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A five-step validation workflow for conditional knockout mouse lines: a practical guide for student researchers

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Abstract. Conditional Knockout (cKO) mouse models are widely used in neuroscience research. Unfortunately, many papers assume that having a cKO mouse genotype is enough to consider the protein absent in the target tissue. Intended deletion can either be obscured or falsely suggested by recombination efficiency, antibody specificity, cellular makeup of the sample or developmental compensation. This Technical Note presents a 5-step process based on the validation principles that were learned during a high-school research placement in a university neuroscience lab; the order roughly reflects their cost, and a student researcher may apply all steps prior to studying phenotypes associated with the cKO line. Steps include (1) genomic PCR confirming recombination in the target tissue, (2) RT-qPCR measuring the level of the transcript in a sample enriched in the targeted cell type or microdissected, (3) in situ hybridization or co-localisation through immunofluorescence of residual expression, (4) Western blots with at least 2 independent antibodies at multiple time points during development for protein level and (5) a null control sample serving as a test for antibody specificity. Each step is accompanied by the question it addresses, approximate cost and time needed for its realization, criteria of passing and typical pitfalls. This note does not contribute any new data; it provides a checklist to avoid a widespread and costly mistake in validation: planning further studies based on an unverified cKO model.

Keywords: conditional knockout, Cre-loxP system, validation workflow, antibody specificity, student research

1. Introduction

With the Cre-loxP system, a gene can be deleted in selected cell types; therefore, cKO mice are very good models for research on how one gene affects the brain [1, 2]. A beginner in neuroscience may think that the procedure is simple. Genotyping comes first, and then researchers compare the cKO group with a control group.

For a long time, many neurogenetics laboratories would validate a knockout in mice by a conventional genotyping PCR on tail DNA and proceed to behavior experiments. Scientists take it for granted that because the tail is genetically a knockout, the brain tissue is a knockout as well; however, having the desired genotype in tail DNA does not imply absence of the protein in the brain tissue. This may happen because of partial cell-specific recombination of the gene by a Cre driver [2], protein longevity, further protein production by other non-target cells, and detection of other proteins by an antibody against the protein of interest [3]. Many

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months' worth of research can revolve around mice that do not pass the knockout validation criteria, leaving scientists in a race to explain their results.

The guide that follows outlines a workflow for validating the deletion in five steps in approximate order from low cost and rapid to high cost and detailed. This workflow is based around one question and three subquestions: Is there a deletion? Where is it? And at what level can it be observed? Evidence related to the reason for discrepancy between the genotype and the status of the protein is reviewed in Section 2; Steps 1 to 5 are listed in Section 3, while two tables highlighting the steps are presented in Section 4; the interdependencies between the steps are covered in Section 5, along with the rationale behind the pass criteria, constraints on the workflow, and student researcher application of the process, with the conclusions presented in Section 6.

2. Literature review

Site-specific recombination technology, particularly the Cre-loxP system, has revolutionized genetic studies of mice through providing a reliable way to manipulate DNA sequences inside animals in vivo [1], in a certain cell type and at a certain time point [2]. The technology does require, however, that certain conditions are met, which cannot be identified by tail genotype. Four factors that can cause a discrepancy between an animal's genotype and the condition of the target protein measured in its tissue have been repeatedly observed in the literature regarding conditional mouse strains. These are insufficient or unexpected recombination, continued presence of the protein and other genes compensating for its removal, dilution of the target cells in a heterogeneous tissue sample and antibody non-specificity.

Recombination itself provides the first problem. Early studies on the tamoxifen-inducible system showed that Cre-mediated deletion of the floxed gene could take place in a number of tissues, yet the level of recombination efficiency differed from one tissue type to another, being highest, about 40%, in the skin [2]. Moreover, recombination could happen in places where it was not desired. According to Song and Palmiter [4], the information regarding breeding and genotyping in the papers involving Cre-lox systems was often incomplete and germline recombination or transient Cre expression in development could be missed by the standard genotyping method. Analysis of data from various laboratories found that germline recombination had occurred in over half of 64 commonly used Cre-driver strains intended for nervous-system-specific recombination, usually biased towards one parent depending on the presence of Cre in either sperm or oocytes [5]. If germline recombination took place, the offspring could inherit the deleted allele in all cells, so that a conditional knockout animal could be a complete knockout [4, 5]. Reporter alleles cannot be considered good surrogates of the gene of interest. Several floxed alleles analyzed at the cellular level displayed considerable variance in their recombination correlation in otherwise genetically identical cell types, which likely depended on chromosomal localization of the allele, the distance between the loxP sites, the sequences adjacent to them, and Cre activity per cell [6]. This meant that recombination of a reporter was not reliable proof of the target allele recombination [5, 6].

Another source of error is the temporal gap between the removal of DNA and the loss of the protein, as well as compensatory effects of other genes. Once the gene is deleted, protein production ceases, but existing proteins remain in place. In one study, the protein turnover rate of approximately 2,500 proteins in mouse tissues was measured, and it was found that there was a difference of four orders of magnitude between them, brain proteins being replaced at a much slower pace than liver and blood ones, and having an average lifespan of 9.0 days versus 3.0 days in the liver and 3.5 days in blood [7]. The consequence is that the protein can still appear in the brain tissue for a while after the gene recombination. Another issue in this regard is genetic

compensation. A phenotypic difference has been noted for knockouts compared to knockdowns in some model organisms like mice, and one suggestion to explain this phenomenon is that gene knockout may induce a compensatory mechanism whose molecular basis, possibly both transcriptional and posttranscriptional, is poorly understood [8]. Protein replacement takes time, hence results for one age do not necessarily apply to another age.

The third variable is the composition of the tissue sample itself. There are numerous types of cells in the brain, and therefore specific subpopulations of neurons cannot easily be distinguished [9]. Conditional deletion could target just a single neuronal subpopulation and then the mRNAs from non-targeted neuronal populations would still be present in the tissue homogenate, possibly obscuring a significant reduction of targeted transcripts. This question has been addressed by genetic approaches. In Translating Ribosome Affinity Purification (TRAP), an EGFP-tagged ribosomal protein is expressed in specific cell populations, followed by purification of polysomal mRNA from them [9]; a similar affinity purification approach targets isolation of nuclei from genetically defined cell types in the mouse brain [10]. Another, complementary approach to the measurement of RNA levels is in situ RNA methods. In RNAscope, single RNA transcripts are visualized and the tissue architecture is preserved; although other approaches like grind-and-bind, for example real-time RT-PCR, do not preserve the histological context, RNAscope does [11].

The fourth potential source is the reagent used for detection. One of the most widely used reagents in research is antibodies; however, no consensus on criteria for validating the antibodies has been reached, while commercial antibodies do not always correspond to the labeling on the tube [12]. Validating the antibody means demonstrating specificity, selectivity, and reproducibility of the antibody under the experimental conditions [12]. An international expert group later identified five conceptual pillars of antibody validation applicable for each specific purpose [3]. Among those, two of them are directly relevant to knockout validation: the genetic approach, in which expression of the protein is eliminated or significantly reduced, and the independent antibody approach, in which the protein is detected using two antibodies targeting non-overlapping epitopes of the same protein [3]. Another challenge relates to measuring the amount of the protein. Single-protein loading controls, like Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) and beta-actin, commonly used in Western blotting, do not reliably measure differences in protein concentrations at the normal level of loading for quantifying rare proteins, while total protein stains were considered adequate alternatives [13].

It is through these four error sources that the queries which must be answered in the validation process become evident. There must be confirmation of recombination in the particular tissue, there must be quantification of transcript loss in the targeted cells, there must be localization of the residual signal, and there must be demonstration of protein loss at the appropriate age using validated reagents.

3. The five-step validation workflow

The five steps follow each other more or less in an increasing order of cost and time; hence, a failed knockout is discovered before making any large investment. For each step, the information provided includes the problem that is answered, the process used to do it, the pass criteria, cost, time taken and common errors made. The pass criteria given here are empirical and are not absolute benchmarks that are true for all genes, brain areas or research questions and need to be altered accordingly.

3.1. Step 1: genomic PCR of the target brain tissue

Question answered—Was the gene deletion actually successfully performed in the brain tissue under analysis?

How it works—While tail genotyping can show what genetic modifications the mouse has, it does not show what modifications have actually occurred in the brain tissue under analysis. To confirm this, a small amount of tissue must be removed from the brain region of interest (e.g., the hippocampus or cortex), and PCR primers specific to the recombined gene must be used to determine that deletion has successfully occurred in this brain region. Testing the target allele itself matters because the recombination of a reporter allele does not reliably indicate the recombination of other floxed loci [5, 6].

Pass criterion—Recombined DNA visualized in the target brain tissue of all cKO mouse samples.

Cost and turnaround—Almost nothing — standard PCR and agarose gel electrophoresis; approximately one day from biopsy to result.

Common mistakes—(i) Relying on the breeder to genotype the mice without verification in the brain tissue of interest. (ii) Utilizing primers that do not discriminate between altered and totally deleted genes, which may result in unanticipated recombination going undetected; the same deletion-specific PCR done using a tail DNA sample, which should not have any deletion, can detect germline recombination [4]. (iii) Removing surrounding brain tissue together with the brain tissue of interest during dissection.

3.2. Step 2: RT-qPCR in specific cell types or microdissected regions of the brain

Question answered—Does the mRNA decrease, and in which cell type or specific subregion of the brain?

How it works—Grinding up a large region of brain tissue will dilute the target mRNA, especially if the target cell is not the most prevalent cell type in that region. To isolate the mRNA of the target cell group, it may help to either microdissect the brain region or use a method that isolates material from the target cell type, such as its ribosome-bound mRNA [9] or its nuclei [10], both of which require mice that express a tagged protein in the target cells. This way, any qPCR results will reflect the degree of mRNA decrease specifically in the cell type or cell group being targeted.

Pass criterion—At least 50% decrease of target mRNA in the target cell type, compared to normal mice.

Cost and turnaround—Moderate — cell isolation takes around one to two weeks, and qPCR takes another week.

Common mistakes—(i) Using the whole brain region as the sample rather than the specific cells or subregions. (ii) Designing primers at a location within the gene which is not the location of the deletion. (iii) Having a small number of mice such that the results cannot differentiate a true partial reduction in expression levels of the mRNA from the single anomalous result from one mouse. In qPCR, the guidelines state that primer sequences and analysis methods must be fully provided as per MIQE; from this, one could look for mistake (ii) [14].

3.3. Step 3: spatial localization by staining (in situ hybridization or immunofluorescence)

Question answered—Where is the leftover mRNA or protein still found?

How it works—Staining the target alongside cell-specific markers, for example by RNA in situ hybridization [11] or immunofluorescence, localizes any leftover mRNA or protein. Finding leftover protein in cells which were not targeted is normal, but finding leftover protein in the nuclei or bodies of cells which were specifically targeted implies that the knockout was not successful in these cells, that protein made before recombination has not yet been degraded [7], or that the antibody detects another protein, possibilities that Steps 4 and 5 help to distinguish. Similarly, finding leftover mRNA in the cell bodies of neurons which were specifically targeted implies the knockout was not successful in these neurons.

Pass criterion—Any leftover signal is demonstrated to only come from non-target cell types across multiple mice and brain sections.

Cost and turnaround—Moderate to high — uses specialized fluorescent stains and microscopes, and takes two to three weeks.

Common mistakes—(i) Becoming alarmed when leftover signal is found in non-target cells. (ii) Looking at a single brain section from each mouse, so normal differences from section to section may stay hidden. (iii) Bleed-through on the microscope, with false positives when two channels of different colors overlap.

3.4. Step 4: western blot of different ages, using a second antibody

Question answered—Is the protein lost in the cKO mice, and at what age?

How it works—Developmental compensation and the half-life of proteins are altered by age. Therefore, a temporal analysis (such as one month versus two to three months) can help in distinguishing a delayed reduction from a failed deletion. Furthermore, the same sample is analyzed using two different antibodies with non-overlapping epitopes to lower the possibility that the original band was a non-specific reaction [3].

Pass criterion—The reduction of the protein is identifiable through the use of the second antibody in the brain area of interest of the mice at the desired age.

Cost and turnaround—Moderate; a routine Western blot in the laboratory needs one to two weeks.

Common mistakes—(i) Utilization of only one mouse age for the experiment; it could have happened that the delayed protein turnover could not have been discovered. (ii) Usage of only one antibody, from the start to the end of the research work. (iii) Excessive protein loading of the gel, which made the differences in results among the studied groups look less impressive than they actually were. (iv) Normalization of protein expression based on a control protein that is variable between mouse groups; total protein staining is an option in such a case [13].

3.5. Step 5: the null-control lane

Question answered—What does a pure-zero sample look like in this assay?

How it works—Running a sample in which the target gene has been completely knocked out demonstrates whether the antibody is recognizing any proteins besides the target protein. A band appearing in the lane of a completely knocked out sample means that the antibody is not specific to the target protein [3, 12], and it should not be used for any knockout validation experiments.

Pass criterion—No specific band appears in the null-control sample, under regular exposure settings.

Cost and turnaround—Depends on whether a null-control sample is available; if one is, adding an extra gel lane takes no extra time or money.

Common mistakes—(i) Skipping this step altogether due to the difficulty of obtaining a null-control sample. (ii) Comparing the null-control image with images of the rest of the gel that were taken under different camera exposure settings.

4. Summary tables

In Table 1, the five steps are placed side by side along with the question to be answered for the respective step, its typical timeline and its decision criterion for passing. The time required for tissue PCR is about one day while for cell-type-enriched qPCR and marker colocalization it ranges from two to three weeks; there is no additional time spent on the null-control lane when the null sample is available. Table 2 outlines the workflow as a checklist for the first week of a student working on a conditional knockout line. It indicates whether the student should confirm, discuss or obtain a mentor's approval for each item. Finally, in the last row, identification and validation of the antibody are documented before beginning any step at the level of protein;

this coincides with the recommendation by the international working group to document antibodies with RRIDs [3].

Table 1. The five-step validation workflow, ordered roughly by resource cost

Step	Question answered	Typical turnaround	Decisive output
1. Tissue PCR	Did recombination occur in the target tissue?	~1 day	Recombined allele present
2. Cell-type-enriched qPCR	Is mRNA reduced in the targeted compartment?	2–3 weeks	≥50% transcript reduction
3. Marker colocalization	Where does residual expression remain?	2–3 weeks	Residual signal mapped to non-targeted cells
4. Time-course Western blot	Is protein reduced at the chosen age?	1–2 weeks	Reduction reproduced with second antibody
5. Null-control lane	Does the antibody detect only the target?	None beyond existing run	Empty null lane

Table 2. A first-week checklist for a student starting work on a conditional knockout line

Item	Action	With mentor
Genotype records	Collect tail genotypes and pedigree for all experimental animals	Confirm
Cre driver scope	Read the driver-line publication; list the cell types it targets	Discuss
Target gene expression	Map reported expression of the target gene across brain regions and ages	Discuss
Assay plan	Decide which of the five steps the project requires, and in what order	Approve
Sample accounting	Plan biological replicates, sex balance, and the statistics to be reported	Approve
Antibody records	Record catalog numbers, Research Resource Identifiers (RRIDs), and validation status for every antibody to be used	Confirm

5. Discussion

5.1. The five steps as one chain of evidence

These five steps do not represent five separate tests of the same phenomenon; on the contrary, they represent a chain of tests from the gene to the protein and, by the very nature of the process described, successively limit the pool of interpretations left open by each of the preceding steps. Step 1 asks whether the recombined allele exists in the tissue being studied; passing this step demonstrates the presence of the recombined allele in that particular tissue, although neither the cells nor their proportion in the whole tissue is established. Step 2 asks whether the transcript level has decreased in the targeted cells, and its criterion concerns the enriched sample and not all cells. Steps 3, 4 and 5 ask successively where the remaining signal is localized, whether the protein is decreased at the age relevant to the planned experiments in the target region, and whether the antibody used

to detect the protein recognizes proteins other than the target one. The success of a step further down the chain can only be taken as evidence of the deletion in conjunction with the steps that preceded it. A decrease in protein measured using an antibody recognizing other proteins as well, or in a sample in which the recombined allele is not demonstrated, does not demonstrate a deletion.

Understood in this fashion, the passes and fails of the protocol reflect the four potential sources of error, incomplete or unexpected recombination, persistence of the protein despite deletion of the gene and the ability of compensatory genes to mask its absence, dilution of the desired cells within the sample, and non-specific recognition by antibodies. A pass at Step 1 and a fail at Step 2 would indicate partial recombination of the cells within the region [2], but it could also indicate dilution of the desired cells in the sample, which is mistake (i) of Step 2. Step 3 would differentiate between these two possibilities because if there is still residual transcript in the cytoplasm of the targeted cells, it reflects incomplete recombination; if it exists only in the non-targeted cells, this would indicate dilution. Passing Steps 1 to 3 and then failing at Step 4 is in keeping with the persistence of protein produced prior to recombination [7], wherein Step 3 identified the mRNA but not the protein, or with an antibody that detects a different protein [3, 12]; a later time point for Step 4 would address the first, while Step 5's null-control lane would address the second.

5.2. The order of the steps and the pass criteria

The steps are listed in rough order of their cost and speed (Table 1). They also move from the gene towards its products. Steps 1 and 2 analyze the DNA and the transcript respectively, Step 3 identifies whatever transcripts or proteins are there, Step 4 analyzes protein while Step 5 analyzes the antibody that was used to identify the protein. Thus any failure will be identified at the appropriate level prior to conducting other steps on the same mice. The cost of the null-control lane in Step 5 is minimal since all it involves is the addition to the Western blot. It is listed last not because it is the most expensive but because its results are interpreted together with those of the Western blot and because it does not involve analyzing the mice but the antibody. The marker colocalization in Step 3 requires two to three weeks and involves special staining and microscopy, whereas the Western blot analysis in Step 4 takes only one to two weeks but might be less expensive than the former; accordingly, the expense pattern serves as a guideline, not an absolute standard.

The criterion used for Step 2, a 50% or greater reduction in the target transcript, is one that can be achieved in practice and one that allows for two known weaknesses of the technique. First, recombination efficiency is known to vary according to tissue type [2] and floxed locus [6], such that not all targeted cells will necessarily possess the deletion. Also, enrichment for any particular cell type using ribosome affinity purification [9] or nuclear affinity purification [10] cannot be assumed to be complete, and so there may be residual expression from nontargeted cells. In addition, the criterion must be considered in conjunction with mistake (iii) of Step 2, since a 50% decrease can only be distinguished from individual differences among mice if enough mice are used. A Step 2 pass is thus an incentive to proceed to Steps 3 and 4 rather than proof that the deletion is complete.

5.3. What the workflow does not establish

The protocol will verify if there was recombination of the gene in the target tissues, if the level of the transcript of the gene is lowered in the target cell types and if the level of the protein is decreased in the target tissues at the age of study. However, the protocol does not verify what else could be affected by this experiment. Genetic compensation, suggested as an explanation of phenotypic discrepancy between knockout and knockdown [8], is not quantified through any of the five steps, which means that even a conditional knockout line that passed all five steps could be different from the control line not only because of the absence

of the protein. The workflow does not indicate at which time the recombination was made. Temporary expression of Cre at a developmental stage is one of many such processes, which may not be discovered by traditional genotyping [4]. A deletion identified in the adult tissue in Step 1 could have been made at the early developmental stages, allowing enough time for the compensation to occur. The time course of Step 4 reveals if there is a reduction of the protein at more than one age but it does not reveal when the deletion occurred.

Further limitations arise with respect to the tissue and the animals. The basis of the time course design is the slower turnover rate of proteins in the brain when compared to proteins in the liver and the blood [7]. In tissues with a higher turnover rate, Step 4 may need fewer time points, while for the slowest turnover rate of brain proteins only one early time point could result in an underestimate of the deletion. Germline recombination [4, 5] cannot be distinguished from conditional recombination in Step 1 using brain tissue samples, because the deleted allele will be present in all tissues in that case; the deletion-specific PCR on tail DNA described in mistake (ii) of Step 1 and the pedigree of Table 2 are steps of the workflow that take this into account. Finally, the proposed workflow was designed for conditional knockout in the mouse brain for one particular gene. This workflow could be used for other types of tissues as well as other recombinase systems [1], but cost, turnaround and pass criteria will have to be reassessed.

5.4. The workflow for student researchers

But it seems fair to raise concerns regarding the necessity of developing a validation list if all processes in it are well-known procedures in scientific practice. However, the fact is that the laboratory in a university has accumulated a lot of experience of work as well as standards in conducting experiments, whereas the one-time visiting student does not possess them yet. Thus, in the beginning of a career in conducting experiments, a beginner will probably encounter the difficulties and errors which a novice will make. A concise workflow arranged roughly in order of increasing costs, along with pass criteria and typical problems, would transfer knowledge from experts to a novice student in the very first week of the project, rather than in the event of failure of an experiment.

Song and Palmiter [4] urge the inclusion of unanticipated recombination events in screening protocols, while requesting the editors and reviewers to demand definite explanations concerning validation of genotyping, and a completed checklist, as well as the deletion-specific PCR screening protocol of tail DNA and pedigree of Table 2, is exactly this type of evidence from which such a statement can be prepared. The same rationale lies behind the inclusion of the other steps of the suggested workflow, ranging from MIQE guidelines on quantitative PCR [14] to principles of antibody validation [3]. What makes the suggested workflow valuable is not any individual recommendation, but their integration into an easily accomplished form by the novice researcher in the first weeks of a project.

One drawback is the fact that this guide provides no new experimental data since all five steps are simply a sum of what people have already been doing and not new at all. It is the claims the researcher is trying to make that determine how much validation should be done, and the process itself cannot replace supervision and consultations from an experienced scientist working in the same field. This paper, on the other hand, aims to provide scientists with a brief yet common guide for their validation processes, one that they should adapt to their specific needs and discuss with their mentor first and foremost.

6. Conclusion

A conditional genotype is a testable hypothesis about protein removal but not evidence of the hypothesis. This five-step workflow is approximately ordered in terms of cost, from low to high. It involves recombination

PCR, qPCR in enriched cellular preparations, colocalization using spatial markers, Western blotting using independent antibodies in a time-course series and a null-control preparation; with this workflow, it becomes possible for a student researcher to test the hypothesis before having to perform costly downstream experimentation. Students who begin their research career using the conditional knockout mouse line are encouraged to validate according to these criteria and discuss them with a mentor in the first weeks of the project. Pass criteria remain empirical; therefore, criteria should be fine-tuned for each gene, brain area and research question at hand.

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