Experimental strategies for the genetic dissection of complex traits in animal models

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Current success in detecting complex trait loci in general, and quantitative trait loci (QTLs) using model organisms in particular, has attracted major biological and biomedical interest. The potential ability to identify genes and their function provides opportunities for new diagnostics and treatments of complex genetic diseases. Despite the success in gene mapping, however, cloning of complex trait loci or QTLs is not straightforward. A major obstacle lies in achieving fine mapping resolution for the detected loci. Compared to the rapid development of sophisticated statistical and molecular tools, development and analysis of experimental designs for various stages in QTL mapping experiments have barely been considered. In this study, novel and existing experimental strategies for QTL analysis are presented and evaluated.

A complex trait is defined as a trait in which a one-to-one relationship between genotype and phenotype does not exist. Risk factors, susceptibility and most quantitative phenotypes fall into the complex trait definition. Until recently, the lack of molecular and analytical tools made the genetic analysis of complex-traits virtually impossible. With current progress, the identification of genes underlying complex traits becomes one of the major challenges of genetics today.

In the past few years, researchers have succeeded in detecting and mapping loci affecting complex traits in humans, model organisms and agricultural species[1–21]. In many instances, complex phenotypes can be assessed quantitatively. Thus, these genes will be referred as quantitative trait loci (QTLs) throughout this paper. Despite major successes in QTL detection, fine mapping has been difficult to achieve. This difficulty is currently a major obstacle to the achievement of both positional cloning and positional candidate gene identification (that is, evaluating candidate genes within a small chromosomal interval to which the gene in question was mapped).

In this study, a mouse model and a dense genetic map are considered for QTL analysis through a three-stage process. Most aspects considered, however, may well apply to other small animals or plants for which inbred strains are available.

In a QTL analysis, three stages can be defined: detection, map estimation and fine mapping. There are conceptual differences between stages that should be addressed for appropriate evaluation of alternative designs. Therefore, each stage will be separately considered in the following sub-sections. Experimental designs for fine QTL mapping, which have been less thoroughly explored, are presented in greater detail (see Box).

Table 1 • Expressions for QTL detection and mapping[a]

	Progeny required for QTL detection	95% CI for QTL map location (in cM)
General expression	$\frac{Z^2_{1-\alpha/2}}{V_{QTL}}$	$\frac{k}{N\delta^2}$
F_2	$\frac{65.5}{2d^2+h^2}$	$\frac{1500}{Nd^2}$
BC	$\frac{60.5}{(d+h)^2}$	$\frac{3000}{N(d+h)^2}$
	$V'_{QTL}=\frac{V_{QTL}}{1-H^2/2}$	$\delta'=\frac{d+h}{\sqrt{1-H^2/2}}$
AIL	$\frac{74.7}{2d^2+h^2}$	$\frac{3000}{Nd^2t}$

[a]Progeny required for QTL detection with power of 50% using standard approximations[22,23]. Confidence interval for QTL map location given as the width of the interval[50]. Both expressions assume a dense genetic map. $Z_{1-\alpha/2}$ = the cut-off point in a standard normal distribution for a probability of $1-\alpha/2$. α=Test significance threshold. For comparative purposes, when genome-wide type I error of 0.05 is considered $\alpha=5.2\times10^{-5}$, 10^{-4} and 1.6×10^{-5} should be used for F_2, BC and AIL, respectively (corresponding to LOD thresholds of 4.3, 3.3 and 4.8, respectively)[32,33]. V_{QTL}=Variance attributed to the QTL, after standardization for a within genotype standard deviation (or variance) of 1.0. V'_{QTL}=Adjusted variance, when d and h are defined in an F_2 population. H^2=Residual heritability (i.e., the proportion of variance explained by genes other than the one tested). d=Standardized allele effect (i.e., half the difference between the means of the two homozygous QTL genotypes divided by the within genotype standard deviation). h=Dominance effect (the difference between the mean of the heterozygous QTL genotype and the average of the two homozygous means, divided by the within genotype standard deviation). N=Sample size. δ=General notation for QTL effect. δ'=Adjusted effect when d and h are defined in an F_2 population. k=Empirical constant. Note that for F_2 and AIL, expressions are appropriate for additive effects, for dominant effects k is reduced up to twofold. t=Number of breeding generations in AIL[45].

QTL detection in a genome scan (stage I)

For an initial genome scan, two classical experimental designs have been used primarily in animal models with inbred strains: the intercross (F_2) and the backcross (BC). Table 1 presents expressions to estimate the number of progeny required for QTL detection as a function of QTL effect and sample size[22,23]. Note that type-I and type-II errors chosen here are somewhat arbitrary and are presented mainly to allow fair comparison among alternative designs. As QTL effects are measured relative to phenotypic variation, the same QTL will have a different effect in a

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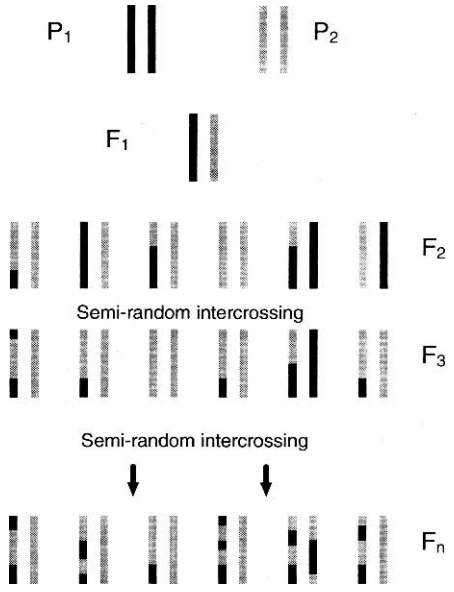


Fig. 1 Production of an advanced intercross line (AIL). Two parental strains are crossed to produce an F_1 and intercrossed to produce a standard F_2. Subsequently, each generation is semi-randomly intercrossed within itself. Although randomness is desired, intercrossing is done to avoid inbreeding as much as possible and thus termed *semi-random* intercrossing. Each generation accumulates recombination at a rate of $r_t=[1-(1-r)^{t-2}(1-2r)]/2$, where r_t is the proportion of recombinants at the t^{th} generation and r is the initial proportion of recombination[45].

BC or an F_2 population. Therefore, for comparison purposes, appropriate adjustments are necessary (Table 1). Additionally, when BC and F_2 are compared, design-specific thresholds should be applied[24,25]. Recombinant inbred (RI) strains have also been used for QTL detection. For most practical situations, however, RI strains are less efficient than F_2 or BC for this purpose. Advanced intercross lines (AILs; Fig. 1) can also be used at the detection stage, although this will require scoring more markers and a higher significance threshold, because of the increase in recombination. Regardless of the experimental design used, numerous statistical and experimental strategies have been developed to increase power and reduce overall costs[26–35].

When one is considering F_2 *versus* BC for QTL detection, it is important to define the precise purposes of the research. If it is desired to have a 'general picture'—that is, number of QTLs segregating and estimates of their additive and dominance effects—an F_2 is preferred. On the other hand, if one's objective is to detect at least some of the major QTL, a BC should be used for greatest efficiency. Table 2 presents a numerical example of the number of progeny required for QTL detection with a BC and an F_2. For additive effects, an F_2 requires about 30% fewer progeny than BC. To detect dominance effects, however, a BC requires about half the progeny of an F_2 population. These results are in contrast to previous conclusions, whereby an F_2 was found to be significantly more powerful than a BC for additive effects. Falconer and Mackay[36], for example, stated that for additive effects, four times as many BC individuals are required to achieve the same power as with an F_2 population (twice as many BC individuals when accounting for genotypic frequencies). The reason behind the discrepancy lies in the fact that previous studies have not taken into account the necessity to adjust for design-specific effects and thresholds. The required significance threshold for a BC is lower[24,25], and the gene effect is higher, because of the reduction in residual genetic variance. Genetic variance caused by gene interactions is also expected to be reduced, making the BC design even more powerful[37]. The general impression is that most QTL detection experiments have been carried out in F_2 populations, no doubt because most early studies were aimed at obtaining a 'general picture', as here defined. To some extent, however, it seems that inaccurate comparisons between BC and F_2 have been misleading.

Table 2 • Progeny required for QTL detection in F_2 and BC[a]

Experimental design	LOD threshold	A/D	% Variance explained	N
F_2	4.3	A	3.0	524
		D	4.5	350
BC	3.3	A	2.0	727
		D	7.7	182

[a]Calculated with expressions given in Table 1, for a QTL with d=0.25, and h=0 (no dominance, A) or h=0.25 (complete dominance, D). For BC, $H^2=0.5$ is assumed to calculate V'_{QTL} for appropriate comparison between BC and F_2 (for symbol definitions see Table 1).

Estimating QTL map location in a genome scan (stage II)

Interval mapping[23] and its variations[26,27,38–40] are commonly used to estimate QTL map location by application of specific software, such as Mapmaker/QTL[23]. The analysis is initially done on the same population as in the first stage, but with different statistical methods (that is, hypothesis-testing *versus* parameter estimation). The most important parameter for evaluating QTL strategies in this stage is mapping accuracy. A number of methods have been suggested to estimate QTL mapping accuracy and construct confidence intervals[23,41–43]. The simplest method for an *a priori* estimate of the expected confidence interval (CI) of QTL map location uses an empirical formula[44]. The general expression presented in Table 1 was empirically developed under a dense genetic map but it is also a close approximation to situations where marker-spacing is up to half of the CI itself. To increase mapping accuracy, an F_2 or BC population should be preferred for additive or dominant effects, respectively (Fig. 2).

The amount of recombination is the main variable that affects stages I and II differently. Whereas for QTL detection less recombination is desired to reduce the total number of markers required for the genome scan, increased recombination improves mapping resolution. RI strains are limited both in regard to progenitor strains and in number; thus, in most cases,

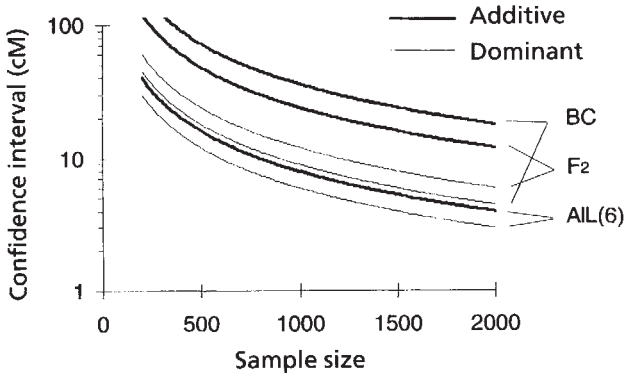


Fig. 2 Confidence interval for QTL map location (on a logarithmic scale) as a function of sample size for a QTL with an additive effect of 0.25 standard deviations. Thick and thin lines correspond to no dominance and complete dominance effects, respectively. For BC, adjusted gene effect was used (see Table 1). AIL(6) represents an advanced intercross line phenotyped at the sixth intercross generation (appropriate expressions given in Table 1).

Box • Strategies for QTL fine mapping

Selective phenotyping. A large F_2 or BC population is produced, and only individuals recombinant at an interval previously defined to contain a QTL are selected for phenotyping. This strategy is based on the rationale that once a gene is mapped to a given interval, only recombinant individuals within that interval contribute to further mapping accuracy. Theoretically, selective phenotyping can be done in a sequential manner; that is, once an interval is defined to contain a QTL, recombinants within that interval are phenotyped. Any number of recombinants phenotyped will reduce the width of the QTL-containing interval; subsequently, a smaller interval can be considered to look for new recombinants. For practical reasons however, probably no more than two steps will be applied. At each stage of selective phenotyping, total number of animals phenotyped is reduced by a factor of 1/2r(1-r) for an F_2 population and by 1/r for a BC population (r being the proportion of recombination between the markers bracketting the interval in question). With selective phenotyping, savings are in phenotyping only; total number of animals produced is equal to that necessary with an F_2 or a BC.

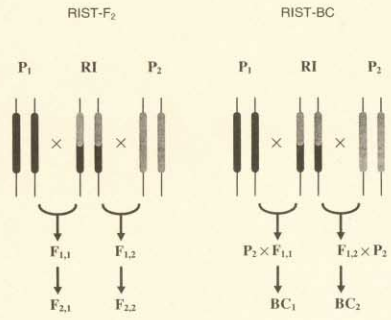
Recombinant progeny testing. Individuals carrying a distinguishable recombinant chromosome at the region of interest are crossed to one of the parental strains to determine the location of the QTL relative to the recombination point. Reducing CI from y cM to x cM will require y/x recombinant individuals, each with a recombination at one of the y/x intervals covering the initial y-cM interval. The expected number of F_2 animals that will be screened, N_s, to detect these y/x recombinant individuals can be approximated as:

$$N_s = \frac{50}{x}\sum_{i=1}^{\frac{y}{x}}\frac{1}{i}$$

For example, if y=25 and x=5 then N_s=23,
or if y=5 and x=1 then N_s=114 (ref. 53)

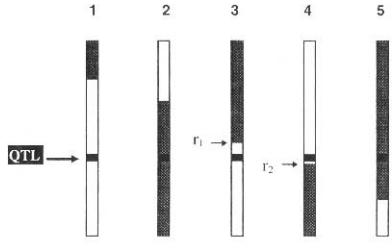
Interval-specific congenic strains (ISCS). As in recombinant progeny testing, N_s individuals are screened to detect y/x recombinant individuals with recombinations equally distributed within the y-cM interval. These animals, however, are now crossed several times with the background parental strain to eliminate alleles from the donor parental strain at all other QTLs affecting the trait. Then animals are intercrossed, and homozygotes for the recombinant haplotype are selected to establish one ISCS. Selection at various stages is done with the aid of DNA markers, significantly reducing the number of generations required with little additional genotyping[53].

Fig. 4 Producing RIST populations. P_1 and P_2 are two inbred strains that served as the parental strains of the RI strains. A selected RI strain, with a recombinant haplotype in the region of interest is crossed with both parental strains to produce two separate F_1 populations, $F_{1,1}$ and $F_{1,2}$. Subsequently, RIST-F_2 and RIST-BC populations are obtained through intercross and backross, respectively.



Recombinant inbred segregation test (RIST). RIST is a novel experimental design aimed at taking advantage of the theoretical high mapping resolution present in RI strains and apply it to QTL mapping. To reduce the QTL-containing interval from y cM to x cM, y/x RI strains are selected with recombinations equally distributed within the y-cM interval. Note that in some cases fewer RI strains will be necessary, as a single RI strain may have more than one recombination in the region of interest. It is expected that for x = 1 cM such strains will be found for 50% of chromosomal regions when a set of 25 RI strains is in hand. The RIST population is constructed as outlined in Fig. 4. The F_2 or BC populations are phenotyped and genotyped with few markers. The $F_{2,1}$ or BC_1 population is genotyped with markers located in the region where P_2 alleles are present in the selected RI strain, and the $F_{2,2}$ or BC_2 is genotyped with markers located in the region where P_1 alleles are present in the selected RI strain. Because the QTL has been previously mapped to this region it will necessarily segregate in one of the F_2 or BC populations but not in the other. Analysis of the two populations will detect the population on which the QTL is segregating and accordingly locate the QTL above or below the recombination point. The overlapping results of all RI strains selected will locate the QTL to the desired interval. For additive effects, RIST-F_2 is preferred. In that case, homozygous genotypes at the marker locus will contribute most of the information. Thus, only homozygous individuals will be selected for phenotyping. When a dominant effect is considered, RIST-BC will be more efficient.

they are not applicable for QTL mapping. Nevertheless, for QTLs of large effects, RI strains may provide significant mapping accuracy because of the fourfold increase in recombination. With AIL (Fig. 1), the CI of map location can be reduced, in theory, by a factor of t/2, where t is the number of the advanced generations. This reduction can be obtained, in practice, up to the sixth or eighth generation if approximately 50 breeding pairs are maintained within each breeding generation[45]. Thus, AILs efficiently provide high mapping accuracy in a genome-wide context at the expense of a lengthier procedure.



$$N_t = c \cdot \left(\frac{Z_{1-\alpha}}{\delta}\right)^2$$

	Recombinant progeny testing	ISCS	RIST	
			F_2	BC
c	16	4	32	16
δ	$\frac{(d+h)}{\sqrt{1-3H^2/4}}$	$\frac{2d}{\sqrt{1-H^2}}$	$\frac{2d}{\sqrt{1-H^2/2}}$	$\frac{(d+h)}{\sqrt{1-3H^2/4}}$

Fig. 3 Genetic chromosome dissection (GCD). A set of recombinant haplotypes (1–5) is analysed, each with N_t individuals, to determine the QTL allelic state. Throughout this process, the smallest common region will eventually be determined by two recombinant haplotypes only. Here, haplotype 3 and 4 locate the QTL into the interval defined by the two recombination points r_1 and r_2. With an estimate of gene effect, two symmetrical hypotheses are tested (i.e., two possible states for QTL alleles). Therefore α represents both type-I and type-II errors. Note that with RIST, each recombinant haplotype is tested with two independent populations. Consequently, each population can be tested with a significance level of $\alpha^{1/2}$. Phenotyping N_t individuals at once is somewhat wasteful. For example, phenotyping only 1/4N_t will detect the QTL allelic state in half the cases with the same type I error. Consequently, it is most appropriate to follow a sequential procedure that can reduce total number of phenotyped progeny to about half[30]. An expression to estimate N_t is given where, c is a constant that depends on the number of genotypic groups present and tested in the population. The adjusted allele effect δ, depends on the design-specific reduction on residual genetic variance (H^2) and on whether the effect is being tested on alternative homozygous or heterozygous versus homozygous. The table at the bottom presents corresponding values of c and δ for recombinant progeny testing, ISCS and RIST, with d and h as defined in Table 1.

Table 3 • Number of progeny required for fine QTL mapping[a]

Experimental design	Number of generations	A/D	Confidence interval reduction from 25 cM to 5 cM: Phenotyped	Confidence interval reduction from 25 cM to 5 cM: Total produced[b]	Confidence interval reduction from 5 cM to 1 cM: Phenotyped	Confidence interval reduction from 5 cM to 1 cM: Total produced[b]
F_2	2	A	ALL	3840	ALL	19200
BC	2	D	ALL	1440	ALL	7200
AIL (F_6)[c]	6	A	ALL	1280	ALL	6400
		D	ALL	480	ALL	2400
Selective phenotyping[d]	2	A	1215	3840	1730	19200
		D	285	1440	345	7200
Recombinant progeny testing	3	A	1540	1590	1540	1640
		D	385	405	385	485
ISCS[e]	6	A	80	300	80	380
		D	80	300	80	380
RIST[f]-F_2	2	A	240	480	240	480
RIST-BC	2	D	ALL	200	ALL	200

[a]Numbers required for CI reduction calculated for a QTL with d=0.25, and h=0 (no dominance, A) or h=0.25 (complete dominance, D). These effects would explain 3.0% and 4.5% of the trait variation in an F_2 population, respectively. Calculations according to expressions given in Table 1 and Fig. 2, assuming sequential sampling and residual genetic heritability of H^2=0.5 (see symbol definitions in Table 1). For recombinant progeny testing, ISCS and RIST a type I error of α=0.025 was used to test each recombinant haplotype. This value corresponds to a probability of 0.05 that one or both of the critical recombinant haplotypes were misclassified and thus the final detected interval does not contain the QTL (comparable to the 95% CI as presented for F_2, BC, AIL and selective phenotyping). [b]Total number of animals produced includes those phenotyped. [c]For the dominant effect it is assumed that the AIL (F_5) generation is backcrossed to the recessive parental strain to produce the population for phenotyping. [d]Proportion of recombination for selected intervals was calculated using Haldane's mapping function. Additive and dominant effects are assumed to be tested in F_2 and BC populations, respectively. [e]Assuming that 40 animals are required for the production stage of each ISCS. [f]In the RIST-F_2 population only homozygotes are phenotyped.

Single-QTL–oriented fine mapping (stage III)

The basis of single-QTL–oriented fine mapping is similar to that of mendelian-gene fine mapping—that is, analysis of recombinants within an interval previously found to contain the gene. When a QTL is in question, however, one must account for additional variation present to be able to accurately determine the allelic state of the QTL for a given recombinant haplotype. With polygenic traits, at a certain point, efforts must be shifted from a genome-wide search (stage II) into single-QTL methods (stage III). Strategies that may be considered for this purpose are described in the accompanying Box. With the exception of selective phenotyping, single-QTL–oriented strategies are similar in concept. This concept (Fig. 3), which may be termed 'genetic chromosome dissection' (GCD), was first introduced by *Drosophila* geneticists[46–49]. With the advent of DNA-level polymorphisms, GCD methods were pioneered with extreme success by researchers using the tomato as a model organism[50–52]. A number of adaptations to rodents have also been suggested[53–55].

Table 3 presents a numerical example of progeny required for fine mapping, under alternative single-QTL–oriented designs. F_2, BC and AIL are also presented for comparative purposes. Although these designs may require more animals, one should keep in mind that the accuracy they provide applies to all QTL segregating in the cross. This may give an indication of the resolution appropriate to switch from stage-II to stage-III methods.

A summarized comparison of the alternative designs for fine QTL mapping is presented in Table 4. Among these designs, only interval-specific congenic strains (ISCS) do not involve a segregating population. Fixed effects, such as litter, residual genetic loci and interactions, may confound the main effect being tested. To avoid this risk, one can detect ISCS by producing a small segre-

Table 4 • Comparison of QTL fine mapping strategies

	Advantages	*Disadvantages*	*Most appropriate for*
SP	Requires only 2 generations.	Requires very large samples, in particular number of animals produced as resolution increases.	QTL with large effects. Target resolution not beyond ~5cM. Major resources available and fast results required.
RPT	Requires only 3 generations. Efficient for dominant effects.	Requires large samples.	QTL with large and dominant effects.
ISCS	Requires few individuals even for small effects. The constructed ISCS can serve as a resource for additional phenotypings.	Lengthy production. Cannot take advantage of dominant effects.	QTL of moderate or small effects.
RIST	Requires only 2 generations. Feasible number of animals required even for small effects.	Requires the availability of RI strains with recombinations in the region of interest.	Whenever appropriate RI strains are available.

SP=Selective phenotyping; RPT=Recombinant progeny testing; ISCS=Interval specific congenic strains; RIST=Recombinant inbred segregation test.

gating population (originated by crossing the ISCS to the background parental strain) and following a similar test to that for the RIST design. ISCS can also be produced in a different manner. As has been suggested[54,55], a single congenic strain for the relevant region can be constructed. Then, with the congenic strain in hand, recombinant progeny testing or ISCS can easily follow.

Discussion

Choosing the most appropriate strategy for a particular QTL-mapping experiment will depend on a number of factors, such as target power or resolution, size and dominance state of the effects, expected number of QTLs and resources available (including time). The comparisons presented here may serve as guidelines for selecting strategies to significantly improve QTL-mapping efficiency. For example, with the novel RIST design (when applicable), a QTL of moderate effect, previously mapped to a 25-cM interval, can be mapped to a 1-cM interval with only a total of 400 individuals and two stages of two generations each (Table 3). In dealing with animal models, particularly mice, there is no limit to the number of population designs that can be produced. Designs in addition to those presented here and optimized combinations of designs should also be considered. In particular, one should take advantage of any specific population resources in hand. For example, recombinant congenics strains were successfully used for detecting epistatic effects[56,57], which are generally difficult to detect[58].

How would linked QTL affect analyses and experimental designs? In a standard F_2 or BC QTL-mapping experiment, the presence of linked QTLs can hardly be distinguished from that of a single QTL of large effect, although some indications of their presence can be observed from the respective lod-curve profile[23,39]. When one proceeds with stage-III methods and as part of the mapping procedure, linked QTLs will be dissected into separate loci, each with smaller effects. This will necessitate increasing the number of animals phenotyped, but not more than what is necessary for unlinked QTLs of similar effects. If chromosomal regions are broken into large numbers of genes, each with a small effect, obtaining good mapping resolution for each gene becomes extremely difficult. The practical consequence in this case is the waste of research resources in going after a major QTL that does not exist. The extent of this problem depends on the relative abundance of alternative scenarios (that is, few or many QTLs), which is difficult to predict. Using AIL at the detection stage can be of help for this problem, as with AIL it is less likely to detect linked loci as a single major gene.

The ultimate target related to QTL analysis is, of course, to get from fine mapping to the gene itself. One should note that as an alternative to linkage analysis, candidate genes can be directly tested for association to particular traits[59–63]. With entire genomes sequenced and all genes known, however, the usefulness of QTL fine mapping strategies is expected to increase even further with application of a positional candidate gene approach. The strategies described in this paper should be integrated with developing technologies (such as high-throughput gene expression analysis, novel molecular genetic tools for genome manipulation, comparative mapping and sophisticated bioinformatics applications) to allow efficient large-scale functional mapping of genes to complex traits.

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