position 2, double α_2 at position 2, leave α_3 unchanged at position 2	+
– No γ_1 at position 3, double γ_2 at position 3, leave γ_3 unchanged at position 3	+++
Round 2 Winner	$\beta_1\alpha_1\gamma_2$
Rounds 3-4:	
Continue to further divide winning subgroups and test as above until single compound is identified.	

Orthogonal Deconvolution

Assignment of 64 functional groups to 3 x 4 orthogonal sets of 16:

	A1 C1	A1 C2	A1 C3	A1 C4	A2 C1	A2 C2	A2 C3	A2 C4
B1	***1***	***2***	***3***	***4***	***17***	***18***	***19***	***20***
B2	***5***	***6***	***7***	***8***	***21***	***22***	***23***	***24***
B3	***9***	***10***	***11***	***12***	***25***	***26***	***27***	***28***
B4	***13***	***14***	***15***	***16***	***29***	***30***	***31***	***32***

	A3 C1	A3 C2	A3 C3	A3 C4	A4 C1	A4 C2	A4 C3	A4 C4
B1	***33***	***34***	***35***	***36***	***49***	***50***	***51***	***52***
B2	***37***	***38***	***39***	***40***	***53***	***54***	***55***	***56***
B3	***41***	***42***	***43***	***44***	***57***	***58***	***59***	***60***
B4	***45***	***46***	***47***	***48***	***61***	***62***	***63***	***64***

Active Subsets:	A1-A2-A3, B2-B3-B3, C3-C2-C1
Selected Compound:	***7-26-41***

Position scanning:

1-N-N	-		N-***1***-N	-		N-N-***1***	+
2-N-N	++		N-***2***-N	-		N-N-***2***	-
3-N-N	-		N-***3***-N	-		N-N-***3***	-
4-N-N	-		N-***4***-N	+		N-N-***4***	+
5-N-N	-		N-***5***-N	-		N-N-***5***	-
6-N-N	+		N-***6***-N	-		N-N-***6***	-
7-N-N	-		N-***7***-N	-		N-N-***7***	++
etc			*etc.*			*etc.*	
Selected compound:			***2-4-7***				

[a] Bold numerals ***1***, ***2***, ***3***, ***4***, ..., ***64*** represent the 64 building blocks. N represents an equimolar mixture of all building blocks. Activity is denoted on a scale from − (inactive) to +++++ (most active).

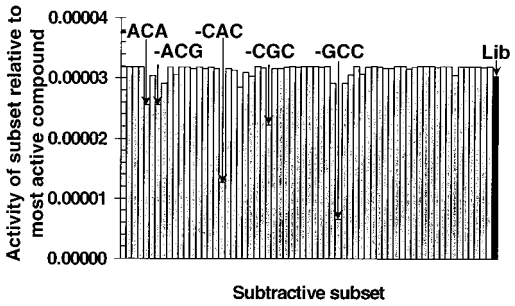

Figure 2. Activities for the 64 subtractive subsets for landscape A in the absence of experimental error. Activities are reported relative to the most active compound in the library which has an activity of 1.0. The solid black bar at the right represents activity of the entire library. Arrows identify the five subtractive subsets with the greatest reduction in activity compared to the parent library.

and the relative difficulty of synthesizing single compounds compared to compound mixtures.

Subtractive deconvolution was simulated for landscapes A and B by testing 64 subtractive pools. Each subtractive pool contained 250 047 (63^3) compounds and contained all possible molecules composed from 63 of the 64 building blocks. Activities of the 64 subtractive subsets for landscape A are plotted in Figure 2. For this landscape, activity of two subtractive subsets (minus ***CAC*** and minus ***GCC***) was reduced more than 2-fold compared to the complete library (black bar in Figure 2). Activity for three others (minus ***ACA***, minus ***ACG***, and minus ***CGC***) was reduced 10−20%, and all other subsets showed less than 10% reduction in activity. When only the three "most important" building blocks, ***CAC***, ***GCC***, and ***CGC***, were used to generate a set of 27 (3^3) compounds, the most active compound in the library was not identified. The selected compound had an activity of only 0.1 relative to the most active in the library. When two additional building blocks, ***ACA*** and ***ACG***, were added and 125 (5^3) compounds were tested, the most active compound in the library was identified. Increasing the number of building blocks from three to five increased the number of compounds to be prepared from 27 to 125 and increased the activity of the selected molecule 10-fold.

Of the five subtractive subsets identified in Figure 2 as having reduced activity, only two (minus ***CAC*** and minus ***GCC***) had activity less than 70% that of the parent library. Thus, in the presence of experimental error, it may have been difficult to identify all five functional groups required to find the most active compound. To assess the effectiveness of subtractive deconvolution in the presence of experimental error, simulations were performed with a 2-fold Monte Carlo error in the activity of each subset. This error represents experimental error in the biological assay for activity or error in compound concentrations within the library. For each simulation, the activity of the 64 subtractive subsets was used to identify the 3, 5, or 10 most "important" functional groups in the library. These few functional groups defined subsets of 27, 125, or 1000 (3^3, 5^3, or 10^3) compounds. A compound was selected from this subset using iterative deconvolution.

Results of subtractive deconvolution in the presence of 2-fold error are plotted in Figure 3. When the subtractive pools were used to identify only three functional groups for follow-up, the likelihood of selecting a compound with good activity was very low. Only 20% of the simulations resulted in selection of a compound with activity better than 0.01. Keeping 5 or 10 functional groups for follow-up substantially increased the likelihood of success. When 10 functional groups were included, the follow-up sublibrary contained 1000 (10^3) compounds and the likelihood of success for subtractive deconvolution approached that of iterative deconvolution on this library.

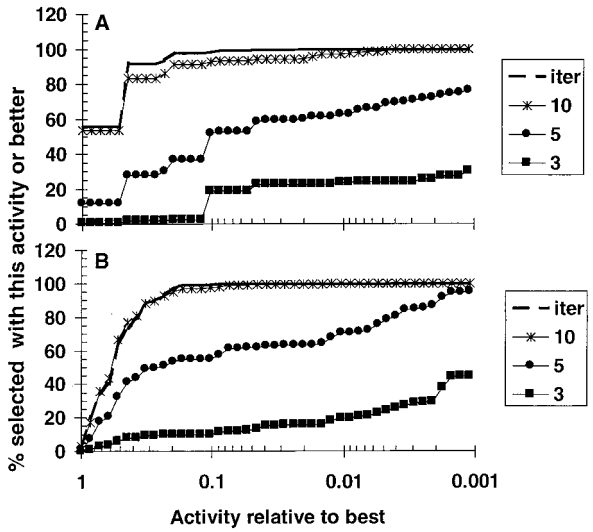

Figure 3. Distribution of activities selected during simulations of subtractive deconvolution for landscapes A and B. Screening of 64 subtractive pools was used to select the 3 (■), 5 (●), or 10 (×) most "important" functional groups in the library. The final compound was selected using iterative deconvolution on the sublibrary defined by these few functional groups. Twofold Monte Carlo error in subset activity was included in the simulations. Results of simulations of iterative deconvolution on the whole library (three rounds with 64 subsets per round) in the presence of 2-fold error are also reported (- - -).

Bogus Coin Deconvolution. The bogus coin strategy has been described by Blake and Litzi-Davis[26] for a library of tetrapeptides and is diagrammed in Table 1 for a library with three positions and 64 functional groups. It begins with synthesis and screening of the entire library as a single mixture. If activity is detected in the library, then the building blocks are divided into three groups and additional subsets are prepared in which the proportion of the first group (α) is decreased, the proportion of the second group (β) is increased, and the proportion of the third group (γ) is unchanged. The effect on activity, decreased, increased, or unchanged, indicates which of the groups was contributing to the activity.

To simulate bogus coin deconvolution, the 64 functional groups were randomly assigned to three groups (α, β, and γ). Three subsets were prepared. In the first subset, the proportion of the functional groups at position 1 was changed. No building blocks from group α were included at position 1; compounds with a functional group from group β at position 1 were included at twice the concentration as compounds with a functional group from group γ at position 1. Similarly, the second and third subsets contained altered proportions of building blocks from groups α, β, and γ at positions 2 and 3, respectively. The change in activity for each of these subsets compared to the parent library (decreased, increased or unchanged) determined which

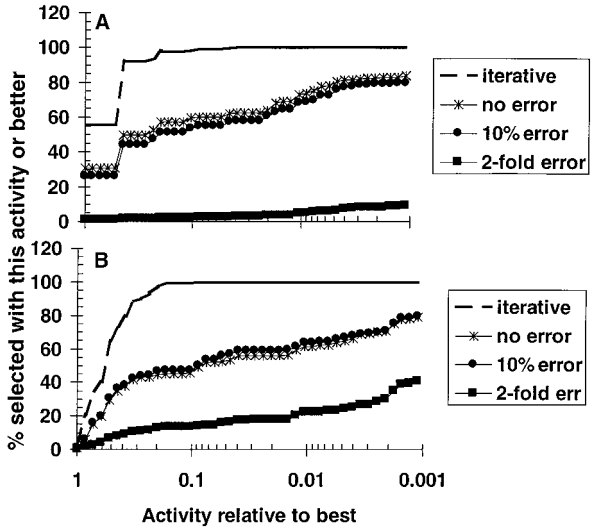

Figure 4. Distribution of activities selected during simulations of bogus coin deconvolution for landscapes A and B. For each of four rounds, building blocks were randomly assigned to three groups and activities were measured for the parent subset and three subsets in which the proportion of the functional groups was altered at one position. Simulations included no experimental error (×), 10% error (●), or 2-fold (■) Monte Carlo error in subset activity. Results of simulations of iterative deconvolution on the whole library (three rounds with 64 subsets per round) in the presence of 2-fold error are also reported (- - -).

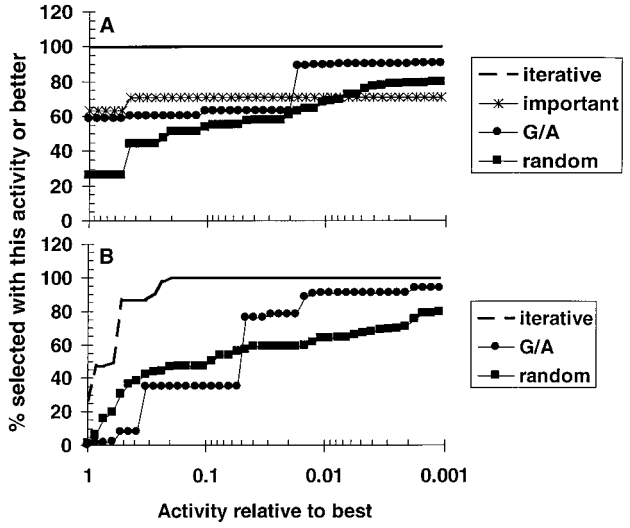

Figure 5. Distribution of activities selected during simulations of bogus coin deconvolution for landscapes A and B. For each of four rounds, building blocks were randomly assigned to three groups (■) or they were assigned using scheme 1 (×) or 2 (●) as described in the text. A Monte Carlo error of 10% in subset activity was included in the simulations. Results of simulations of iterative deconvolution on the whole library (three rounds with 64 subsets per round) in the presence of 10% error are also reported (- - -).

group (α, β, or γ) belonged at that position. The parent for the second round contained only building blocks from the winning first round group at each position. To generate the three subsets for the second round, each winning group was again divided randomly into three groups, proportions were altered as above, and the process was continued until a unique compound was selected.

Results of simulation of bogus coin deconvolution for both landscapes in the presence and absence of experimental error are plotted in Figure 4. Even with no experimental error (× in Figure 4), the bogus coin strategy was much less likely than iterative deconvolution to identify a compound with good activity. In an attempt to discover why the bogus coin strategy was so unsuccessful, simulations that resulted in selection of an inactive compound were examined. Failure of this strategy to select a compound with good activity was due to the presence of more than one active compound in the library. Landscape A contained two compounds with the best activity and 10 more with good activity. Many of the failures occurred when a functional group from one of the best compounds was randomly assigned to group α and the corresponding functional group from the other best compound was assigned to group β. When the concentration of α was dropped to zero and β was doubled, the net change in activity was small so γ was selected, even though it contained neither of the functional groups from the two most active compounds.

When 2-fold experimental error was added to the simulations, the success rate of bogus coin deconvolution was greatly reduced (squares in Figure 4). This was expected because this strategy depends on an increased activity when the concentration of an active molecule in a subset is doubled. In theory, doubling the concentration of an active molecule in a subset will result in a 2-fold increase in activity. In practice, the increase was often less because active compounds occurred in all subsets. Thus, with 2-fold error in subset activity, the increase in activity was not always detected and the incorrect group was often selected. A Monte Carlo error of 10% (circles in Figure 4) allowed detection of smaller changes in activity resulting in a success rate similar to that with no error.

As mentioned above, failures of bogus coin deconvolution were due to building blocks from different active molecules appearing in different groups, especially in the omitted (α) and doubled (β) groups. We hypothesized that the likelihood of success for bogus coin deconvolution would be improved if compounds with similar activities were assigned to the same group. To test this hypothesis, we evaluated two schemes for keeping compounds with similar activities together. The first scheme assigned building blocks from the most active compounds to the α group in every round. This scheme was unrealistic because one does not normally know the composition of the most active compounds until deconvolution is complete. It did, however, provide us with an opportunity to evaluate the effect of keeping active compounds together. When functional groups from the most active compounds were assigned to the same group during bogus coin deconvolution (× in Figure 5A), the likelihood of selecting a compound with good activity improved over bogus coin deconvolution with random assignment to groups (squares in Figure 5A). The second scheme to keep active compounds together was based on knowledge of the functional characteristics of the building blocks and may be more realistic. The free energy contribution of a GU base pair is similar to that of an AU pair in an RNA duplex.[59,60] We simply assumed that building blocks in which G replaced A would contribute similarly to activity of the molecule, and those building blocks were grouped. For example, the building blocks CUA and CUG were assigned to the same group as were AUC and GUC. When this special pooling scheme was applied to

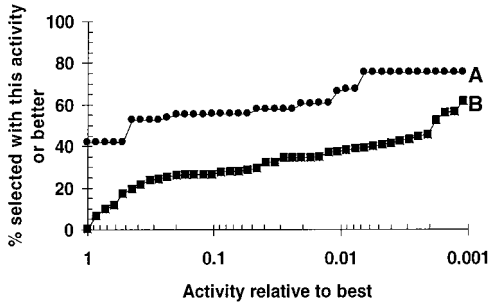

Figure 6. Distribution of activities selected during simulations of deconvolution with 3D orthogonal pooling for landscapes A (●) and B (■). Activities were measured for three sets of 64 subsets. Each subset contained 4096 compounds. The selected compound was the unique compound defined by the intersection of the most active subset from each set of 64. A different random assignment of building blocks to subsets was used for each simulation. No error in subset activity was included in the simulations.

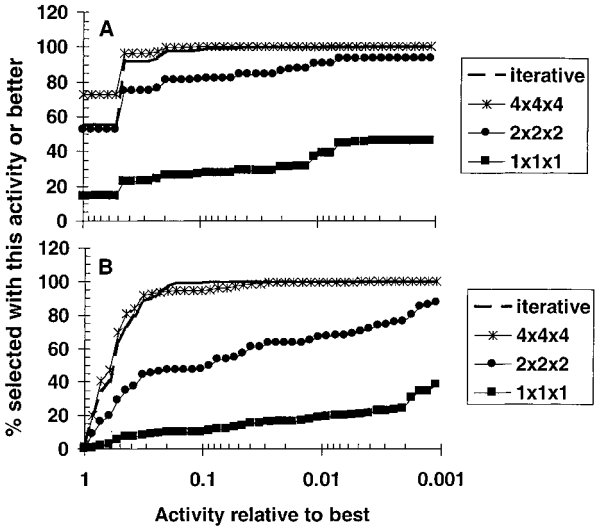

Figure 7. Distribution of activities selected during simulations of deconvolution with 3D orthogonal pooling for landscapes A and B. Activities were measured for three sets of 64 subsets. Each subset contained 4096 compounds. The most active four (×), two (●), or one (■) subset from each set of 64 was used to identify 64 (×), 8 (●) or 1 (■) unique compounds for synthesis and testing. The selected compound was the most active of these unique compounds. A different random, assignment of building blocks to subsets was used for each simulation. Twofold Monte Carlo error in subset activity was included in the simulations. Results of simulations of iterative deconvolution on the whole library (three rounds with 64 subsets per round) in the presence of 2-fold error are also reported (- - -).

landscape A (circles in Figure 5A), the likelihood of selecting a compound with good activity was greater than that for bogus coin deconvolution with random assignment to groups (squares in Figure 5A). Similar improvement was not observed for landscape B (compare circles to squares in Figure 5B). Even with pooling schemes designed to keep active compounds in the same group, the success rate of bogus coin deconvolution was much less than that of iterative deconvolution.

Orthogonal Pooling. Orthogonal deconvolution has been reported by Deprez et al. for a tripeptide library.[54] A three-dimensional variation was described by Feldner.[55] The building blocks are assigned to three orthogonal groups. Three series of subsets are prepared and synthesized, and the selected compound is the single unique compound that appears in the most active subset from each series.

To simulate orthogonal deconvolution for our library, the 64 functional groups were randomly divided into four ***A*** groups, four ***B*** groups, and four ***C*** groups as shown in Table 1. Thus, each functional group belonged to one ***A*** group, one ***B*** group, and one ***C*** group the intersection of one ***A*** group with one ***B*** group and one ***C*** group defined a single functional group. Sixty-four (4^3) ***A*** subsets of 4096 (16^3) compounds were prepared. Each contained one of the four ***A*** groups at each of the three positions. Similarly, 64 ***B*** subsets and 64 ***C*** subsets containing one of the four ***B*** or ***C*** groups at each position were prepared and tested. Because the ***A***, ***B***, and ***C*** groups were orthogonal, the intersection of the most active ***A*** subset with the most active ***B*** subset and the most active ***C*** subset identified a single compound, and that single compound was selected.

Results for deconvolution by 3D orthogonal pooling are plotted in Figure 6. Even in the absence of experimental error, this strategy was unsuccessful. Frequently, for landscape A, the most active ***A*** subset contained one of the most active compounds and the most active ***B*** subset contained the other most active compound. The intersection of these two subsets contained neither of these two most active compounds and no compound with good activity at all. Thus, a compound with poor activity was selected. To correct this problem and improve the success rate of orthogonal pooling, more than one active subset from each group was followed. Following the two most active ***A***, ***B***, and ***C*** subsets required testing of 8 (2^3) unique compounds and following four ***A***, four ***B***, and four ***C*** subsets required testing of 64 (4^3) unique compounds. When 8 or 64 unique compounds were tested, the likelihood of success for orthogonal pooling improved. For landscape A, in the absence of experimental error, following the two most active ***A***, ***B***, and ***C*** subsets was sufficient to identify the most active compound most of the time (data not shown). In the presence of 2-fold experimental error, the likelihood of success for orthogonal pooling approached that of iterative deconvolution when four ***A***, four ***B***, and four ***C*** subsets were followed (Figure 7).

Position Scanning. Position scanning is a strategy which has been used successfully with peptide[29,56,57,61−63] and non-peptide[51,64] libraries. Position scanning on a library with 64 building blocks and three positions is diagrammed in Table 1. A set of mixtures is synthesized for each position. Each subset contains all compounds with a single building block at one position and all building blocks at the other positions. The most active compound is deduced by selecting the functional group from the most active subset at each position.

For this library, 192 subsets (three sets of 64 mixtures) were prepared and tested. Each subset contained 4096 (64^2) compounds. Results of position scanning simulations for landscapes A and B are plotted in Figure 8. For landscape A, with only a few active compounds, position scanning was only slightly less successful than iterative deconvolution; for landscape B the difference between iterative deconvolution and position scanning was greater. We previously simulated position scanning for an oligonucleotide library with four building blocks and nine positions.[47,48] Positions scanning with nine positions and four functional groups was unsuccessful

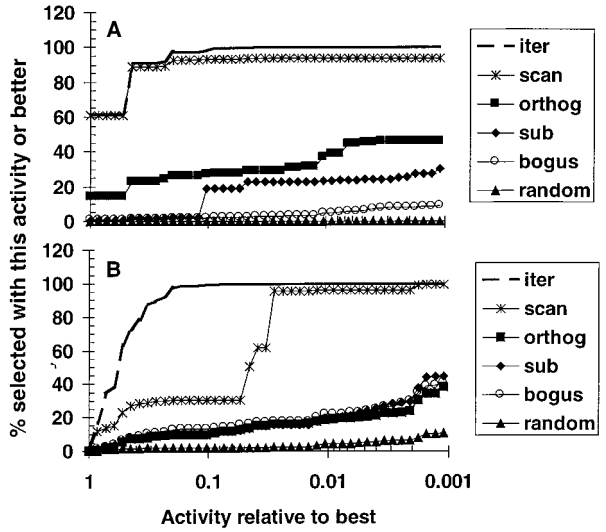

Figure 8. Activity distributions for compounds selected from landscapes A and B during simulations of deconvolution using a variety of deconvolution strategies. Strategies evaluated were iterative deconvolution (- - -), position scanning (×), 3D orthogonal pooling following only the most active subset from each series (■), subtractive deconvolution following only the three most important functional groups (◆), bogus coin deconvolution (○), and random selection of a compound from the library (▲). Twofold Monte Carlo error in subset activity was included in the simulations.

due to multiple pharmacophore alignments with good activity.[48] The detrimental effects of alternate alignments were reduced when position scanning was performed with three sets of 64 subsets rather than nine sets of four subsets.

Simulations of Subset Synthesis. Simulations presented above allow one to evaluate each deconvolution strategy for the likelihood that a molecule with good activity will be identified. When comparing strategies for deconvolution, it is also important to assess the effort required to prepare and test the sample mixes. The synthetic effort required for each of the deconvolution strategies was evaluated for three different types of synthesis diagrammed in Figure 9. The first type of synthesis was the split/mix procedure in which each reaction added a single functional group at a single position to a mixture of compounds (Figure 9B).[21,65,66] This synthetic strategy allows for near quantitative yields. Each reaction is driven to completion with a large excess of functional group. The second type of synthesis was competitive coupling of monomer mixtures,[23,51,57,64] in which many functional groups were simultaneously added at a single position to a mixture of compounds (Figure 9C). The disadvantage of this strategy is that one must rely on well-characterized kinetics to ensure equal concentrations of each compound in a mixture. The advantage, however, is that fewer coupling reactions are required. The third type of synthesis considered was competitive addition of functional groups simultaneously at all positions of a compound (Figure 9D).[22,52,67,68] The advantage of this strategy is that one single coupling reaction produces each mixture and orthogonal protection of reactive sites on the scaffold is not necessary.

For each deconvolution strategy and our library with three positions and 64 building blocks, the number of steps required to prepare subsets for deconvolution was counted. Table 2 lists the number of coupling steps for

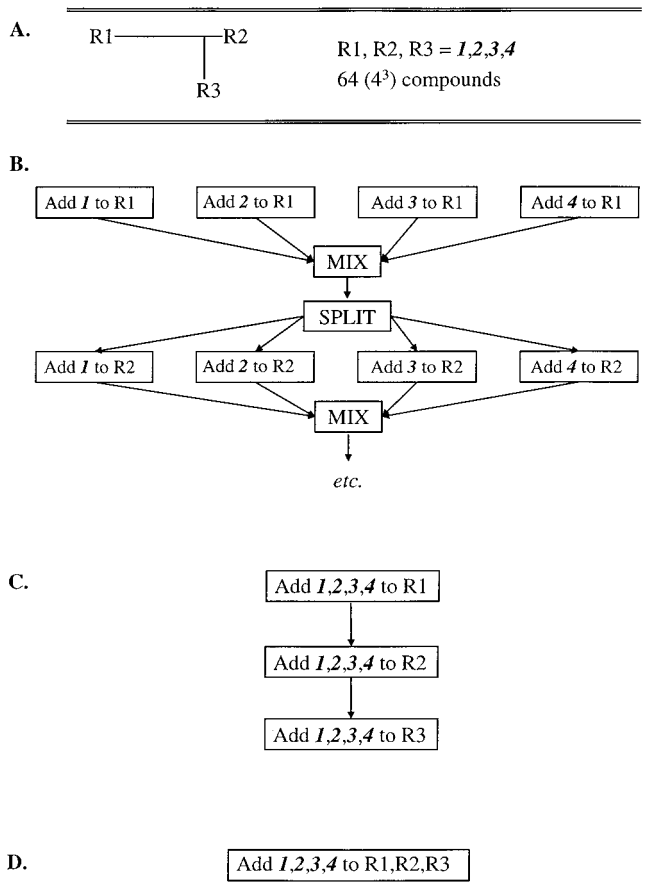

Figure 9. Synthesis methods for a library with three positions and four functional groups. (a) structure of the library, (b) split/mix synthesis, (c) competitive coupling, one position at a time, and (d) competitive coupling, simultaneous addition to all positions.

each deconvolution strategy and each synthesis procedure. Although steps of splitting and/or mixing compounds are not enumerated, they are roughly proportional to the number of couplings, and thus, the values in Table 2 provide a rough estimate of the relative difficulty of the synthetic effort required for each deconvolution procedure. Also listed in Table 2 are the number of testing steps and the number of iterative rounds of synthesis and testing required. Each round requires additional subset synthesis followed by the screen for biological activity and adds to the time required for deconvolution.

Discussion

The success of iterative deconvolution has been demonstrated both experimentally[24−45] and with computer simulation.[47,48] The disadvantage of iterative deconvolution is the iterative synthesis and testing required. The set of first round subsets can be used for any assay. Subsets for subsequent rounds, however, depend on the identity of the most active subset from the first round. Thus, a separate deconvolution must be performed for each biological assay against which the library is screened. Although clever schemes for synthesis of multiple mixtures[21,69,70] and robotic automation[71,72] lessen the synthesis burden of iterative deconvolution, deconvolution strategies with smaller synthesis requirements are attractive.

Several strategies for "streamlined" deconvolution have been described. These strategies reduce the

Table 2. Synthesis and Testing Requirements for Different Deconvolution Procedures Applied to a Library of 262 144 Compounds with Three Positions and 64 Possible Functional Groups at Each Position

deconvolution procedure	no. of coupling steps required for synthesis						no. of samples to be screened		no. of follow-up rounds
	split/mix		competitive coupling to one position at a time		simultaneous competitive coupling to all positions				
	first round	subsequent	first round	subsequent	first round	subsequent	first round	subsequent	
iterative[a]	192	384	66	321	NA[b]	NA[b]	64	128	2
subtractive	12288	39	195	39	65	27 compds[c]	65	27	1
bogus coin	705	369	12	39	NA[b]	NA[b]	4	12	3
3D orthogonal	3904	3	252	3	NA[b]	NA[b]	128	1	0
position scanning	12672	3	387	3	NA[b]	NA[b]	192	1	0

[a] The synthetic effort for iterative deconvolution depends on the deconvolution order. Results are presented for fixing at the last synthesis step in the first round. [b] Strategies labeled NA (not applicable) cannot be used with simultaneous addition because they require a fixed position. Synthesis by simultaneous addition, by definition, requires the same set of functionalities to be added simultaneously to all positions of the molecule. A hybrid strategy can be devised that used simultaneous additions for some positions and split/mix for others.[67] Results for hybrid strategies will lie between those for the constituent parts and are not listed here. [c] The final step in deconvolution requires synthesis and testing of 27 unique compounds. These compounds may not be directly accessible from the unprotected scaffold used for simultaneous addition at all positions.

synthetic effort required for deconvolution either by reducing the total number of samples to be synthesized or by eliminating intermediate rounds of deconvolution. The intermediate rounds are costly because a separate series of syntheses is required for each biological assay and because of a significant time delay between first round screening and final identification of a sample.

Previously we found a model of RNA hybridization and computer simulation of deconvolution useful for evaluation of pooling strategies for iterative deconvolution and position scanning. The model gave us the opportunity to simulate numbers and types of deconvolution that were experimentally inaccessible.[48] The results of the simulations identified key issues and questions which we were then able to evaluate experimentally.[73] The same approach has been used for this work. The model library was modified to contain compounds with three positions and 64 building blocks rather than nine positions and four building blocks as was used previously. The library with fewer positions and more building blocks was used because streamlined strategies evaluated here are most efficient when applied to libraries with many functional groups. In addition, most chemical libraries contain two to four positions for functionalization, so we believed the library with three positions may be more realistic than that with nine positions.

Results in the last columns of Table 2 confirm that the strategies presented are more streamlined than iterative deconvolution. Position scanning and 3D orthogonal pooling put all the synthesis and testing into the first round. Once the first round subsets have been prepared, leads can be identified in several assays with no further synthetic effort. Similarly, subtractive deconvolution determines the exact composition of the selected compound during the first round. The second round is needed only to find the structure. The advantage of bogus coin pooling is the very small number of subsets required. Only 16 screens were necessary to identify a single compound from a library of 262 144! Thus, each of these strategies offers some efficiency over iterative deconvolution.

The magnitude of this advantage in efficiency depends on the synthetic strategy employed. When competitive coupling was used, bogus coin required only 51 coupling steps for complete deconvolution, compared to 200-400 for the other strategies (see Table 2). This synthesis advantage was reduced, however, if coupling kinetics precluded competitive addition and split/mix synthesis was required. In fact, iterative deconvolution used fewer couplings than any other deconvolution strategy when split/mix synthesis was employed (Table 2). Subtractive deconvolution is ideally suited for libraries synthesized by simultaneous addition of functional groups to all positions. No other deconvolution method can be used, and minimal synthetic effort is required. This strategy is limited, however, to libraries with the same sets of functional groups at all positions. Advantages of efficiency in synthesis can also be reduced by requirements for analysis. Split/mix procedures were designed to insure mixtures contained equal concentrations of all components. Thus mixtures prepared by split/mix methods may need less analysis than those prepared by competitive coupling reducing the advantage of strategies like bogus coin or subtractive deconvolution suggested by Table 2.

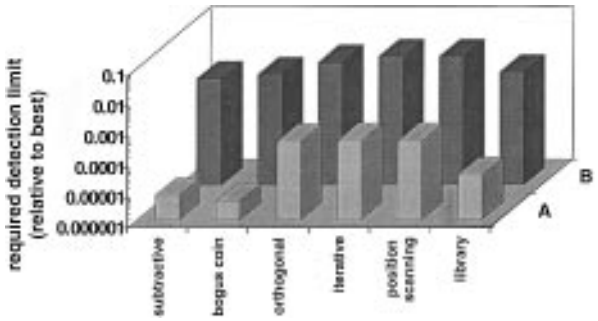

Figure 10. Minimum sensitivities required for each deconvolution strategy. Reported values are the activity of the least active round 1 subset selected during simulations of deconvolution on landscape A or B.

Another consideration when selecting a deconvolution method is the sensitivity and accuracy of the biological screening assay. To make a correct selection, activity of the selected subsets must be detectable and quantitatively reproducible. Figure 10 plots activity of the selected first round subset for each of the strategies evaluated. These values represent the detection limit required for successful deconvolution using each library. For landscape A, subtractive deconvolution and the bogus coin approach required a substantially more sensitive assay than the other three strategies. An assay without sufficient sensitivity may preclude use of subtractive or bogus coin deconvolution strategies.

Probably the most important consideration in selection of a deconvolution strategy is the likelihood of

selecting the most active or one of the most active compounds in the library. Five different deconvolution strategies were simulated on two different libraries. For landscape A, iterative deconvolution and position scanning were much more successful than the other three strategies, especially in the presence of experimental error. For landscape B, position scanning was substantially less successful than iterative deconvolution but still more successful than the other three approaches (see Figure 8). As shown in Figures 3, 5, and 7, modifications to subtractive, bogus coin, and orthogonal deconvolution, such as following up more than one subset, improved the likelihood that these strategies would find a compound with good activity. In no case, however, was the modified strategy more successful than iterative deconvolution. Failures of these streamlined strategies were due to the presence of more than one active compound in the library and the inability to correctly distinguish between subsets with similar activities in the presence of error.

In our simulations, five deconvolution strategies were evaluated for two molecular landscapes using three criteria. The first criterion was the effort required for synthesis and testing. The second was assay sensitivity required for successful deconvolution and the third was the likelihood of identifying a compound with good activity. Although the two landscapes were selected to represent a broad range of real libraries, the relative advantages of each strategy depends on the library tested. A small library or one with fewer functional groups and more positions is more easily deconvoluted using iterative deconvolution or position scanning because strategies such as subtractive deconvolution or bogus coin offer less advantage when there are few functional groups. Conversely, if the library contains one compound that is much more active than any others, the likelihood of success with the more streamlined strategies increases. Our simulations on two relatively large libraries, one with several active compounds (landscape A) and one with very many active compounds (landscape B) demonstrated that iterative deconvolution and position scanning had the fewest sensitivity requirements and the greatest likelihood of success. Depending on the method of synthesis, however, iterative deconvolution can require much more synthetic effort than the other strategies. All these criteria must be considered when selecting the deconvolution method for a combinatorial library.

Experimental Section

Simulations of Pooling and Deconvolution. The targets for landscapes A and B were, respectively, 5′-GUGUGGGCA-3′ and 5′-UGGGCA-3′. Methods for calculation of free energies for library sequences binding to target RNA have been described previously.[47] We define the activity of a molecule as the reciprocal of the concentration needed to bind 50% of the target molecules

$$\text{activity} = 1/\text{IC}_{50} = K_\text{A} = \exp(-\Delta G°_{37}/(RT))$$

where K_A is the association constant for the molecule, $-\Delta G°_{37}$ is the binding free energy, R is the gas constant (0.001 987 (kcal/mol)/K), and T is temperature (310.15 K).

Pooling strategies were simulated by dividing the library into subsets according to the pooling scheme. Activities of each subset were calculated as the average activity of the compounds in the subset.[47,51] This calculation assumes no synergism or antagonism between compounds within a subset. A result of this averaging procedure is that the reciprocal of the activity of a mixture is the *total* concentration of compounds in the mixture needed for 50% binding.

For bogus coin pooling, each simulation included a different random assignment of the building blocks to three pools. The cutoff used for a change in activity was 50%. Activity of the subset was "unchanged" unless it increased to more than 1.5 times that of the parent or dropped below 0.67 that of the parent.

Experimental error in subset activity was included in the simulations by assuming the observed activities had a log normal distribution about the true activity. We assumed log(activity) had a normal distribution with a mean equal to log(true activity) and a standard deviation equal to log(2) for 2-fold error or log(1.1) for 10% error. Observed activities for each subset were generated using standard Monte Carlo techniques.[74] Typically 500 simulations were performed for each set of conditions. Reproducibility of the results of Monte Carlo simulations were assessed by performing two sets of 500 simulations using identical conditions. When the two sets of simulations were compared, percent selected at each activity (see, for example, Figure 3) differed by 2% or less at all activities. Thus, we estimate the inaccuracy in percent selected during our simulations to be 2% or less.

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