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Systems Genetics

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Systems Genetics

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Preface

Systems genetics is actually an old field with a new name. RA Fisher [1], S Wright [2-4], and JBS Haldane [5, 6]—the three leading figures of the *modern synthesis* who brought genetics into alignment with evolutionary biology—are the intellectual founders of what we would now call systems genetics. They used other terms—population genetics, statistical genetics, and quantitative genetics. We can add one more scientific progenitor, CH Waddington, a founder of what is now called systems biology and a key figure who helped align developmental biology with genetics [7].

The advantage of the term *systems genetics*, and the reason for its rapid rise in prominence, is that it emphasizes the concept “system” rather than the resource type (*population*), the measurement type (*quantitative*), or the method of analysis (*statistical*). Our colleague Grant Morahan coined the term in 2004 to refocus attention toward sets of related phenotypes, sets of gene variants, and sets of environmental factors and away from more restricted terms that were then in use—*genetical genomics*, *complex trait analysis*, and *QTL analysis* [8–10]. A short definition of systems genetics and its relations to other approaches may help.

Genetics can be divided roughly into three ways of looking at relations between genetic and phenotypic variation:

1. *One-to-one relations*—in other words, classical Mendelian genetics—the study of qualitative traits linked either to spontaneous mutations or to targeted modifications of genes.
2. *One-to-many relations* between single phenotypes and sets of loci or gene variants—in other words, QTL mapping, genome-wide association, and complex trait analysis.
3. *Many-to-many-to-many relations* among (a) sets of correlated and interacting phenotypes at different levels (metabolites, mRNAs, protein, organelles, cells, tissues, organ systems, and classic phenotypes and outcome measures), (b) sets of gene variants, and (c) sets of environmental factors and treatments.

The latter is the ultimate goal of systems genetics, but the reality is that we need to be working on problems at all three levels concurrently. No doubt about it: the amazing complexity and adaptability of biological systems needs to be dissected into manageable units for analytic and economic reasons. Results that make headlines and that are most highly rewarded tend to be the 1-to-1 simplifications—gene X causes aging, gene Y causes schizophrenia. But what is just as obvious now is that the yin of “dissection,” “analysis,” and “reduction” needs its complement—the yang of “assemble,” “synthesis,” and “integration.”

The main motivation is not merely a scholastic intellectual balance—improved health care, agricultural productivity, and the design of robustly engineered biological systems absolutely require a deep understanding of the range of action of the whole.

The good news is that we finally have powerful tools both to dissect and to assemble biological systems with rapidly improving range, precision, and throughput. The duality of

genetics can be balanced. Generating millions of precisely measured genotypes and molecular phenotypes—our biological parts list—is now practical for thousands of cases, in principle, under many conditions. Human cohorts of millions of subjects, all sequenced and accompanied with comprehensive health records, will soon be routine. For assembly and integration of these parts, we have the computer scientists, bioinformaticists, mathematicians, statisticians, and public funders to thank for every faster and more sophisticated ways to evaluate how best to put pieces together and how to predict outcomes with some level of precision. We now can even look forward with angst to *ab initio* creation—making new biological systems from scratch. We are on the cusp of amazing capabilities.

The chapters in this volume will give you a hands-on appreciation of the range of activity and methods in systems genetics. This volume does not cover the whole range of activity; our contributors are drawn from a small but vibrant community of rodent experimental geneticists. Most of us are focused on mouse models with the goal of translational impact to better understand and cure human diseases. Most of us grew up in this new genomics era of QTL mapping, and a dominant theme of many protocols is how best to track down genetic causes of heritable variation across a wide range of systems and traits. But if you stand back and envision the whole activity represented in this volume, you will see how protocols and results can be snapped together to build more holistic models in a true systems spirit. We are now well poised to implement ever more powerful methods and models.

We thank our many colleagues, collaborators, and the 100 contributors to this volume. Both of us were frankly surprised by the highly enthusiastic responses given to our requests for protocols in this new area—no thumbscrews required. That is an excellent sign. And in keeping with the theme of systems integration, we expect that there will be strength in numbers and complementarity—that readers will, we hope, find real synergy in using collections of these protocols.

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Chapter 1

Resources for Systems Genetics

Robert W. Williams and Evan G. Williams

Abstract

A key characteristic of systems genetics is its reliance on populations that vary to a greater or lesser degree in genetic complexity—from highly admixed populations such as the Collaborative Cross and Diversity Outcross to relatively simple crosses such as sets of consomic strains and reduced complexity crosses. This protocol is intended to help investigators make more informed decisions about choices of resources given different types of questions. We consider factors such as costs, availability, and ease of breeding for common scenarios. In general, we recommend using complementary resources and minimizing depth of resampling of any given genome or strain.

Key words Genetic reference population (GRP), Recombinant inbred (RI), Collaborative Cross (CC), Congenic lines, Consomic and chromosome substitution lines, Recombinant congenic strains, RI intercross (RIX) and RI backcross (RIB) progeny, Heterogeneous stock (HS), Diversity outcross (DO), Hybrid diversity panel (HDP), Reduced complexity cross (RCC), Gene-by-environment interactions ($G \times E$)

1 Introduction

A large number of innovative resources for systems genetics have been developed over the last 15 years [1]. There are at least two reasons for this burst of activity. The first catalyst was the introduction of far easier, cheaper, and more comprehensive methods of genotyping [2, 3] that we already take for granted. State-of-the-art genotyping for recombinant inbred (RI) strains consisted of ~1600 microsatellite markers (dinucleotide repeats) in 2001 [4]. Over the next 5 years this number increased to more than 10,000 SNPs [5], and we now rely on genotypes at more than 100,000 SNPs using Affymetrix or Illumina platforms [6, 7] at modest cost—well under \$0.01 per marker. The second reason was rapid progress on ways to map quantitative traits with progressively higher precision and power [4, 8–16], culminating in the establishment of the Complex Trait Consortium in 2002 [17]. A good problem we now face is selecting wisely from the many options and resources that are now available. Any choice is a major commitment. This protocol highlights factors researchers should consider and balance.

2 Methods

2.1 *Guidance on Using This Protocol*

The goal of this protocol is to step through the decisions associated with selecting resources for both QTL mapping and systems genetics. The first issue is to define classes of questions. Different questions benefit from different types and mixtures of resources—the cliché “different horses for different courses” applies. In **Part 2.3** we review current murine resources used in QTL mapping and systems genetics. In **Part 2.4** we consider one multipurpose experimental design that will work reasonably well for a range of questions. Consider this design a starting point for your discussions and decisions. We provide some notes on the pros and cons of the resources, many in a simple question-and-answer format. Since everyone has their own biases, ask others for their opinions.

These are among the main considerations or themes that go into the choice of resources for systems genetics:

1. Cost and availability (strains, hybrids, cases).
2. Phenotype diversity, heritability, and genetic architecture.
3. Marker density, mapping precision, and power.
4. Sequence diversity and genetic blind spots.
5. Selective phenotyping or genotyping.
6. Complexity of QTL intervals.
7. Population structure, admixture, and analytic methods.
8. Depth of genetic, omics, and phenome data resources.
9. Robustness, replicability, extensibility, and translatability.

To foreshadow our conclusions: Most researchers currently rely on a single type of resource or cross, and while there are good historical reasons for this focus, this is no longer an optimal or advisable strategy. We now have such a range of powerful genetic resources optimized for different purposes that it makes sense to take advantage of combinations of complementary crosses and even multiple species [18–22]. Analytic methods do get more complex when using combinations of resources, but some of the same methods used to handle admixed human cohorts in genome-wide and phenome-wide association studies (GWAS and PheWAS) have now been adapted to handle combined experimental cohorts [23–25].

Our other conclusion is that a mapping resolution of about 1 Mb will usually be adequate to transition to validation, including translational analysis of human GWAS and PheWAS data sets [22, 26, 27], analysis of knockout (KO) and knockin (KI) phenotypes, bioinformatic and omics dissection, and pharmacological intervention. This is especially true in an era of super high precision but mechanistically unanchored GWAS. The need for high precision mapping in mouse has been supplanted by an acute need for

powerful resources to understand and accurately predict genome-to-phenome (G2P) relations under a wide range of environments and treatments.

2.2 Types of Questions Guiding the Experimental Approach

We consider four main types of questions:

2.2.1 Type 1 Questions

The classic forward genetic question—what are the polymorphic genes and sequence variants that modulate a phenotype or disease risk? This is by far the most common question our research community has dealt with over the last two decades and will probably remain so for the next several decades. Almost all human GWASs have this same simple reductionist motivation—a generalization of the classic Mendelian approach but applied to messier and continuously variable quantitative traits.

The repeated mapping of large numbers of QTLs and their causal QT genes (QTGs) quickly leads to complex systems-level questions—a transition we now are beginning to see in human GWAS. This shift has happened gradually over the past decade. The pioneering work by Wakeland and colleagues on the family of gene variants that contribute to autoimmune disease is a fine example [28]. The work of Hunter and colleagues on metastasis networks [29, 30] and of Morahan and colleagues on type I diabetes [31] provide two other examples of this movement from QTL analysis to complex systems genetics. This shift is leading to the discovery of new biomarkers, diagnostics, mechanisms, and treatments.

Type 1 questions are usually approached in two steps: the first involves mapping QTLs to confidence intervals of 0.5–5 Mb, while the second and more problematic step involves proving to your own satisfaction (and that of reviewers and readers) that a polymorphic candidate gene has been validated as a source of trait variance [12, 15]. Almost all of the technical motivation and innovation in the late 1990s and early 2000s in the field of QTL mapping addressed mapping precision, with less explicit consideration given to statistical power. There was, and still is a good reason for this focus on precision: once the right gene has been identified, it becomes possible to switch from genetic causality defined by loci and LOD scores, to actionable molecular mechanisms modulated by differences of protein expression or sequence. Thanks to many human GWASs, we now understand much better how to control the risk of false discovery using populations that incorporate more and more recombinations and complex admixture. One goal of this protocol is to help you get to a sweet spot with a balance of power and precision. A second goal is to help ensure that the results are robust and translatable.

- 2.2.2 Type 2 Questions** Questions related to $G \times E$ and treatment effects on phenotypes. These types of questions will be crucial to those interested in systematic manipulations of diet, environmental stressors, age, pathogens, drug exposure, and differences in social interactions. Mice and other inbred and isogenic model organisms are extremely well suited to evaluate complex experimental effects in the context of QTL mapping. The ability to impose well-controlled perturbations across large cohorts is among the strongest motivations to use model organisms. This kind of design is already the most common and critical in agricultural genetics.
- 2.2.3 Type 3 Questions** Questions related to the global genetic modulation of single traits or of systems of correlated phenotypes. These types of large-scale questions often fall under the heading of “genetic architecture.” This term encompasses the analysis of many components of heritable and nonheritable variation, particularly the number and effect sizes of loci, independence and interactions among loci, and the roles of the environment, epigenetics, parental effects, and developmental noise [32]. Oddly enough, before it became easy to map QTLs, these types of hard questions were at the heart of quantitative genetics. In fact, major branches of statistics had their birth in questions of genetic architecture, including ANOVA and path analysis [33, 34]. The diallel cross—the production of a matrix of F1 hybrids from inbred strains—is one of the mainstays of this type of quantitative genetics [35]. Recent examples include studies by Airey et al. [36], Crowley et al. [37], and Percival et al. [38] who have used diallel sets of RI strains and the founders of the Collaborative Cross (CC).
- 2.2.4 Type 4 Questions** Type 4 questions are related explicitly to predicting G2P relations. Given summed effects of gene variants (Type 1 questions), $G \times E$ interactions (Type 2), and the architecture of all sources of variance (Type 3), can we assemble predictive models of disease risk as a function of age, environment, diet, and drugs? This is the core question and quandary of precision health delivery. Precision medicine will have a short grace period, but if geneticists, molecular biologists, statisticians, and computational scientists have not delivered something impressive to match the hype, this term and the field risk being dismissed as a misnomer in the same way that *artificial intelligence* (AI) was dismissed and left unfunded for long periods. We need great experimental resources to generate and help validate predictions efficiently. The next section provides quick definitions and commentaries on the pros and cons of the important resources.
- 2.3 Pros and Cons of Resources and Crosses** We list of some of the major types of resources, from most simple to most complex in terms of level of genetic variation and complexity. The types of crosses and how they are generated are shown schematically in Fig. 1 with numbers that correspond to subsections.

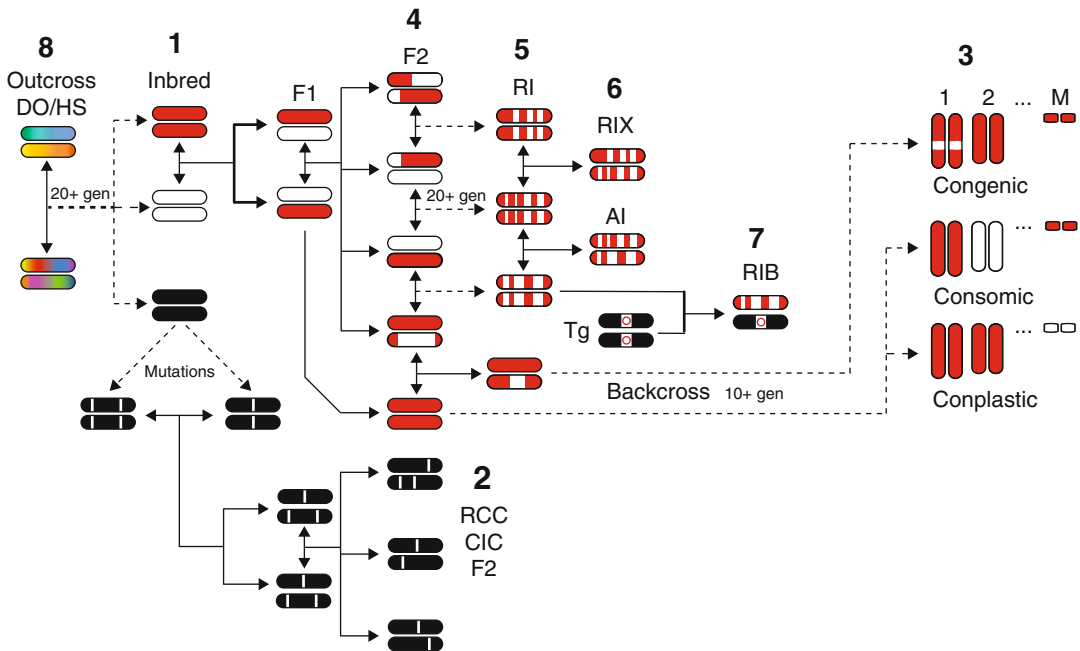


Fig. 1 Breeding schema for different genetic resources and populations. Breeding schemes used to generate the resources. Short bars symbolize pairs of chromosomes. The colors (here, arbitrarily chosen as *red*, *white*, or *black*) denote the haplotypes/genotypes of the chromosomes. The large numbers within the figure correspond to Section 2 subheadings. **Tg**—a transgenic line. Note that AI progeny are not generated from RI lines, but they have chromosomes with recombination patterns similar to those of RIX. For all other abbreviations see text of Section 23. Adapted from [1] with permission

2.3.1 Single Fully Inbred Strains (Fig. 1.1)

Single full inbred strains such as DBA/2 and C57BL/6, are often the starting point for in vivo studies. We usually do not think of inbred strains in isolation as a resource for systems genetics, but a family of knockouts can be bred into a single isogenic strain [39] or a single KO can be crossed into a hundred different inbred strains [40] to generate interesting cohorts.

Large sets of distinct inbred strains incorporate a great deal of genetic variation (three are shown in Fig. 1.1), and collectively may also be used as a core resource for systems genetics [41]. Genome sequence data are available for more than 36 inbred strains [42] (www.sanger.ac.uk/science/data/mouse-genomes-project) most of which are also part of the Mouse Phenome Project [43]. Such collections of inbred strains—often termed diversity panels—provide a quick and ready resource for profiling how traits vary across a wide range of genomes, but there are not enough easily available strains to map QTLs effectively. Power is low and false discovery rates (FDRs) are high. However, sets of common inbred strains combined with sets of RI strains are an excellent joint resource for systems genetics—a combination called a hybrid diversity panel to which we return below.

The most commonly used inbred strains have often been split into sets of substrains. These will carry different sets of a few spontaneous mutations that have been picked up over decades of maintenance in different colonies. In mice, C57BL/6J and C57BL/6N are the genetic background strains used for almost all KO, KI, and transgenic modifications (www.mousephenotype.org). Thanks to powerful sequencing technologies, sets of related substrains now provide an interesting new resource for G2P mapping. We describe this novel approach below.

2.3.2 *Reduced Complexity Cross (RCC) or Coisogenic Cross (CIC)* (Fig. 1.2)

Both RCCs and CICs are novel types of “postgenomic” intercrosses between very closely related substrains [44, 45] or even coisogenic pairs. For example, genomes of the C57BL/6J and C57BL/6N substrains differ at a total of about 36 known coding variants [42] but these substrains also differ for a surprisingly large number of phenotypes, including responses to several drugs and treatments [46–49]. BXD29/TyJ and BXD29-Tlr4^{clps-2J}/J constitute a coisogenic pair that differs at two or three loci [50]. How is it possible to map an F2 that has almost no sequence variants? Once two substrains have been sequenced deeply (>30-fold coverage), there will almost always be a large enough number of spontaneous noncoding mutations to assemble a sparse genome-wide panel of SNPs and indels for mapping sources of phenotypic differences.

While the mapping precision of an F2 RCC or CIC will be poor (intervals of 20 Mb or more), the small number of segregating variants within any interval means that it can be practical to identify candidate QTGs and even QT nucleotides (QTNs) efficiently [51]. Kumar used this approach to define a mutation in *Cyfp2* that controls response to cocaine and methamphetamine [44]. The utility of an RCC in mapping and even in systems genetics points out that the key variable in “cloning” QTLs is not mapping precision per se but the number of polymorphic genes and sequence variants within a QTL’s confidence interval. A 5–10 Mb interval containing only a single sequence variant will be far more easily reduced to cause and mechanism than a highly polymorphic 0.1 Mb QTL containing five genes and hundreds of sequence variants [44, 52].

2.3.3 *Consomic and Congenic Whole Genome Panels* (Fig. 1.3)

By backcrossing two inbred strains to each other while tracking genotypes of progeny over several generations, it is possible to effectively transplant whole chromosomes from donor strain *A* into recipient strain *B*. A full set of consomic strains will consist of 22 lines, each with one swapped chromosome plus the recipient control strains. There are now two sets of consomic strains—crosses of A/J or PWD/Ph into C57BL/6J [14, 53]. Buchner and Nadeau [54] have considered the pros and cons of consomic sets and their efficiency relative to other resources.

A whole genome congenic panel is basically a finer-grained version of a consomic set, but now each strain contains only a piece of

a single donor chromosome [55]. The main utility of consomic and congenic sets is their high power to map phenotypes to single chromosomes. They have been used more recently to study epistasis and epigenetic effects [54]. Their main disadvantage is that mapping QTLs requires the production of a secondary F2 intercross or a set of interval-specific congenic strains. Whole chromosome effect sizes will almost inevitably decrease during this process [56].

2.3.4 Off-Target Mutations and Isogenic Strains

One important factor to consider before using congenic and consomic strain sets is their sensitivity to spontaneous mutations that will accumulate gradually and progressively on the recipient (non-transplanted) background chromosomes. Spontaneous mutations or allele conversion events that arise on these other 20 chromosomes can cause variant phenotypes, and these new phenotypes risk being misattributed to putative variants on the donor chromosome—essentially off-target effects [57]. It is therefore useful—sometimes even essential—to verify that traits map to the introgressed chromosome by making a small F2 from the consomic or congenic stock. Tracking down off-target variants is difficult because there are no known polymorphisms with which to map the other chromosomes. Sequencing consomic strains and using RCC methods is the obvious, but costly solution.

This raises a broad issue that applies to all crosses that are carried for many generations, including standard inbred strains, RI strains, AI progeny, and HS stock: what is the relative impact of inevitable *de novo* mutations on the measured phenotypes and results of different types? The good news is that for most of these resource types, new mutations will be unique to one strain or one case and will not segregate across the whole cross. Provided that the analysis and results are statistical collectives based on a large sample of strains or cases, then rare mutations, even those that are fixed in single strains, will simply be lumped as another source of error variance. In contrast, in situations in which mapping and other results depend on a single case and control—as when using congenic and consomic lines—there is a risk of misattribution of effects.

2.3.5 F2 Intercrosses and Backcrosses (Fig. 1.4)

The F2 intercross has been used widely in systems genetics, starting with the work of Damerval [58], Schadt, Luskis, and colleagues [59, 60]. Their main advantage is the ability to make large numbers of progeny quickly from almost any stock (usually inbred strains). F2 intercrosses and N2 backcrosses have a structure that makes mapping and the analysis of covariance among traits simple. There is no need to correct for population substructure (*see Note 1*)—a problem that arises in almost any multi-generation cross (e.g., heterogeneous stock (HS), AIs, and RI strains). It is practical to enhance the complexity and utility of an F2 intercross for systems genetics and for standard QTL mapping by making a four-way F2—for example by crossing A×B F1s to C×D F1s to produce AB×CD F2 progeny. This type of F2 is being used in an experimental study of life span in mice [61].

2.3.6 *Advanced Intercrosses (Fig. 1.6)*

AIs are simple extensions of F2s in which all subsequent generations are randomly bred, but with careful avoidance of sib matings [9, 10]. The number of recombination events per AI case climbs steadily as the depth of the pedigree increases. At the eighth generation (about 2 years of breeding), 100 AI progeny, if made correctly, will provide about the same mapping precision as 500 F2 for Mendelian traits [9]. The countervailing problems with AIs are (1) the more complex logistics of using more than 100 breeders for up to ten generations has a high cost, (2) the variable kinship among AI progeny needs to be factored into any kind of mapping or other statistical analysis, (3) the need for a significantly higher density of markers, and, perhaps most seriously (4) the loss of power associated with the increased number of recombinations per animal. A solution to some of these issues, first pointed out by Darvasi and Soller [9], is to generate RI strains from AI stock—the so-called Advanced RI (ARI) strains—and both the CC and many of the new BXD strains are actually ARIs.

Trade-Offs. There are important trade-offs between mapping precision and mapping power—the ability to detect QTLs with effects that account for a defined percent of the trait variance assuming a given sample size. As pointed out by Lander and Botstein [8], the longer the genetic map, the higher the thresholds for statistical significance. The relation is complex, but Table 1 provides a rough guide of tradeoffs. One column is marked **Recs/case** or recombinations per case, and a second column is marked **LOD Threshold**, or the linkage score that will often be needed to achieve genome-wide significance. **Recs/case** is an index of the potential precision of a resource, whereas the LOD score in this context is an inverse index of statistical power. High **Recs/case** are good for precision, but high LOD score requirements are bad for power.

The goal of course is precision with power. The simplest way to get both is to type larger and larger numbers of cases. A better solution is to combine complementary resources—one optimized for power such as a conventional F2 or conventional RI strains, and one optimized for precision—such as the Collaborative Cross (CC), a Hybrid Diversity Panel (HDP), AI, HS, or DO stock. The reason why joint resources are not used widely yet is because (1) many of the resources are new, and (2) the computational aspects of the analysis are more involved. But we now have powerful algorithms [23–25] that can handle dense genotypes and complex cohorts and covariates. Some of these are available online in the new version of GeneNetwork.

2.3.7 *RI Strains (Fig. 1.5)*

RI strains were originally made for mapping highly penetrant Mendelian traits [62, 63], but they were eventually adopted for the analysis of complex traits [64]. RIs are now a key resource in systems genetics. Their main advantage relative to F2s and HS is that

Table 1
Resources for systems genetics

Type of cross	Recs/case	LOD Threshold	\$/Geno typing	\$/Case ^a	Isogenic	Inbred	Phen-ome	GXE	Breeding	References
Consomic and congenic sets	1	1–2	0	140	Yes	Yes	Yes	Easy	Variable	[14, 55]
Reduced complexity cross	25	1–2	25	20	Almost	Almost	Hard	Hard	Easy	[44, 45]
F2 intercross, 2-way or 4-way	25	2.5–3	25	15	No	No	Hard	Hard	Easy	[8, 16]
Advanced intercross	100	4–5	100	100	No	No	Hard	Hard	Hard	[9, 10]
RI strains and advanced RI Strains	50 to 80	3–4	0	140	Yes	Yes	Yes	Easy	Variable	[4, 8, 22]
Advanced intercross RI strains	80	4–5	0	140	Yes	Yes	Yes	Easy	Variable	[4, 8]
RI Intercross F1s (RIX, RIB)	100 to 200	4–6	0	50	Yes	No	Hard	Easy	Easy	[36, 38, 40]
Hybrid diversity panel (HDP)	1000	6+	0	20–150	Yes	Yes	Yes	Yes	Easy	[18, 19]
Collaborative cross (8-way RI)	135	4–6	0	195	Yes	Yes	Yes	Easy	Variable	[13, 17]
Diversity outcross (DO HS)	400+	5–7	100	55	No	No	Hard	Hard	Easy	[84, 85]
Outbred stock (e.g., CD-1, CF-1)	1000	6+	100	7	No	No	Hard	Hard	Easy	[68, 79]

^aCosts do not include shipping

each unique genometype (genetic individual) is represented by a stable inbred strain that can be replicated in large numbers—essentially a sexually reproducing clone. RIs are therefore an excellent resource for studies that benefit from replication across individuals (e.g., dosing and toxicity studies of genotypes) or across environments (i.e., studies on $G \times E$), and for the gradual assembly of deep phenome data that can be used in G2P analysis. In mice, there are now sufficient numbers of RI strains to allow for comparatively precise and well-powered QTL mapping. There are currently two major types of RI strains in mice:

1. Classic two-parent RI strains. There are a total of about 340 of these types of mouse RI strains, including ~150 BXD available as live stock and many other small RI families: AXB/BXA (29 live), AKXD (20 cryopreserved), BXH (12 live), BRX58N (7 cryopreserved), CXB (12 cryopreserved), ILSXIIS (60 cryopreserved), LGXSM (~18), NXSM (15 cryopreserved), SWXJ (13 cryopreserved).
2. The Collaborative Cross (CC). This is a unique eight-way RI set of about 100 strains that is now in widespread use for QTL analysis and systems genetics [13, 17]. These strains are available both from UNC Chapel Hill and the Jackson Laboratory.

Classic RI strains that are derived from standard F2 intercrosses harbor more recombinations per genome—about 40–50—than do backcrosses (10–15), or F2 intercrosses (20–30) and therefore deliver better QTL precision than one might expect even with modest samples size (Fig. 1, note the alternating red and white haplotype blocks that make up the chromosomes of the RI strains). The ability to resample individuals also reduces the impact of non-genetic trait variance—effectively boosting heritability [65]. Pandey and Williams [66] computed the empirical precision of *cis*-acting expression QTLs (*cis*-eQTLs) in the BXD family across the whole genome at different mean LOD scores and at different marker densities (their Fig. 8.6). With a cohort of 67 strains and using only two samples per strain, eQTLs with LOD scores of between 3 and 5 were located within ± 2 Mb of the parent gene. Those with LOD scores above eight were typically within ± 1 Mb. Corresponding empirical mapping precision based on *cis*-eQTLs can now be easily computed for many resource types across the whole genome using data sets and queries built into GeneNetwork ([67], this volume). Examples of doing this for a large AI ($n=811$) and a well matched AI-derived RI set ($n=40$) are given in **Note 2**.

The CC RI strains are capable of even better mapping precision than standard RIs for two reasons. First, the recombination load (the crossover probability) of CC strains is 1.75 times higher than that of typical two-parent RI strains due in part to the rounds of intercrossing required to merge all eight genomes

(Table 5 of ref. 16). Second, the inclusion of multiple parental genomes within the CC means that it is possible to carry out a fine-grained haplotype contrast analysis that can effectively reduce QTL intervals and numbers of QTG candidates [68]. Haplotype contrasts of the same general type can also be exploited using combinations of conventional RI families, inbred strains, and F2 crosses [18, 25, 63, 69].

The most important disadvantage of conventional RI strains and other standard two-parent crosses is that they segregate for only a fraction of all known polymorphisms. For example, the BXD family segregates for a total of ~5.2 million sequence variants—about 44% of common variants among standard inbred strains [70]. Some stretches of the genome will be almost completely identical by descent [6] and these regions will not normally contribute much to trait variance. This disadvantage however may also be viewed as an advantage when trying to dissect a QTL, since the load of polymorphisms within an interval will be about sixfold lower than that of the corresponding interval in the CC or DO stock, and thus the number of viable candidate genes may be much reduced. As shown by Li and colleagues, phenotypes that map into these genetic blindspots can be particularly easy to map to QTNs [52].

A practical disadvantage of RI strains is that they often have poor breeding performance compared to many F2s and outbred stock. While BXD strains average 4–5 pups per litter, some are hard to maintain and can be sensitive to housing conditions. Many CC lines have even lower fecundity. This is one reason why many inbred strains are so much more expensive than outcross or HS animals (Table 1) and why they are often cryopreserved rather than kept as live stock. This issue was also a factor motivating the creation of the DO: The DO provides a way to stabilize recombination events that were at risk of extinction (Gary A. Churchill, personal communication). Speaking of the obvious, a final disadvantage of RI strains is that they are inbred—an anomalous genetic architecture that will not only decrease fitness but will often increase trait variance relative to isogenic F1 hybrids due to the loss of heterosis and allele buffering.

2.3.8 Advanced RI Lines

There are also several interesting variants of RI strains. The first of these are highly recombinant RI strains generated from AI progeny [9]. Many of the new BXD strains (BXD43 and higher) are AI-derived [4, 71], as are all of the LGXSM strains [72]. Instead of directly inbreeding siblings of an F2, progeny are crossed to avoid sib matings for as many as 30 generations, prior to the inbreeding phase (another 20 generations). The main benefit of using AI stock for making RI strains is a significant increase in potential QTL mapping precision (*see Note 2*), but as usual, with loss of power.

2.3.9 RI Backcrosses (Fig. 1.7)

The second variant involves making a set of F1 intercrosses between RI strains and a single inbred strain—usually one that carries interesting modifier alleles with a dominant or additive effect. For example Hunter and colleagues crossed 18 AKXD RIs to an FVB strain carrying a dominant cancer gene variant to map modifiers of metastasis [29]. They refer to this cross as an RI backcross (RIB) because the 18 sets of F1s are similar to a backcross—those chromosomes inherited from the RI parent are recombinant, whereas those inherited from the other strain are not. This idea can also be generalized across multiple RI sets and inbred strains. For example, Bennett and colleagues crossed an APOE transgenic strain to more than 31 common inbred strains and 66 BXD, AXB/BXA, BXH, CXB RI strains [40] to study the genetic architecture of atherosclerosis.

2.3.10 RIX Panels (Fig. 1.6)

RIX panels are a clever new extension of RI strains that have some interesting advantages over RI strains and HS. Given a set of 10 RI strains, it is simple to cross all of them to each other: 1×1 , 2×1 , 3×1 , 3×2 and reciprocal crosses 1×2 , 1×3 , 2×3 , and so on. From only 10 starting strains one can produce a full diallel set made up of 100 isogenic sets of F1. In a full diallel we do not gain much precision by resampling the same parental haploid genome in different combinations (1×2 , 1×3 , 1×4 , etc.). While no new recombination event occur in making these F1s, one does expose an interesting range of phenotypes, such as those exploited by Rasmussen and colleagues [73] to develop mouse models of Ebola infection.

What makes RIX particularly attractive now for both mapping and systems genetics is that we have several large sets of RI strains—more than 100 BXDs and close to 100 CC lines. While it is not practical to generate or study a full 200×200 matrix of 40,000 RIX progeny and founders, it is practical to sample all 200 of these RI genomes by making 100 nonoverlapping sets of RIX litters: 1×2 , 3×4 , ... 198×199 , and 199×200 . And two different RI sets can be crossed (e.g., BXD1 to CC001). A set of 100 disjoint (nonoverlapping) RIX progeny solves a number of problems—(1) efficient sampling of large RI families that exploits all recombination events in the parental RIs; (2) much lower inbreeding coefficients than inbred parents; (3) genetic complexity much more like that of human populations; (4) ability to study parent-of-origin and dominance effects; (5) fully defined genomes; (6) deep replication of any particular RIX to increase phenotype precision; (7) more direct analysis of $G \times E$ using precisely the same genotypes under two or more conditions; and as a (8) powerful resource to test predictive models of G2P relations.

Disadvantages of RIX sets include the following: (1) they can be costly to generate compared to HS or DO stock; (2) there will be a loss of genetic variance associated with the heterozygosity of RIX progeny compared to homozygous parents [74]; (3) breeding and cohort logistics are somewhat more complicated and

expensive; and (4) it will be difficult for a community of researchers to define a single reference set of RIXs to use for collaborative phenotyping because there are such huge numbers of potential RIX that can be made.

2.3.11 Hybrid Diversity Panels (HDP)

An HDP is an aggregate of RI strains and common inbred strains that are usually phenotyped together and used as a single joint mapping resource [19, 69, 75, 76]. They are used for at least two reasons: (1) to achieve comparatively high mapping precision (intervals of 1–5 Mb) that can match those of HS and DO stock using as few as 100 inbred strains; (2) to make it possible to assemble large phenomes that can be used for $G \times E$ analysis. A HDP does not have a rigid definition, and a mouse HDP could and should include CCs, BXDs, and even RIX. Depending on its membership of isogenic genotypes, an HDP will share some of the same problems of any one RI family, but to a lesser degree. For example, the issue of genetic blind spots will be less serious except for a few regions of the genome that tend to be identical-by-descent even in the CC. The main problem of an HDP is the generally low to moderate fecundity of members and their high acquisition costs.

2.3.12 Outbred Stock (OS), Heterogeneous Stock (HS), and Diversity Outcross Stock (DO)

Outbred stock (OS)—often referred to as Swiss Webster stock [77, 78]—are the progeny of nine albinos (two males and seven females) imported from a colony in Lausanne to New York in 1926. They were subsequently distributed to researchers and commercial vendors worldwide as “standard laboratory” mice. As expected given this history, OS do not incorporate much genetic variation. Genomes of 66 OS colonies studied by Yalcin and colleagues [79] were heterozygous at no more than 34% of polymorphic loci, and a significant number of colonies were almost fully inbred. The theoretical attraction of some OS colonies is their potential high mapping precision with LD blocks that are only a few hundred kilobases.

HS and DO could be considered variants of OS, but here we use a modern definition of HS and DO as special stock generated from well-structured intercrosses and outcrosses among diverse sets of inbred progenitor strains. HS are almost always maintained using larger colonies—50 or more breeding cages—and breeding schemes that minimize mating of closely related individuals. One original motivation to make HS was to produce new models by intercrossing diverse strains, and then selectively breeding progeny for high and low phenotypes in responses to drugs, alcohol, and other treatments [80, 81]. The Northport HS (HS-Npt) made by intercrossing A/J, AKR/J, BALBc/J, CBA/J, C3H/HeJ, C57BL/6J, DBA/2J, and LP/J is a good example [82]. HS have also been used for high precision QTL mapping [83].

The DO is an example of a modern HS made by intercrossing early generations of the CC [84, 85]. DO mice are significantly

more diverse even than HS-Npt or outbred stock for the simple reason that three of the progenitors of the DO and CC—PWK/Ph, CAST/Ei, and WSB/Ei—are inbred strains derived from highly diverse wild *Mus* species and subspecies. DO cohorts are now at the 22nd generation (G22) of outcrossing. The DO segregate for well over 40 million common sequence variants with minor allele frequencies above 10%. These animals breed well and incorporate four to sixfold more genetic variants than the number of common variants in human populations.

There are two key advantages of DO and HS: (1) they have a genetic complexity that equals or exceeds that of most human populations. They are excellent models for precision medicine; (2) like AI cohorts, they gradually accumulate large numbers of recombinations and therefore can resolve QTLs with high precision; (3) the high genetic diversity among parental strains ensures that phenotypes will be highly variable and that most regions of the genome will be polymorphic; and (4) they usually have excellent breeding performance, a feature that reduces costs.

The main disadvantages of HS and DO stock is the inevitable flip side: the high recombination load and map expansion will reduce statistical power per case and the high genetic complexity and numbers of haplotype can make it difficult to resolve single linked QTGs and QTNs. The last and most obvious experimental disadvantage is that HS and DO animals are genetically unique. This means that it is more difficult to acquire phenomes for these types of resources or to use them as effectively in G×E studies.

2.4 A Multipurpose Design for Systems Genetics

In this section we consider some of the designs that can now be used to address the four types of questions in Subheading 2.2. In the first section below (2.4.1) we consider Type 1 questions with a focus on mapping precision. In the second section (2.5), we start to wrap everything together by considering a single adaptable design for systems genetics that will be good for discussion purposes. We comment on ways to modify or extend this multipurpose design using a Question and Answer format. Much of the text is summarized in Tables 1 and 2.

2.4.1 Genotypes and Genetic Maps: What Mapping Resolution Is Needed?

The goal is usually to get down to about 1 Mb precision as efficiently as possible. Assume we are completely naive—we only know what traits interest us and that traits are somewhat variable among individual mice belonging to a few strains or stocks. We do not have estimates of heritability and we do not yet know what strains or crosses would be most useful.

One of the best resources in this situation is to study phenotypes in a small number of strains and F1 hybrids between these strains. This made sense several decades ago [75] and it makes even more sense today [86] because these initial “survey” data can eventually be wrapped into a mapping study with all other resources—whether

Table 2
A design for systems genetics using mouse resources

Group	Types	Type notes	N	Reps	M	F	Months
Group 1A	8	Fully inbred strains	48	6	3	3	2.4
Group 1B	4	4 F1s using 8 genotypes	24	6	3	3	1.2
Group 1C	8	Your choice	48	6	3	3	2.4
Group 2A	40	CC or BXD, AXB (exploratory)	160	4	2	2	8
Group 2B	40	CC or BXD, AXB (selective)	160	4	2	2	8
Group 3A	40	RIX (semi-exploratory)	160	4	2	2	8
Group 3B	40	RIX (selective)	160	4	2	2	8
Group 4A	100	DO or HS (predictive)	100	NA	50	50	5
Group 4B	100	DO or your choice	100	NA	50	50	5
Sums	380		960				48

HS, DO, CC, or RIX. For example, a study of 6 individuals each of 18 isogenic groups, such as sets of fully inbred strains some of their F1s, will answer questions related to trait heritability, trait dominance, and if you are lucky, even give you hints about genetic complexity and architecture. It may be possible to evaluate if the trait or disease phenotype is controlled by a small number of QTLs (the oligogenic model) or by hundreds of QTLs (the polygenic or “infinitesimal” model) [75]. This 120-case study will also enable you to perfect phenotyping and learn much more about sources of technical error, sex differences, and selecting better resources for the next stages.

The main risk in this type of pilot study is batch effect and phenotype drift. Systematically phenotyping strains *A* through *R* at a steady pace of one genotype per week over 4 months is a poor experimental design, since temporal variance and drift will masquerade as a heritable difference among lines. Interleave the phenotyping to study ten different genotypes with one or two individuals each for the first phase of the experiment and then repeat cycles as needed. An interleaved design may not be feasible in all situations, in which case consider re-phenotyping well-known strains throughout a study to check for drift.

2.4.2 Mapping Precision

While more mapping precision is always a good thing, there is not much justification to refine maps down to much less than confidence intervals of 1–2 Mb. Intervals of this size can now be efficiently dissected using an impressive and diverse array of data resources—including of course, full genome sequence for all genes in all strains. A small number of candidate genes and variants can

now often be tested efficiently using genetically engineered mice, fish, flies, worms, or human GWAS data sets, in vitro analyses, or even phenome-wide association [22].

Another reason not to obsess about precision much below 1 Mb is the fuzzy functional definitions of genes. This is highlighted by a recent analysis of one of the strongest loci that modulates obesity in humans—SNPs within intron 1 of the *FTO* gene. While the position of linkage is not in question, these SNPs apparently tag variants in a long-range enhancer of *IRX3*—a small transcription factor 0.5 Mb distal [87]. This emphasizes that functional validation is critical, and that the law of diminishing return can kick in with some force under 1 Mb. We consider a 0.5–1 Mb as a reasonable goal that can usually be achieved efficiently using a combination of resources described below. This is not quite as precise as what can be achieved with large GWAS, but unlikely human studies we can efficiently transition to molecular mechanisms.

2.4.3 Assumptions

To develop this multipurpose design we assume almost nothing other than that the traits of interest are heritable and genetically complex, and that the initial focus is not on $G \times E$ or treatment effects, developmental stages and ages. We will come back to extensions that these types of questions toward the end of this section.

Sample size and costs of stock. As our starting parameter, we budget for 240 individuals per year over a 4-year period—960 cases total at a pace of 20 per month and 1 per day. This is a modest throughput that should be adaptable to almost any type of study, even electrophysiology, advanced imaging and behavioral methods. The cost of mice may range from as little as \$20 per case to as much as \$200. Standard inbred strains such as those used to generate the CC cost between \$20 (C57BL/6J) and \$200 per animal (WSB/EiJ) with an average of \$102. The average price for most of the resources discussed in this chapter is currently about \$150 per case. An experiment using 240 cases/year will typically require a budget of ~\$40,000/year. Housing costs are variable, but it is safe to assume 25–50 cages will incur a cost of \$10,000–\$20,000/year. If cases must be genotyped (e.g., F2, HS, and DO stock), then factor in a charge of as much as \$100 per case (Table 1).

Sex balance. Whenever possible males and females should be used in roughly equal numbers and concurrently. Not only is the use of both sexes becoming a mandate, but results will also be more interesting and robust in terms of their translational relevance. Finally, sex differences can provide mechanistic insight. The inclusion of both sexes in a design does not double the required sample size, even when using isogenic cohorts of RIs, RIXs, or HDPs. A balanced sample of just one or two males and females across multiple genotypes can be a powerful design to detect sex differences. Of course, sparse sampling does not address sex differences within any single strain, but this is a topic that may be worth revisiting in a second phase of work.

While it may look tidy in a Methods section, it is not necessary to get numbers of cases balanced precisely either by sex or genotype. Do not obsess about filling every cell in a design uniformly. If you must obsess about anything, make it (1) batch confounds, (2) drift in phenotyping standards, and (3) quality control for electronic records and case identifiers. When possible consider whether litter effects are a confounding factor in phenotype variation. This is a particular risk for RIX designs in which one single litter may be used for each genotype.

2.5 Experimental Design for Systems Genetics

2.5.1 Stage 1:
Heritability, Technical Robustness of Assays, Effects of Sex, and Genetic Architecture (Table 2)

The main purpose of phase I is to make sure you understand more about the main sources of variance of phenotypes. It is well worth a 3–6 month pilot to make sure the phenotyping methods and assays work well. The data from this initial work will eventually be useful for mapping.

Group 1A: Six individuals each of eight inbred strains. It would make great sense to start with the parents of the CC. Depending on your field of study you could add or substitute AKR, BALB, DBA/2J, FVB, or other common strains.

$$n = 48$$

Error-checking: Since assignment errors can destroy your results, keep track of coat color, and even better, save tails of animals for *post hoc* genetic verification. This is important for all stages of the work.

Group 1B: Six individuals from each of four F1 hybrids made using strains A through H (AB, CD, EF, GH, or the reciprocals AB, BA, CD, DC). The parental strains for the F1s can be selected based either on greater genetic differences or on contrasting phenotypes.

$$n = 32$$

Group 1C: Six individuals from each of eight additional types based on the initial results above, or to encompass other interesting strains selected from the Mouse Phenome Project (phenome/jax.org) or based on any interest you have in RCC methods. You could also use this set of 48 cases to resolve problems or seize opportunities. This set could include F1 hybrids.

$$n = 48$$

Question 1: Is six samples per type really enough? ANSWER: If you are not examining different environmental factors, then yes. In fact, you probably should not do 6 per type at any one time or from only 1 or 2 litters, but break work into analysis of 2–4 cases for each of 12 types, and generate data over several batches. You may want to run pairs of males and females (littermates even) in single batches, since you are likely to be used paired *t* tests. If you find that the batch effects are large, then you have learned something important and may need to rethink the design of the larger study. If you find that there is variation as a function of age, you have also learned

something important. Furthermore, after phenotyping six per type, you will have a good idea if any particular phenotype needs to be resampled to higher *N*s. See **Note 3** that discusses some of the factors that should be considered when selecting number of biological replicates.

Question 2: Should I use wild strains such as PWD/PhJ, CAST/EiJ, or WSB/EiJ? **ANSWER:** Yes, unless there is some specific contraindication, such as cost, availability, or wildness. There is no reason to not expose yourself to the remarkably wide range of phenotypes at this stage. (Make sure you unbox wild strains carefully or you will have stories to tell.)

Question 3: Should I use HS or DO stock initially? **ANSWER:** No, not unless you have already used these types of resources or need them to address a specific hypothesis. You cannot estimate heritability from a single cohort of HS animals.

Question 4: Should I phenotype pairs of closely related substrains? **ANSWER:** Probably not at this stage unless you already know that there are significant differences in related phenotypes among substrains. If you are interested in exploiting RCC methods then include pairs or trios of substrains in Group 1C. Genetic variance will be lower in substrain contrasts, so you will need to increase sample size to 8–12 per type.

Question 5: Why are F1 hybrids useful? **ANSWER:** For at least these three reasons: (1) F1 hybrids are used to evaluate effects of gene variants on phenotypes in organisms with a more typical heterozygous genome. F1 hybrids are isogenic so they have many of the advantages of inbred strains. (2) F1 hybrids also enable us to evaluate whether phenotypes are dominant or recessive. (3) Reciprocal F1s can be used to study parent-of-origin effects on phenotypes. Note that some of these advantages do not apply to F1s between closely related substrains.

2.5.2 Stage 2: Low Resolution Mapping and Systems Genetics

The purpose is to understand the genetic complexity of phenotypes by low-resolution mapping but with good power. If there are a few QTLs with large effects then even a cross with 40 genotypes will highlight one or two loci. Since we rely on RI strains for this first analysis, it should be possible to compare all new data with all previously generated phenotypes and QTLs. We can be confident to find some interesting leads, generate new hypotheses, and perhaps even gain mechanistic insight.

Group 2A: Four each of 40 RI strains. Use four each if heritability is <0.4 , otherwise consider using two each of 80 strains, particularly if you suspect that trait variance is controlled by a major effect locus. You can always return to the RI strains to boost your samples size.

Group 2B: Same as above, but using a new set of 40 RI strains. You will now already know if you have detected suggestive or significant QTLs. If the answer is yes, then you can selectively phenotype those RI strains that have recombinations between the right haplotypes in the right regions. You might also want to replicate any outlier strains detected in Group 2A. If the results from Group 2A do not yet provide compelling candidates, then just forge ahead with more or different RI strains.

$$n = 160$$

Question 6: Could I not use RIX in Group 2B? **ANSWER:** Yes, since you will have RI strains available, this is an option. However, the RIX will not provide you much more genetic signal unless you use different RI parents to make the RIXs. RI and other fully homozygous strains have twice the genetic variance of F1 hybrids. This gives them a power advantage at early stages of mapping.

Question 7: Should I use BXDs, AXBs, or the CC strains? **ANSWER:** The CC will almost always be a good choice, as they are likely to exhibit the highest phenotypic variance in any target phenotype. BXDs and AXBs will provide better mapping power *per case* due to their lower genetic complexity, but this benefit can be neutralized by less phenotypic variance. If the parents of the RI panels differ markedly and your focus is more on systems genetics than mapping precision (e.g., C57BL/6J vs DBA/2J), then the BXD may be the best first choice for the simple reason that so much data has been accumulated for these strains. Availability of RI strains can sometimes be the main constraint.

Question 8: Can I mix CC stains with other RI panels? **ANSWER:** Yes, and this is precisely the motivation for resources such as the HDP. It is probably a good idea to sample at least 16 strains in any one RI set so that you can evaluate whether or not a locus is segregating and so that you can estimate trait covariance to some degree among phenotypes within single RI families.

Question 9: Should I use consomic or congenic panels for this work? **ANSWER:** No; not unless your screen in part 1 included PWD/Ph and A/J and suggested that these strains differed markedly from C57BL/6J. These are the strains that have been used to make consomic sets. Consomic strains can have good power if you sample each of 20 strains with 6 or more cases, but to achieve mapping precision (± 5 Mb), you will have to generate your own derivative crosses, and effect sizes of loci can evaporate during the production of congenics [56].

Question 10: How do I handle outlier strains in the initial QTL analysis? **ANSWER:** Transform data so that outliers do not have an overwhelming effect on maps and other statistical results. You can winsorise high and low outliers or use a logarithm transform. Replicate outliers if you suspect technical error.

2.5.3 *Stage 3: High-Resolution Mapping and More Systems Genetics*

Group 3A: Four each of 40 sets of RIX progeny that are produced by crossing within or even across sets of RI strains. You will need 80 RI strains to make 40 nonoverlapping RIXs. Vendors may be willing to do this for you if the strains are not available to you. At this point you will almost surely have a small set of reasonably well mapped loci. You will also have enough data to decide if you want to reevaluate your questions. Are you really after QTGs, do you want to test a specific intervention, or do you want to try your luck at G2P prediction using a set of molecular and genetic biomarkers? This first set of 40 RIX progeny should enable you to do all three.

$$n = 160$$

Group 3B: Same as above but this set could be generated to test an intervention or age (using Group 3A as a control). Or this RIX group could be created selectively to test multilocus interactions or parent-of-origin effects.

$$n = 160$$

2.5.4 *Stage 4: High-Resolution Mapping, Predictive Validation, and Systems Genetics*

The combined results of the three stages should have left you with a set of loci mapped to less than 2 Mb. If that is not the case, then this final stage should help achieve that goal. Ideally, you might want to select DO stock on the basis of genotype, and that may be a service that will soon be available. This would be most useful if only one specific haplotype contrast is generating trait variance (e.g., a 1 vs 7 split of haplotype effects).

Group 4A: DO or HS. DO stock will probably be most accessible and also generally most suitable.

$$n = 100$$

Group 4B: Your wildcard. You could continue with a second set of 100 DO mice if the first results strengthened results. Or you could use the DO mice you still have to selectively cross animals with specific combinations of alleles. This would require selective genotyping of specific SNPs. DO mice are a wonderful source of genetic variance, but you may want to select or trim back some of those variants. This will position you well to predict phenotypes based on combinations of haplotypes at two or three loci.

Alternatively, use this group of cases for further studies on the effects of treatment, age or stage (see Group 3B).

$$n = 100$$

Question 11: How do I genotype DO or HS? **ANSWER:** Even in the most demanding situation of mapping DO, HS, and wild caught populations, markers need only be about 100 kb apart [79], and since the mouse genome is about 2.5 Gb, 100,000 well chosen

markers will be more than adequate. Virtually any population, no matter how complex its genetic architecture can now be typed using the latest version of the mouse universal genotyping array (the GigaMUGA) or by sparse sequencing for about \$100/case [88].

For selective genotyping of a handful of markers in DO or RCC F2 intercrosses you can use standard protocols that will probably require acquiring sets of PCR primers. Costs may be as high as \$1/genotype/case. If you require a few hundred markers per case then a good ballpark cost for custom genotyping is under \$0.10–0.20 per genotype per case—or \$20–40 for 200–2000 markers for an F2 progeny. Finally almost all inbred, RI strains, an RIX progeny are already well typed and there is no cost at all.

Question 12: Is there a strong justification to use all of these types of resource—RIs, RIX and HS/DO? **ANSWER:** These resource types perform many of the same functions. However, $G \times E$ will be easier to study using RI and RIX. RIX progeny made using CC RIs are genetically similar to DO animals, but incorporate fewer recombinations per animal. Data from RIX cases can also be used to build up a phenome database and are potentially more useful for large collaborative teams, but this advantage may remain theoretical for the next several years. DO/HS animals are logistically far easier to obtain and provide you with access to the ultimate breadth of genetic and phenotypic diversity. They are the closest you can get to a wild-type mouse population short of capturing your own. If you results from Stages 1 to 3 are supported in DO populations, then you can be sure that results will have the maximum replicability and perhaps even translatability to human populations. You may also be able to computationally and genetically “extract” specific disease models from RI, CC, and DO stock.

3 Future Directions and Conclusions

Thanks to the massively reduced cost and increased scope of omics technologies, it is now feasible for small collaborative groups—and even single research groups—to execute large studies in systems genetics. We can anticipate that the use of this new systems paradigm will accelerate in the coming years with the advent of new and improved methods of quantifying an individual’s proteomes, metabolomes, metagenomes, and epigenomes as a function of cell type, tissue, age, and state. It is great to have the core animal resources that are needed to take advantage of this rapidly expanding set of omics technologies.

What we have not considered in this chapter is the analytic and synthetic tools needed for high-content systems genetics. How do we actually map aggregated data from 1000 cases with complex substructure? How do we build predictive models and test their fit to empirical data? These questions are taken up in many of the chapters in this volume.

4 Notes

1. What is *population substructure* and how does it make statistical analysis and mapping trickier? We all have learned that observations used in many statistical tests should be independent. In genetic crosses all F2 progeny are usually treated as independent observations. But what if there are strong litter effects, or batch confounds due to technical errors. These effects can introduce variance into a cross that can obscure the detection of the genuine effects and produce spurious linkage. Similarly, in an AI cross, one mating pair may produce 50 siblings whereas another mating pair produces only 5. In this case we have known and unbalanced pedigree substructure that needs to be corrected even when doing something as simple as computing a correlation coefficient. Large GWASs sometimes combine data from different ethnicities and it is also essential to correct statistically for the kinship relations among members. In some cases we can use the genotypes of cases to compute a matrix of kinship similarity, and use this matrix to correct for the population substructure. If we know the litter and batch identifiers we can also adjust for these nuisance variables in a statistical model.

In large RI sets such as the BXDs and CC, there is cryptic substructure that may not show up easily in genotypes but that that may still be important. The BXDs for example, were generated in multiple cohorts between 1970 and 2013 using the same parental strains—C57BL/6J and DBA/2J, but of course, 43 years of breeding history will add many new variants to both parents and some of these are already well known to have important effects [89].

2. To estimate empirical precision for QTLs across a population in GeneNetwork (www.genenetwork.org) you first need to select an expression data set from the pull-down menu. In this example, select **Species** = *Mouse*, **Group** = *B6D2 AI PSU*, **Type** = *Muscle mRNA*, and **Data Set** = *PSU B6D2 AI Muscle...*

Enter this query into the **Get Any** box:

cisLRS = (23 46 50)

where cisLRS is the linkage statistic specifically for the *cis*-acting eQTLs. The first two values in parentheses are the minimum and maximum LRS values to return ($LRS = LOD \times 4.61$), and the final number is the size in megabases of the acceptance window used to define how close a gene must be to the QTL peak to be considered *cis*-acting. In this case the acceptance window is very broad, and the peak LRS can be anywhere 50 Mb on either side of the gene.

This search will generate 2086 hits. You can resort and download the results as an Excel table using the **Download Table** button. In this large F2 intercross with more than 800 cases

generated by Ari Lionikas and colleagues between C57B/6J and DBA/2J, the mean offset between 2000 *cis*-eQTLs with LOD between 5 and 10 and their genes is 7.0 ± 0.21 Mb.

If you try precisely the same set of operations with a matched BXD Advanced RI data set (*EPFL/LISP BXD CD+HFD... Exon Level*) you will find that the mean offset between 4400 *cis*-eQTL in this data set is 2.0 ± 0.06 Mb. The latter ARI data set is based on ~320 cases (1 array with 4–5 pooled samples for each of 40 strains under two conditions—high fat and standard chow diet [21]).

3. Genetic studies usually benefit more by increasing the n of genotypes that are phenotyped than by increasing the n of replicates per type (e.g., Fig. 1b in ref. 90, and *see* ref. 65). All else being equal, a studying of 160 types without replication should be superior in terms of QTL results to one of 40 strains and 4 replicates of each. This is obvious for Mendelian traits such as coat color, but it also holds true for quantitative traits—even those with low heritability. However, at an early stage of a study it is vital to understand heritability and technical confounds and in some cases, replication is easy and cheap. For this reason, it is a good idea to begin work with six to eight replicates of a few “reference” genomes. When using isogenic cases we recommend two replicates minimum, one per sex. Bumping this up to two per sex per strain will improve the comfort level of many reviewers, although to keep them happy you will probably need 6–8 per group. There are also some good reasons to study six or more cases per genotype even after heritability is known: such as studies of genetic control of variation itself [91] or pharmacological effect thresholds.

One way to think about the diminishing returns of high replication rates is to compare t scores and z scores required to achieve statistical significance for simple two-sample comparisons using different sample sizes. The z score assumes variance of the population is known and the critical value to reject the null at alpha 0.05 is $z = 1.96$. In contrast, the t score estimates variance from the sample itself, and the critical values start at a woefully high 12.71 for $n = 2$, but drops toward the asymptote of 1.96 very quickly: 3.182 for $n = 4$, 2.757 for $n = 6$, and 2.201 for $n = 12$.

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