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Protocol 2: Using the Collaborative Cross to study the role of genetic diversity in cancer-related phenotypes

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Abstract

Human populations are genetically diverse and often a single mouse model can only represent a small subset of the human population. Studying genetic diversity directly can improve the predictability of mouse cancer models for the biology of cancer and the effect of carcinogens and therapeutics in humans. The Collaborative Cross (CC) is a panel of inbred mouse lines that captures 90% of the genetic diversity of the *M. musculus* strain and can identify regions of the genome that are responsible for variation in cancer phenotypes across the population.

Materials:

The materials necessary depend on the cancer model being tested, whether carcinogen, pathogen, genetically engineered mouse model, or syngeneic tumor graft model. Statistical methods for analyzing results and mapping genome regions that control variable expressivity of phenotypes are rapidly evolving (Threadgill and Churchill 2012). Several websites provide up to date information on methods for use of the CC (<http://csbio.unc.edu/CCstatus/index.py>; <http://compngen.unc.edu/wp/>; <http://churchill.jax.org/research/cc.shtml>; <http://mus.well.ox.ac.uk/mouse/>). It is important that a qualified statistician be involved in the design and analysis of CC experiments.

Reagents:

1. CC mice across as many different lines as available from the Systems Genetics Core, University of North Carolina (<http://csbio.unc.edu/CCstatus/index.py>). Approximately 100 independent lines should be used in initial experiments. Additional lines can be chosen to refine mapping of the sources of genetic variability (Williams et al. 2002).
2. Mouse model of cancer (see Protocol 1 of this chapter as well as other protocols throughout this book)
 - a. carcinogen induction of tumorigenesis (e.g. skin or colon tumors)
 - b. viral induction of tumorigenesis
 - c. genetically engineered mouse model of cancer with dominant inheritance pattern (e.g. oncogenic transgene or heterozygous tumor)

suppressor that spontaneously develops tumors), preferably on an inbred strain background.

- d. mouse tumor cell lines derived from well-defined, inbred mouse strain and wild-type mice from the syngeneic strain
3. Genotyping reagents (see Protocol 1)

Equipment:

1. Computer with database to follow mouse breeding (see Protocol 1) and statistical software

Method:

Three mouse cross designs are described as examples, but the details will depend on the individual model being tested. This protocol focuses on considerations for the design of mouse crosses to focus on genetic effects. Statistical methods will depend on whether the phenotype is discrete or continuous and whether the CC lines being used are fully inbred and sequenced. In the first example individual CC mice are tested without requiring additional breeding. This example could be used for testing the variability in response to a carcinogen or pathogen (Durrant et al. 2011; Bottomly et al. 2012; Ferris et al. 2013), for looking at variability in drug pharmacokinetics or toxicity (Gelinaz et al. 2011), or examining cancer-related physiology, such as variation in body weight (Mathes et al. 2011b), hematopoietic measures (Kelada et al. 2012), metabolic measures (Thaisz et al. 2012), diet, or exercise (Mathes et al. 2011a). The second example describes crossing a genetically engineered mouse cancer model onto CC lines to identify genomic regions that enhance or suppress a tumor phenotype or test novel therapeutics in genetically engineered mouse models. This approach can help identify the optimal strain background to study the cancer phenotype of interest. The third example describes how to use tumor lines from inbred mouse strains (e.g. see Gursel et al. 2011 and Zemp et al. 2013) in hybrid CC mice to generate cancer “patient populations” for preclinical testing of novel therapeutics. The following are general considerations for all experiments.

1. Controlling for sex and cross design: In all of the following examples, the goal is to understand how genetic heterogeneity affects a tumor related phenotype, and it is therefore important to control for all other variables very carefully. The most efficient studies will maximize genetic diversity across as many different CC lines as possible, with minimal biological replicates. As described in more detail in the topic overview and Protocol 1, sex and parental inheritance of the strain background should be carefully controlled, choosing either one sex or cross design for the study, or equally balancing the sexes and/or reciprocal crosses.
2. Controlling for seasonal effects and other long-term confounders: There is evidence that changes in animal caretakers over time and seasonal effects can introduce bias, particularly in behavioral and metabolic studies (Chesler et al. 2002a; Chesler et al. 2002b). The seasonal effects may be due in part to changes in the availability of grain sources for mouse chow through the year. Soy meal or

alfalfa in chow can introduce differential exposure to estradiols (Thigpen et al. 1999; Brown and Setchell 2001; Thigpen et al. 2004; Thigpen et al. 2007). The study of different CC lines should be balanced (or replicated) across the seasons, particularly in complex breeding experiments that may not be able to be completed within a month's time.

3. Controlling for circadian effects: There is also strong evidence that daily circadian rhythms can affect responses to treatments (Filipski et al. 1999; Granda et al. 2001; Filipski et al. 2002; Granda and Levi 2002; Mormont and Levi 2003; Filipski et al. 2004; Filipski et al. 2005; Granda et al. 2005; Li et al. 2005; Li et al. 2006; Levi et al. 2007). It is therefore important to control the time of day that an intervention is performed, so that certain genetic backgrounds are not grouped in the morning, for example, while others are grouped in the afternoon. This could give the erroneous conclusion that an effect is related to a certain region of the genome, as opposed to the timing of treatment.
4. Handling of wild-derived lines: CC mice carry different genetic contributions from the wild-derived strains CAST/EiJ, WSB/EiJ, and PWK/PhJ, and can more difficult to handle than many classical inbred strains commonly used in cancer research (Philip et al. 2011; Threadgill et al. 2011). To avoid losing jumpy mice during handling and cage changes, it is helpful to place the cages to be opened in the bottom of a large sterile bin or tub with high sides before opening the cage. This helps to contain any mice that escape. Before ordering CC strains researchers should ask questions about the behavioral peculiarities of the lines they plan to buy from the supplier and warn animal caretakers in quarantine and housing facilities of what to expect (Threadgill et al. 2011).
5. Pilots in the eight CC founder strains: It is often helpful in the later analysis to perform the same experiments in the 8 founder strains available from Jackson laboratories (<http://jaxmice.jax.org/>; A/J cat #000646, C57BL/6J cat #000664, 129S1/SvImJ cat #002448, NOD/ShiLtJ cat #001976, NZO/H1LtJ cat #002105, PWK/PhJ cat #003715, CAST/EiJ cat #000928, and WSB/EiJ cat #001145). In this case, it may be useful to have biological replicates in 5–10 mice from each founder strain. This experiment establishes that the phenotype being examined is under the control of genetic factors present in the CC and supports the expansion of the experiments to 100+ CC lines (Campbell et al. 2012). This data also helps to determine the direction and size of the phenotypic shifts caused by different haplotypes and can be used to narrow the mapped genomic regions (Yalcin et al. 2005; Thaisz et al. 2012; Ferris et al. 2013).
6. Statistical considerations for experimental design: As mentioned above, modeling of the CC suggests that using the largest number of independent lines is more important than including many biological/technical replicates. For mapping the genomic regions underlying variable expressivity, it has been shown that better results are obtained using a haplotype inference method that can independently assign linkage to one of the eight founder strains (Aylor et al. 2011), as opposed to a single marker association analysis that distinguishes

different SNPs, but cannot definitely assign them to one of the eight founder strains (Figure 1A). Haplotypes can be inferred using a Hidden Markov model (<http://mus.well.ox.ac.uk/mouse/>) (Mott et al. 2000).

Example 1: Carcinogen/Pathogen/Cancer-Related Physiology (Figure 1B)

1. Import as many CC lines as possible from the repository as a single group to control for long-term biases of season and animal caretaker changes. Import inbred founder strains from Jackson laboratory if needed.
2. Once mice have cleared any necessary institutional quarantine procedures, allow animals to acclimate to the mouse facility, light-dark cycle, and diet before beginning the procedures. Different procedures may require different periods of acclimation (e.g. 1–2 weeks).
3. Perform manipulations and measurements on mice at consistent times of day (e.g. inject therapy for testing pharmacokinetics during morning hours and collect serums and body weights at predetermined timepoints).
4. Analyze linkage between phenotypic variation and founder haplotypes across the CC lines (see references for different approaches) and determine statistical significance of findings using permutation testing (Churchill and Doerge 1994; Doerge and Churchill 1996) or other correction for multiple hypothesis testing.

Example 2: Genetically Engineered Mouse Model of Cancer (Figure 1C)

1. To allow time for breeding and to control for seasonal effects, import batches of CC breeders (e.g. 20, 50 or 100) depending on the available space for breeding cages. Choose one sex for the CC line and the opposite sex for the genetically engineered cancer model.
 - a. Using a cancer model on a well-defined inbred strain background will dramatically save on the cost of genotyping because the SNP genotypes of the CC lines are being established (<http://csbio.unc.edu/CCstatus/index.py?run=Pseudo>) and the SNP genotypes of the F1 hybrids with the inbred strain can be inferred.
 - b. While experiments can be designed using more complex cancer models, generally models that can be bred as single dominant allele in the first generation (e.g. *Nf1*–/–; *Trp53*–/–*cis* (Reilly et al. 2000) or S100 β -v-erbB (Weiss et al. 2003)) will be most practical for these experiments (Reilly 2009).
 - c. Some cancer models (e.g. breast cancer models (Lifsted et al. 1998)) may develop cancer prior to the weaning of pups. In these models it is best to breed female CC mice to males from the cancer model.
 - d. CC mothers will pass on different maternal effects (e.g. mitochondria, milk composition and early post-natal nutrition) that must be taken into account in the interpretation of results. To avoid these effects, and to

examine the role of Y chromosome polymorphisms on phenotypes, breed males from the CC lines to females from the cancer model.

2. Breed CC mates to cancer model mates (1–2 mating pairs per CC line, in case one pair fails to breed) and genotype progeny for the genetically engineered alleles from the cancer model (see Protocol 1). Keep 1–2 male and 1–2 female progeny carrying the genetically engineered allele from each CC line for phenotyping.
3. If it is necessary to breed CC progeny in batches due to cage space constraints, stagger the breeding to every 2–3 months around the calendar year (e.g. September, November, and January) and breed a second litter from each line in a different season (e.g. February, April, and June). These genetic replicates will allow the birth month to be included as a covariate in the analysis and help control for seasonal effects.
4. Collect phenotype data according to the cancer model.
5. Analyze linkage as described in Example #1 (4).
6. Test whether sex or birth month interact with the identified loci.

Example 3: Syngeneic Tumor Graft Model (Figure 1D)

1. Import as many CC lines as possible as described in Example #1 (1). Choose one sex for the CC line and the opposite sex for the inbred strain syngeneic with the tumor cell line to be tested (Strain X).
 - a. Because inbred strains can undergo genetic drift over time, it is important to verify that the tumor cell line can be successfully grafted into Strain X before setting up crosses to the CC panel.
2. Breed CC mates to Strain X (1–2 mating pairs per CC line, in case one pair fails to breed), keeping the strain of the mother or father constant. Select one sex of the offspring to use in grafting experiments. Use 1–2 animals from each CC line.
 - a. Tumor lines can grow at different rates in male and female hosts (Perrin et al. 2007), so it is important to pick one of the sexes as the graft recipients.
3. Implant tumor cells into the CC × Strain X hybrid according to the model and measure the desired tumor parameters (e.g. rate of growth, response to therapy, etc)
4. Analyze linkage as described in Example #1 (4).

Discussion:

The development of the CC has been accelerating rapidly over the past decade, with new methods and tools still in flux. The structure of this panel of lines offers many benefits for understanding how genetic heterogeneity will affect tumorigenesis and treatment (Collaborative Cross Consortium 2012). To get the most out of these rather costly

experiments, it is critical that studies be designed to minimize all confounding variables. By incorporating controlled genetic heterogeneity into mouse models of cancer, it is hoped that these models will be more predictive of tumor behavior and response to therapy in humans. This has the potential to greatly reduce to clinical costs of developing new therapeutics and to help develop predictive markers for prevention and early detection of cancer.

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Figure 1:

Examples of methods for using CC to study genetic heterogeneity in mouse models of cancer. (A) Single marker-based association (red box) cannot conclusively assign a particular point in the genome to a particular founder strains. Founder alleles can give opposite effects on the phenotype and cannot be appreciated by this method. Inferring the founder haplotype (blue box) across regions of the genome allows one to assign a particular point in the genome to a particular founder haplotype (in this case the B6 strain). (B) In the case of physiological measurements or individual manipulations that do not require breeding of the CC lines, it is most efficient to use single animals obtained from as many different CC lines as possible. (C) When a mouse model of cancer needs to be bred to different CC lines, resources should be balanced between using multiple mice from each litter and using as many independent CC lines as possible. Because these breeding and aging experiments tend to extend over longer periods of time, it may be important to gather progeny from a given

CC line during different times of year to control for seasonal effects. (D) To test the effects of genetic background on tumor growth and response to therapy, hybrid hosts can be made by breeding CC lines to inbred mice of the strain of mouse tumor cell line models. The resulting F1 hosts will carry the same antigens as the tumor line and should not reject the graft.

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