# Efficient Pooling Designs for Library Screening

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**Abstract**

We describe efficient methods for screening clone libraries, based on pooling schemes which we call "random $k$-sets designs". In these designs, the pools in which any clone occurs are equally likely to be any possible selection of $k$ from the $v$ pools. The values of $k$ and $v$ can be chosen to optimize desirable properties. Random $k$-sets designs have substantial advantages over alternative pooling schemes: they are efficient, flexible, easy to specify, require fewer pools, and have error-correcting and error-detecting capabilities. In addition, screening can often be achieved in only one pass, thus facilitating automation. For design comparison, we assume a binomial distribution for the number of "positive" clones, with parameters $n$, the number of clones, and $c$, the coverage. We propose the expected number of *resolved positive* clones—clones which are definitely positive based upon the pool assays—as a criterion for the efficiency of a pooling design. We determine the value of $k$ which is optimal, with respect to this criterion, as a function of $v$, $n$ and $c$. We also describe superior $k$-sets designs called $k$-sets packing designs. As an illustration, we discuss a robotically implemented design for a 2.5-fold-coverage, human chromosome 16 YAC library of $n = 1,298$ clones. We also estimate the probability each clone is positive, given the pool-assay data and a model for experimental errors.

# 1  Introduction

Much of the current effort of the Human Genome Project involves the screening of large recombinant DNA libraries in order to isolate clones containing a particular DNA sequence. This screening is important for disease-gene mapping and also for large-scale clone mapping [Olson, *et al.,* 1989]. More generally, efficient screening techniques can facilitate a broad range of basic and applied biological research. Whenever the objective is to find "needles in a haystack", a reliable test indicating whether or not at least one needle

occurs in a specific part of the haystack can greatly facilitate the isolation of the needles [Du and Hwang, 1993], [Dyachov, 1979]. Such tests are called "binary group tests". The most reliable binary group tests routinely used to screen groups of clones for a particular DNA sequence employ either a hybridization-based assay or a PCR-based, STS assay.

Each group of clones is called a "pool", a group test is called a "pool assay", and a collection of pools is called a "pooling design". A convenient specification of a pooling design is a binary clone-*by*-pool incidence matrix: if a clone occurs in a pool the matrix element equals unity. Figure 1a depicts an incidence matrix for a small pooling design.

Any clone containing a DNA sequence of interest is termed a "positive". Pools yielding a positive assay are termed "positive" pools. For the time being we assume there are no experimental errors, in which case each positive pool contains one or more positive clones. After the pools have been assayed, the clones fall into one of four conceptual categories, illustrated in Figure 1b. If a negative clone occurs in at least one pool containing only negative clones then its negative status can be determined from the pool assays and it is called a "resolved negative". The remaining negative clones are called an "unresolved negative". A positive clone is called a "resolved positive" if it occurs in at least one pool with no other positive clone and no unresolved negative clone, otherwise it is called "unresolved positive". Thus,

the status of the resolved positive and resolved negative clones is resolved by the experiment. Although the experimenter will in principle be unable to distinguish the unresolved positive from the unresolved negative clones, as will be seen, it may be useful to separately analyze these two types of clones.

The expected number of unresolved negative clones was previously proposed as a criterion for selecting pooling designs [Barillot, *et al.,* 1991]. However, the library-screening objective can range from the isolation of a small proportion of the positive clones to the isolation of all of the positive clones— hence, we propose the expected number of resolved positive clones as a criterion of design optimality. For example, a cDNA library typically contains multiple copies of many sequences, but the experimenter may want to identify only one of these clones. In Section 3 we calculate both these expectations for several proposed and implemented pooling designs, under the assumption of a binomially-distributed number of positive clones. Confirmatory tests are ordinarily performed on the candidate positive clones. The average number of such tests that would be required to determine the status of all the clones equals the expected number of positive clones plus the expected number of unresolved negative clones. The probability that a design yields a one-pass solution (i.e. the status of all clones is resolved) may also provide a useful criterion for design optimality. This criterion, introduced in [Balding and Torney, in press], will be discussed in a future publication

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[Balding, *et al.*, submitted].

Our focus is on one-stage designs in which all of the pools are assayed in one pass. The use of one-stage designs facilitates automation because a robot can be fully programmed from the outset, irrespective of intermediate results. In addition, one-stage designs require the construction of fewer pools over multiple screenings, since the same pools are used for each screening. Standard pooling designs for large libraries have several stages, and they often involve row-and-column pools, *viz.* Figure 2 and [Amemiya, *et al.*, 1992], [Chumakov, *et al.,* 1992], [Evans and Lewis, 1989], [Green and Olson, 1990], [Sloan, *et al.,* 1994]. The examples of Section 3.2 illustrate the differences between these approaches for a hypothetical tenfold-coverage, human-genome library with 72,000 clones.

We propose "random $k$-sets" designs for one-stage library screening. In these designs, each clone occurs in $k$ pools, and all choices of the $k$ pools are equally likely [Dyachov, 1989]. Random $k$-sets designs are easy to specify for any number of pools, and they are efficient, in terms of the expected number of resolved positives, in comparison to alternative designs. The expected numbers of resolved negative and resolved positive clones are given in Section 2.4. Using these formulas, the optimal choices of $k$ for a given application can be determined. Numerical results are given for a range of clone library sizes and coverages in Section 3. (Although performance will vary accord-

ing to the specific instance of the random design, measures of performance are narrowly distributed between different instances for large designs; thus, expected measures of performance provide a useful guide). We also discuss techniques for constructing superior $k$-sets designs, called "$k$-sets packing" designs, in Section 2.3.

In Section 2.5 we describe techniques for ranking candidate positive clones, given the pooling design, the pool-assay data, and a model for experimental errors. Ranking methods are employed to illustrate the performance of random $k$-sets designs in the presence of pool-assay errors, through computer simulation.

We have previously described efficient techniques for manual construction of pools [McCormick, *et al.,* 1993b]. The designs proposed here are intended for implementation using robots. We employed the Packard 204 multiPROBE robot to pool a 1,298-clone, 2.5-fold-coverage, chromosome-specific YAC library, implementing a four-sets design on 47 pools.

## 1.1 Notation

The number of clones in the library is denoted by $n$. The number of pools in a pooling design is denoted by $v$, and the number of pools in which a clone occurs is denoted by $k$. The number of clones in a clone library which cover any particular location of the cloned region is assumed to be binomially-

distributed with expectation $c$ (called the coverage). The standard notation $\Pr(A|Z)$ is used for the conditional probability of event $A$, given event $Z$. We use the notation $\binom{a}{b}$ for the binomial coefficient $a!/(b!(a-b)!)$, and we introduce $B$ for the binomial probabilities

$$B(a, b, t) = \binom{a}{b} t^b (1 - t)^{a-b}. \tag{1}$$

# 2    Methods

We developed several computer programs as part of our pooling methodology, described below. Copies of these programs are freely available from the author for correspondence.

## 2.1    Pooling and Screening Methods for a 1,298-Clone YAC Library

A complete-digest, Cla I library of 1,298 clones was generated from human chromosome 16 [McCormick, *et al.,* 1993a]. Based on an average cloned-DNA size of 200 kilobasepairs and a chromosome size of 98 megabasepairs [Morton, 1991], the coverage of this library was conservatively estimated at $c = 2.5$. A four-sets packing design on $v = 47$ pools was used; see Section 2.3 for its characterization. Thus, each clone occurred in four of the pools. (With this value of $v$, two sets of pools and controls can be put on one 96-well dish). Deep-96-well mitrotiter dishes were filled with YPD medium using the

Dynatech plate-filler; each well contained 1.9 ml of medium. These dishes were incubated at 30° C for 48 hours to check for contamination prior to inoculation. Then, 14 uncontaminated dishes were inoculated by hand stamping from a selective-medium copy of the library, lacking uracil and tryptophan. The inoculated dishes were incubated at 30° C for 72 hours, achieving stationary growth. We subsequently used a Packard multiPROBE 204 robot to construct the pools. The robot cycled through the following procedure, using all four liquid-handling tips: suspend the yeast in a well, aspirate 0.8 ml, dispense 0.4 ml in each of two specified pool tubes, and sterilize and clean the tips. (A larger syringe would have allowed us to aspirate the full well volume and would have reduced the overall time for pooling). The sterilizing and tip cleaning began with a five-second rinsing with five ml of distilled water. Next, one ml of 0.525% sodium hypochlorite solution was aspirated and discharged. Then, the tips were rinsed with five ml of sterile water, and the tips were placed in individual cups to clean the outside as well. (These rinsing and sterilizing steps account for about half of the run time. They were sufficient to prevent cross-contamination on the basis of a yeast-growth assay). Final pool volumes of approximately 46 ml were collected in 50 ml centrifuge tubes. Pools were maintained at room temperature during the pooling run, which took approximately 12 hours. Human intervention was required hourly to replace the dishes from the arrayed library.

Agarose gel plugs containing yeast cells were prepared from each pool. The plugs were treated with zymolase to make spheroplasts, and they were treated with ESP (500 mM EDTS, 1% sarcosyl, and 1 mg/ml proteinase K), to digest away proteins. The plugs were dialyzed extensively against TE. The DNA was purified from the agarose using the Geneclean kit. GeneAmp PCR reagent kits were used for PCR screening of the pool DNA. (50 ng of pool DNA and 2.5 units of Taq polymerase and 1.5 mM $MgCl_2$ were the noteworthy features of the 50 $\mu$l reaction volume).

## 2.2    Methods for Programming Robots

Two software systems, written in 'C', were developed to facilitate use of the Packard multiPROBE 204 robot. These systems were both used in the implementation described in Section 2.1.

Our "scheduling" software can readily be adapted for an arbitrary robot. This system enables the implementation of arbitrary pooling designs, *viz.* Figure 1a, creating a list of volumes for well-to-pool transfers. In addition, it lists the location, size, and quantity of "source" and "destination" plastic-ware. The scheduling system can be used to coordinate the simultaneous use of multiple robots engaged in constructing a set of pools from one library. The output of the scheduling system is used by a robot instruction "interpreter", described next.

The interpreter program reads the scheduling input file and generates a complete list of commands for the Packard robot. The basic actions of the robot are aspirating, dispensing, and rinsing. Each of these actions requires many steps and hence many robot commands. When the scheduling program provides the necessary parameters, the interpreter program generates these commands, which can then be implemented using HAM, the Packard-robot software controlling the monitor and the robot.

For example, the scheduling program supplies the well number and the volume of aspiration, and the interpreter program generates the appropriate list of robot commands. The interpreter determines whether multiple tips can aspirate or dispense simultaneously. It also records all commands and actions and enables termination of the run at any time.

## 2.3  $k$-Sets Design Generation—$k$-Sets Packings

A four-sets design on 47 pools was constructed using a pseudo-random number generator. The design was based on a random four-sets design; however, a prospective four-set was discarded if it had more than two elements in common with another four-set already in the design. In addition, we ensured that each pool contained approximately the same number of clones (109 to 111). In Section 3.3 our performance criterion is used to compare the design we generated using these constraints to the average perfomance of a random

four-sets design.

Designs with a bound on the size of the intersection between two $k$-sets are referred to as "$k$-sets packing" designs. In general, it is unsatisfactory to have two identical $k$-sets in a random $k$-sets design: if one of them is positive it would be impossible to establish the status of the other. Similarly, it is undesirable for two clones to coincide in a large number of pools (i.e. for the sets of pools each occurs in to be similar). Bounding the intersection ensures that a negative clone cannot be unresolved due to a small number of positive clones.

## 2.4   Mathematical Methods

In this section we give expressions for the measures of design performance—expected numbers of resolved negative and resolved positive clones—for random $k$-sets designs. We assume that each clone is positive with probability $c/n$, independently of the other clones, which yields a binomial distribution for the number of positive clones. Although this may not be strictly realistic, it provides a reasonable approximation for the purposes of design comparison [Clarke and Carbon, 1976], [Lander and Waterman, 1988]. The expressions we derive are readily modified to accommodate an arbitrary probability that there are $p$ positive clones, provided that all $p$-subsets of the clones are equally likely to be the positive clones. Such a modification would be useful when

screening cDNA libraries, for example. Here we assume no experimental error. Pool-assay errors are treated in Section 2.5.

Recall the definition of random $k$-sets designs: each clone occurs in precisely $k$ of the $v$ pools, and each of the possible $\binom{v}{k}$ subsets of $k$ pools has equal probability to be the pools in which a particular clone will occur.

We begin by formulating $\bar{N}$, the expected number of unresolved negative clones—negative clones occurring only in positive pools; thus, $n-c-\bar{N}$ is the expected number of resolved negative clones.

$$\bar{N} = \sum_{p=0}^{n} (n-p)B(n,p,c/n)\sum_{i=0}^{k}\binom{k}{i}(-1)^i z_i^p, \tag{2}$$

where

$$z_i = \binom{v-i}{k}\binom{v}{k}^{-1},$$

and $B$ denotes the binomial probabilities (1). Interchanging the order of summation in (2) and discarding some terms of order $n^{-1}$ yields

$$\bar{N} \approx (n-c)\sum_{i=0}^{k}\binom{k}{i}(-1)^i \mathrm{e}^{-c(1-z_i)}. \tag{3}$$

Insight into the behavior of (2) can be obtained from the "independent pools" approximation. The inner summation is an inclusion-exclusion formula for the probability, $K_k^{(p)}$, that all of the $k$ pools in which a negative clone occurs contain at least one positive clone, given that there are $p$ positive clones. The probability that a given pool is negative is $(1-k/v)^p$, and the independent

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pools approximation uses

$$K_k^{(p)} \approx \left(1 - \left(1 - \frac{k}{v}\right)^p\right)^k.$$  (4)

The approximation (4) would be exact if pool outcomes were independent. Because pool outcomes are negatively correlated, the independent pools approximation gives an upper bound for $K_k^{(p)}$ and hence $\bar{N}$. The bound is tight for $p$ not too small and improves as $v$ increases. A recursive expression for $K_k^{(p)}$ is given in Appendix A (Equation (8)).

We now formulate $\bar{P}$, the expected number of unresolved positive clones; thus, $c - \bar{P}$ is the expected number of resolved positive clones. The details of the calculation are given in Appendix A. Recall that, in the absence of pool-assay errors, a positive clone is unresolved if it occurs only in pools containing either another positive clone or an unresolved negative clone.

After substitution and performing the summations over $p$ and $u$, Equation (17) becomes

$$\bar{P} = c\left(1 - \frac{c}{n}\right)^{n-1}\alpha^{(1)} + c\sum_{x=k}^{v}\sum_{y=x}^{v}\binom{v}{x}\binom{x}{y-k}\binom{v-x}{y-x}\binom{v}{k}^{-1}Q_{x,y}$$  (5)

in which

$$Q_{x,y} = \sum_{i=v-x}^{v-k}\binom{x}{v-i}(-1)^{i-v+x}\sum_{j=0}^{y-x}\binom{y-x}{j}(-1)^j R_j,$$

$$R_j = \left(\left(\frac{z_i c}{n}\right) + \left(1 - \frac{c}{n}\right)(1 - \beta(1-\xi_j))\right)^{n-1} - \left(\left(1 - \frac{c}{n}\right)(1-\beta(1-\xi_j))\right)^{n-1},$$

and $\alpha^{(1)}$, $\beta$ and $\xi_j$ are defined at, respectively, (9), (15) and (16), and $z_i$ is defined below Equation (2).

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It is seen that (5) can be evaluated with $\mathcal{O}(v^4)$ summands. To evaluate (2) and (5) for large $v$, we used MAPLE V's extended-floating-point precision capabilities [Char, *et al.,* 1992]. In addition, we wrote our own extended-precision FORTRAN routines, which typically required about one percent as much c.p.u. time to obtain an equally accurate result. The evaluation can also be done without using extended precision: the alternating signs can be eliminated by using the recursive Equations (8) and (13). In this case the summations over $p$ and $u$ must be approximated numerically.

We have also derived approximations for $\bar{P}$ that are more easily evaluated. If the number of positive pools were fixed at the expected number, $\omega$:

$$\omega = v \left( 1 - \left( 1 - \frac{k}{v} \right)^p \right),$$

then the probability a negative clone is unresolved negative is $\mu$, given by

$$\mu = \binom{\omega}{k} \binom{v}{k}^{-1},$$

Averaging over the number of unresolved negatives, with a Poisson approximation to the binomial distribution, and using the "independent pools" approximation gives

$$\bar{P} \approx \sum_{p=1}^{n} pB(n,p,c/n) \left( 1 - \left( 1 - \frac{k}{v} \right)^{p-1} e^{-\mu(n-p)k/\omega} \right)^k,$$

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A more accurate approximate formula is obtained if pools are not assumed to be independent:

$$\bar{P} \approx \sum_{p=1}^{n} pB(n, p, c/n) \sum_{i=0}^{k} \binom{k}{i} (-1)^i z_i^{p-1} e^{-\mu(n-p)(1-\zeta_i)}, \qquad (6)$$

in which

$$\zeta_i = \binom{\omega-i}{k} \binom{\omega}{k}^{-1}.$$

Equation (6) was used to generate Figures 3 and 4; standard "double precision" floating-point operations were sufficient. We found, over the domain of Figure 3a, the largest difference from the exact minimum number of pools, from Equation (5), was about six percent of the number of pools. Also, the approximate result typically differs from the exact result by no more than one pool.

## 2.5 Methods for Ranking Clones

Although we focus on ranking the clones according to the probability that each is positive—given the pool-assay data and a model for the experimental errors—ranking sets of candidate positive clones can also be a desirable objective. In the absence of errors, it is usually possible to identify many resolved positive clones, but it can also be important to rank the remaining clones according to the probability that they are positive. These objectives

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can be achieved in the following framework.

Bayes' rule can be used to estimate the probability that an individual clone is positive, given the vector of pool-assay outcomes, denoted $V$, and a model for the experimental errors. In this context, Bayes' rule can be written as follows:

$$\Pr(I_i^+|V) = \left(1 + \frac{\Pr(V \& I_i^-)}{\Pr(V \& I_i^+)}\right)^{-1}, \tag{7}$$

in which $I_i^+$ (respectively $I_i^-$) denotes the event that clone $i$ is positive (respectively negative). To evaluate exactly the ratio on the RHS of (7) would require a calculation for each possible subset of the clones, taken to be $P$, the set of positive clones. Because $2^n$ is typically very large, this is not feasible, and, therefore, we sampled subsets to estimate the ratio. These estimates were then used to rank the clones. In our preliminary studies of this approach, we selected subsets in which each clone has an equal probability of appearing. For the example described in Section 3 with 33,000 clones, we sampled approximately 330,000 subsets for each clone, both for the numerator and denominator of the ratio. To reduce both the noise of and the computational work required for the sampling, individual clones were added to and removed from these two groups of subsets—one used for the numerator and the other for the denominator. However, a much more efficient approach is Gibbs sampling of $P$, and the Hastings-Metropolis al-

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gorithm have also been implemented, facilitating the estimation of Equation (7) [Bernardo and Smith, 1994], [Bruno, *et al.,* 1994].

The model for experimental errors enters into the evaluation of the RHS of Equation (7). For each set $P$ of selected clones, taken to be the positive clones, we find the union of the pools in which any of these clones occur. Call this set $\Upsilon$. In the pool-assay data, $V$, let $v_{-|+}$ be the number of negative pools in $\Upsilon$, and let $v_{+|-}$ be the number of positive pools not in $\Upsilon$. Furthermore, a two parameter error model is adopted, with the error-rate parameters for false-positive and false-negative pool assays equal $\lambda_{+|-}$ and $\lambda_{-|+}$, respectively. These errors are taken to be independent; thus, the probabilities on the right-hand side of Equation (7) are evaluated using

$$\Pr(V|P) = \lambda_{+|-}^{v_{+|-}}(1 - \lambda_{+|-})^{v-|\Upsilon|-v_{+|-}}\lambda_{-|+}^{v_{-|+}}(1 - \lambda_{-|+})^{|\Upsilon|-v_{-|+}},$$

in which $|\Upsilon|$ denotes the cardinality of $\Upsilon$.

# 3   Results

## 3.1   Random $k$-sets

In this Section we employ our criterion—the expected number of resolved positive clones—to select optimum parameters for random $k$-sets designs. For libraries of varying number of clones, $n$, and coverage, $c$, the minimum number of pools required for random $k$-sets designs to achieve an expected

number of resolved positive clones equal to 0.5 $c$ and 0.95 $c$ is illustrated in Figures 3a and 4a, respectively. Figures 3b and 4b depict the corresponding optimum values of $k$—maximizing the expected number of resolved positive clones for the number of pools plotted in Figure 3a and 4a. The latter plots depict a number of values of $c$ at which the optimum value of $k$ changes abruptly. At these values of $c$, the product of $c$ with the probability that there are $j$ or fewer positives is comparable to the desired expected number of resolved positive clones. As $c$ increases through each of these "transitions", the optimal pooling design resolves the cases with one additional positive clone, in order to achieve the desired expected number of resolved positive clones. These transitions are most pronounced for small values of $c$—for example, where the optimal designs go from resolving only one positive to resolving two positives, over a small range of $c$ centered on 0.69. These transitions influence the minimum number of pools, contributing to the irregularity of the contours in Figure 3a and Figure 4a in this region. For most values of the variables $c$ and $n$, the dependence of the expected number of resolved positive clones upon $k$ is not pronounced in the vicinity of the optimum value of $k$. Thus, the optimum $k$ provides a rough guideline for efficient pooling designs, as can be seen in the following examples.

Given a goal of five resolved positive clones, on the average, a tenfold-coverage, human-genome library of 33,000 clones [Cohen, *et al.*, 1993] could

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be accommodated on 170 pools (Figure 3a). The optimum value of $k$ is ten, which would result in an average of 1,941 clones per pool. The expected number of unresolved negative clones is approximately 44. If, instead, the goal were to have an an average of 9.5 resolved positive clones, then 253 pools would be required (Figure 4a). The optimum value of $k$ would also be ten and there would be an average of approximately 1,304 clones per pool. The expected number of unresolved negative clones is approximately 2.8. Therefore, on the average, 3.3 confirmatory tests would be required to resolve the status of every clone.

It may also be desirable to bound the average number of clones in a pool in order to avoid high pool-assay error rates. This can be achieved by constraining $k$, the number of pools containing any one clone. Suppose it were desirable to have fewer clones per pool, say, approximately 1,000, and also to achieve an average of five resolved positive clones for the library described in the previous paragraph. Then, from Equation (5), 191 pools would be required with $k = 6$, and the average number of clones in a pool would be approximately 1037.

Experimental error necessitates more pools to achieve comparable results. We performed some computer-simulation experiments for a tenfold-coverage library of 33,000 clones with a false-negative error rate of 0.1 and a false-positive error rate of 0.01—rates consistent with our preliminary experiments

on pools containing approximately 110 clones, described in Section 3.3. We used a random ten-sets design, and an arbitrary set of ten clones was selected to be the positive clones. The result of the Bayes' ranking, described in Section 2.5, was that five of these ten clones were ranked in the top ten. Thus, it is feasible to identify the positive clones—even with appreciable experimental error.

## 3.2   Comparison with other designs

To facilitate comparison, we consider a tenfold-coverage, human-genome library of 72,000 clones for which the following row-and-column pooling design has been implemented [Chumakov, *et al.,* 1992]. The library is partitioned into 94 lots, all but one containing eight 96-well dishes and the remaining one with six. The rows and columns of eight microtiter dishes are combined to construct 20 pools. In addition, eight more pools, each containing all of the clones from one dish, are constructed. Thus, the total number of pools is $28 \times 94 = 2,632$. For this design, a lot contains a resolved positive clone only if it contains only one positive clone. Therefore the expected number of resolved positive clones is approximately $10e^{-10/94} \doteq 9.0$, and the expected number of unresolved negative clones is approximately 3.3 [Barillot, *et al.,* 1991]. The following designs have been proposed for screening the same library, using approximately one-tenth as many pools.

[Barillot, *et al.,* 1991] proposed novel pooling designs for a library with the aforementioned parameters. One of these designs assigned each clone to a lattice point in a cubic, integer lattice with 43 points on each axis. Each pool would contain all of the clones with each coordinate: thus $3 \times 43 = 129$ pools would result. To gain more information about the positive clones, a linear transformation was used to obtain a new configuration of the clones on the lattice points and, thus, another set of 129 pools, specified by the new coordinates. Thus, each cubic configuration yields three groups of 43 pools with the property that each clone occurs in one pool from each group. In general, this property of the Barillot *et al.* designs distinguishes them from the designs we propose.

Computer simulation was used for the 258-pool cubic design to determine that the expected number of resolved positive clones is nearly 8.8. Also, the expected number of unresolved negative clones is approximately equal 13.3. (We assumed that 72,000 of the lattice sites were initially selected uniformly at random for the first configuration).

For the same library, using the results of Section 2.4, the optimum choice of $k$ for a random $k$-sets design on 258 pools is 11. In this case the expected number of resolved positive clones is 9.1 and the expected number of unresolved negative clones is 5.3. However, if we choose $k = 6$ to achieve pools comparable in size with the "cubic" design, then the expected number of re-

solved positive clones is 7.3, and the expected number of unresolved negative clones is 12.6. The $k$-sets packing designs, described in Section 2.3, would yield larger expected numbers of resolved positive clones.

## 3.3  Screening results

The following theoretical and computational results bear on the predicted performance of our four-sets packing design. As above, this library is assumed to have a binomial number of positive clones with expectation 2.5. Computer simulation was used to estimate the expected number of resolved positive clones for the four-sets packing design at approximately 1.47—versus 1.36, from (5), for random four-sets designs. Similarly, the expected number of unresolved negative clones for the four-sets packing design is 3.98—versus 4.68, from (3), for a random four-sets design. Thus, to identify all the positives would require confirmatory testing of 6.48 clones, on the average.

The four-sets packing design was implemented for the 1298-clone, human chromosome 16, YAC library. We observed numerous false negative and also false positive pool assays, precluding the identification of clones on the basis of being either resolved positive or unresolved positive or negative. Twenty-two STSs were screened against the pools to achieve closure of the chromosome 16 framework map. We ranked the clones according to the probability of being positive, based on the pooling results, as described in Section

2.5. We set the error rates $\lambda_{+|-}$ and $\lambda_{-|+}$ equal 0.14 and 0.06, respectively. These rates are primarily based on comparing the frequency of positive pools with that predicted using the coverage. After performing confirmatory testing on the top eleven clones (on average), an average of 1.8 positive clones were identified. Six of the twenty-two STSs yielded no positive clones.

# 4 Discussion

Improved methods for screening clone libraries will allow more efficient use of currently available biological resources. We propose using $k$-sets designs for unique-sequence screening of large clone libraries. These designs are efficient, flexible, easy to specify and can allow screening in one-pass, thus minimizing human intervention. When possible, we also advocate using the $k$-sets packing designs, discussed in Section 2.3, which can yield further substantial gains in efficiency. The automated implementation of a four-sets packing design for a YAC library containing 1,298 clones, over a period of 12 hours, demonstrates the utility of commercially-available robots.

In addition to the expected number of unresolved negative clones, proposed by Barillot *et al.,* we propose a new design performance criterion: the expected number of resolved positive clones. Optimizing a pooling design according to either criterion will be sensitive to the upper tail of the distribution for the number of positive clones, which we have assumed to be

binomial for design comparison. For some applications, the determination of average behavior might not be adequate; thus, it may be useful, for example, to estimate the probability that $j$ positives are resolved positive for a pooling design, given that there are $p$ positives. In any case, pooling designs which have a high probability of achieving the screening objective are useful, even if there is no guaranteed performance for a particular STS. On the other hand, to guarantee that the status of all of the clones is resolved would require many more pools than given in Figure 4a.

We computed the smallest number of pools required for a random $k$-sets design to achieve a given expected number of resolved positive clones. Figures 3a and 4a depict the number of pools required for libraries of coverage $c$ with $n$ clones, and Figures 3b and 4b depict the optimum values of $k$. When implementing any pooling design, it could be informative to compare the total numbers of pools and the number of pools containing each clone to those plotted in Figures 3 and 4 because, after optimization, these designs come close to achieving optimal performance. One could use the Bayes ranking in several ways to find the best parameters for a random $k$-sets design in the presence of experimental error. For the time being, we selected a design from Figure 3 and simulated its performance in the presence of a realistic level of experimental error.

Data on pool-assay errors is clearly important in the design of pooling

experiments. Pools with a smaller proportion and concentration of target DNA might be less likely to yield the correct PCR product. Also, some primers could be more prone to fail to produce products than others. False-positive results could result from cross-contamination. It is not clear that our simple model of experimental error—with independent probabilities for false-negative and for false-positive pool assays—is adequate. We will assess the fidelity of our pooling and screening experiments while assaying the four-sets packing pools for our physical map of human chromosome 16.

Experimental error blurs the distinctions between clones in our four categories and motivates both the consideration of error-correcting pooling designs [Balding and Torney, in press] and effective ranking of the candidate-positive clones [Bruno, *et al.,* 1994]. In general, an automated ranking algorithm will also propose a set of clones for confirmatory testing. It could propose several candidate clones and use the results from these confirmatory tests when proposing further candidate clones. One criterion for evaluating ranking algorithms could be the average number of proposed clones required to identify $i$ of the positive clones or all of them, in the event that there are fewer than $i$ positives. Or it could be the sum of the probabilities of being positive assigned to positive clones in computer simulations. One can optimize the design of the pooling experiments, given the selection of a ranking technique. In addition, the relative costs of confirmatory testing and of

missing positive clones could be employed as part of design optimization.

It may often be possible to generate designs with better performance than random $k$-sets designs. Such designs could include $k$-sets packings, in which there is a bound, $t$, on the number of pools in which two clones coincide. By varying the value of $t$, $k$-sets packing designs can provide an extremely powerful and flexible approach to library screening. When $t = k$, we have random $k$-sets designs which are efficient and very easy to construct. As $t$ is decreased, we gain even greater efficiency at the cost of additional computations in design generation. At the other extreme we have maximum-size $k$-sets packings which are, we believe, maximally efficient but often difficult to construct. Further, we have shown [Balding and Torney, in press] that packings which achieve the maximum possible size are best possible in some cases, in terms of maximizing the probability of a one-pass solution. Such designs can also be optimal subject to guaranteed error-detection requirements. The magnitude of the improvement one can achieve by constraining the intersections is exemplified by the predicted performance of the cubic row-column pooling design [Barillot, *et al.,* 1991]. Thus, we are exploring combinatoric optimization techniques for the construction of $k$-sets packing designs. Some preliminary methods were used for optimizing the pooling design for the Cla I, YAC library described in Section 2.3. We plan to improve these by applying techniques such as the method of conditional expectations

to de-randomize generation of the designs [Alon and Spencer, 1992].

In summary, although our preliminary results should prove useful, much exploratory work remains—based upon a better understanding of the prevalent experimental errors—to achieve superior pooling designs, further reducing the labor and increasing the efficiency of large-scale, library-screening experiments. Furthermore, pooling the clones from a pre-existing map will involve new challenges because of the prior knowledge about the joint probability distribution for positive clones.

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**Figure Legends**

Figure 1a

Title: "Incidence Matrix for a Pooling Design"

A binary incidence matrix for a particular pooling scheme with six pools and five clones. A clone occurs in a pool if the corresponding matrix element equals unity and does not occur in a pool if the corresponding matrix element equals zero. Pool number 1 contains clones number 1, 3, and 4, pool number 4 contains only clone number 5, et cetera.

Figure 1b

Title: "Terminology and Example of Categorization"

The binary incidence matrix of Figure 1a is depicted, but now clones 1 and 2 are taken to be positive clones and clones 3, 4 and 5 are taken to be

negative. The '1's for the positive clones are replaced by '+'s, the '1's for the negative clones are replaced by '-'s, and the '0's are omitted. In the absence of experimental error, all the pools containing either of the positive clones would be positive; thus, only pool 4 would be negative, as depicted above the horizontal line opposite 'Pool Assay'. This hypothetical pooling experiment would resolve the status of clones 1 and 5, and the remaining clones could be either positive or negative. Clone 5 is a negative clone occurring in pool 4, which contains no positive clone, thus it is a resolved negative clone. Clones 3 and 4 are negative clones occurring only in pools containing positive clones; thus, they are both unresolved negative clones. Because clone 1 is a positive clone occurring only in pool 6, which does not contain either another positive clone or an unresolved negative clone, it is a resolved positive clone. Clone 2 is a positive clone occurring only in pools containing either other positive clones or unresolved negative clones; thus, it is unresolved positive.

Figure 2

Title: "Row-and-Column Pools of the Clones in a 96-well dish"

A row and column design with 96 clones and 20 pools. Two clones are positive (row 3, column 3 and row 5, column 8) and hence the four indicated pools would be positive, in the absence of experimental error. There are two unresolved negative clones (row 5, column 3 and row 3, column 8) and 92 resolved negative clones. The two positive clones are unresolved positive.

Figure 3a

Title for Figure 3a: "Minimum Number of Pools with the Expected Number of Resolved Positives Equal $0.5c$"

The $x$ axis is $n$, the number of clones to be pooled; $1000 \leq n \leq 100,000$, and the $y$ axis is $c$, the coverage parameter; $1/4 \leq c \leq 16$. Both axes have a logarithmic scale, and the tics facing the inside of the plot are at integer values while those on the outside are at values corresponding to half integers. The smallest value of $v$ such that a random $k$-sets design can achieve the target expected number of $0.5c$ resolved positive clones is depicted. Equation (6) was used to generate the data for this plot.

Figure 3b

Title for Figure 3b: "Optimum $k$, Expected Number of Resolved Positives Equal $0.5c$"

The $x$ axis is $n$, the number of clones to be pooled; $1000 \leq n \leq 100,000$, and the $y$ axis is $c$, the coverage parameter; $1/4 \leq c \leq 16$. Both axes have a logarithmic scale, and the tics facing the inside of the plot are at integer values while those on the outside are at values corresponding to half-integers. The values of $k$ achieving the maximum expected number of resolved positive clones are depicted, with the value of $v$ depicted in Figure 3a. These expected values slightly exceed $0.5c$. For this domain of $c$ and $n$, optimal values of $k$ fall between 6 and 12. Equation (6) was used to generate the data for this

plot.

Figure 4a

Title for Figure 4a: "Minimum Number of Pools with the Expected Number of Resolved Positives Equal $0.95c$"

The $x$ axis is $n$, the number of clones to be pooled; $1000 \leq n \leq 100,000$, and the $y$ axis is $c$, the coverage parameter; $1/4 \leq c \leq 16$. Both axes have a logarithmic scale, and the tics facing the inside of the plot are at integer values while those on the outside are at values corresponding to half-integers. The smallest value of $v$ such that a random $k$-sets design can achieve the target expected number of $0.95c$ resolved positive clones is depicted. Equation (6) was used to generate the data for this plot.

Figure 4b

Title for Figure 4b: "Optimum $k$; Expected Number of Resolved Positive Clones Equal to $0.95c$"

The $x$ axis is $n$, the number of clones to be pooled; $1000 \leq n \leq 100,000$, and the $y$ axis is $c$, the coverage parameter; $1/4 \leq c \leq 16$. Both axes have a logarithmic scale, and the tics facing the inside of the plot are at integer values while those on the outside are at values corresponding to half-integers. The values of $k$ achieving the maximum expected number of resolved positive clones are depicted, with the value of $v$ depicted in Figure 4a. These expected values slightly exceed $0.95c$. For this domain of $c$ and $n$, optimal values of $k$

fall between 7 and 12. Equation (6) was used to generate the data for this plot.

# A   Appendix: Derivations

The inner summation in (2), which equals the probability, $K_k^{(p)}$, that $k$ specified pools contain one or more positive clones when there are exactly $p$ positive clones, can be evaluated via the recursive formula

$$K_j^{(p)} = \sum_{i=0}^{j} \binom{j}{i}\binom{v-j}{k-i}\binom{v}{k}^{-1} K_{j-i}^{(p-1)},\tag{8}$$

for $j \leq k$, where $K_{j-i}^{(0)} = 1$ if $j = i$, otherwise $K_{j-i}^{(0)} = 0$. Equation (8) is numerically advantageous because it involves no subtractions.

We turn now to the expected number of unresolved positive clones, $\bar{P}$. Let $\alpha^{(p)}$ denote the probability that a selected positive clone is unresolved, given that there are exactly $p$ positive clones. When $p = 1$, the positive clone will be unresolved only if a negative clone occurs in precisely the same $k$ pools, so that

$$\alpha^{(1)} = 1 - \left(1 - \binom{v}{k}^{-1}\right)^{n-1}.\tag{9}$$

(In practice, a $k$-sets design would usually be generated so that no two clones occupy precisely the same pools and hence $\alpha^{(1)} = 0$, as described in Section 2.3. Here, however, it is convenient to consider standard random $k$-sets designs.)

For $p \geq 2$, we determine $\alpha^{(p)}$ by conditioning on the values of three random variables $U$, $X$, and $Y$, where $U$ is the number of unresolved negative clones, $X$ is the number of pools which would be positive if the selected positive clone were removed, and $Y$ is the number of positive pools. Thus, $Y{-}X$ is the number of pools containing the selected positive clone but no other positive clone. The selected positive clone will be unresolved positive either if $Y{-}X$ is zero or if each of these $Y{-}X$ pools contains at least one unresolved negative clone. Thus,

$$\alpha^{(p)} = \sum_{u=0}^{n-p} \sum_{x=k}^{v} \sum_{y=x}^{v} \Pr(A|U{=}u, X{=}x, Y{=}y)\Pr(U{=}u, X{=}x, Y{=}y), \qquad (10)$$

where $A$ denotes the event that every pool in which the selected positive clone occurs contains either a positive clone or an unresolved negative clone. There is an implicit conditioning on the number $p$ of positive clones in each term of (10).

To evaluate (10), we use the equality

$$\Pr(U{=}u, X{=}x, Y{=}y) = \Pr(U{=}u|X{=}x, Y{=}y)\Pr(Y{=}y|X{=}x)\Pr(X{=}x).$$

Now

$$\Pr(X{=}x) = \binom{v}{x} L_{v-x}^{(p-1)},$$

where $L_j^{(p)}$ denotes the probability that $j$ specified pools are precisely the negative pools. By the inclusion-exclusion principle,

$$L_j^{(p)} = \sum_{i=j}^{v-k} \binom{v-j}{i-j}(-1)^{i-j} z_i^p, \qquad (11)$$

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in which $z_i$ is the probability, introduced at (2), that a given clone occurs in none of $i$ specified pools. Therefore

$$\Pr(X=x) = \binom{v}{x} \sum_{i=v-x}^{v-k} \binom{x}{v-i}(-1)^{i-v+x} z_i^{p-1}. \tag{12}$$

As was the case for $K_j^{(p)}$, a recursive formula for $L_j^{(p)}$ is also available:

$$L_j^{(p)} = \sum_{i=j}^{j+k} \binom{i}{j}\binom{v-i}{k-i+j}\binom{v}{k}^{-1} L_i^{(p-1)}, \tag{13}$$

in which $L_i^{(0)} = 1$ if $i = v$, otherwise $L_i^{(0)} = 0$. Parenthetically, the inclusion-exclusion principle can be used to derive the $K_j^{(p)}$ from the $L_j^{(p)}$ and vice-versa.

$$K_j^{(p)} = \sum_{i=0}^{v-j} \binom{v-i}{i} L_i^{(p)};$$

$$L_j^{(p)} = \sum_{i=v-j}^{v} \binom{j}{i-(v-j)}(-1)^{i-(v-j)} K_i^{(p)}.$$

Given $X=x$, the distribution of $Y$ is Hypergeometric:

$$\Pr(Y=y|X=x) = \binom{x}{y-k}\binom{v-x}{y-x}\binom{v}{k}^{-1}. \tag{14}$$

Further, given $Y=y$, the distribution of $U$ is conditionally independent of $X$ and is binomial:

$$\Pr(U=u|Y=y) = B(n-p, u, \beta), \tag{15}$$

where $B(a, b, t)$ denotes the binomial probabilities (1) and where $\beta$ is the probability that a given negative clone is unresolved negative, so that

$$\beta = \binom{y}{k}\binom{v}{k}^{-1}.$$

Finally, for $u \geq 0$,

$$\Pr(A|U=u, X=x, Y=y) = \sum_{j=0}^{y-x} \binom{y-x}{j}(-1)^j \xi_j^u, \tag{16}$$

in which

$$\xi_j = \binom{y-j}{k}\binom{y}{k}^{-1}.$$

The RHS of (16) is, essentially, $K_{y-x}^{(u)}$, noting that each of the possible $\binom{y}{k}$ subsets of the $y$ positive pools has equal probability of being the pools which contain a particular unresolved negative clone.

Equation (5), follows from (9) and (10), together with (12), (14), (15), and (16);

$$\bar{P} = \sum_{p=1}^{n} pB(n, p, c/n)\alpha^{(p)}, \tag{17}$$

where $B(a, b, t)$ denotes the binomial probabilities (1).