Analysing the outcome of CRISPR-aided genome editing in embryos: Screening, genotyping and quality control

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The application of CRISPR/Cas9 technology has revolutionised genetics by greatly enhancing the efficacy of genome editing in the early embryo. Furthermore, the system has enabled the generation of allele types previously incompatible with in vivo mutagenesis. Despite its versatility and ease of implementation, CRISPR/Cas9 editing outcome is unpredictable and can generate mosaic founders. Therefore, careful genotyping and characterisation of new mutants is proving essential. The literature presents a wide range of protocols for molecular characterisation, each representing different levels of investment. We present strategies and protocols for designing, producing and screening CRISPR/Cas9 edited founders and genotyping their offspring according to desired allele type (indel, point mutation and deletion).  

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Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeat; Cas9, CRISPR associated 9; ddPCR, droplet digital polymerase chain reaction; DNA, deoxy-nucleotide acid; DSB, double stranded break; HDR, homology directed repair; indel, insertion/deletion; ND, not determined; NHEJ, non-homologous end joining; nt, nucleotide; PAM, protospacer adjacent motif; PCR, polymerase chain reaction; QC, quality control; sgRNA, single guide RNA; ssODN, single-stranded oligo-deoxynucleotide; WT, wild-type.  
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1. Introduction

Originally identified as a bacterial adaptive immune system, the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR Associated 9 (Cas9) system has recently been adapted and re-deployed to efficiently modify eukaryotic genomes [1]. Since the system’s first description as a genome editing tool, a wide range of allele types have been obtained. Modifications reported range from simple indels [2], precise introduction of point mutations [3], insertion of loxP sites or tag sequences [4], or larger cassettes such as cDNA for expression [5]. For each of these experiments, the mechanism of editing involves scanning of the DNA by the CRISPR/Cas9 complex prior to cutting and repair [6]. The complex molecular events involved in the process are starting to be unravelled and this knowledge exploited for a more efficient application of the system [7].

Although not highlighted in the initial reports of genome editing by CRISPR/Cas9 [3], we and others have described that the CRISPR-aided mutagenesis is an unpredictable process. Founder animals are often mosaic and sequence changes additional to those intended can be found associated at the site of repair (illegitimate repair) [8]. The literature offers a wide range of protocols for analysing the outcome of CRISPR aided-mutagenesis, but many do not allow for a comprehensive characterisation of the complexity of mutagenesis events generated with the system, nor for the identification of unforeseen additional sequence changes. Table 1 summarises different techniques that can be employed to characterise mouse mutants generated by the microinjection of CRISPR/Cas9 reagents and shows examples of references that apply them.

Here we describe the use of the CRISPR/Cas9 technology for the modification of the mouse genome to produce indels, point mutations and deletions and analysing the outcome of these experiments. We have drawn on our experience of the generation and quality control of large numbers of mouse mutants, many of them produced within the International Mouse Phenotyping Consortium (IMPC) [13], and delineated methods for efficient screening, genotyping and ascertaining of the quality of new alleles produced. We detail strategies to analyse the genetic complexity of the animals produced by CRISPR/Cas9 editing according to the type of allele to be generated. We propose that F0 animals (born from microinjection) can be screened for the presence of an allele of interest, but the genotype of new mutant lines is proven in the subsequent generation only. The approach we propose can be employed for the mutagenesis of other species.

2. Materials and methods

2.1. Mutation design and generation of reagents for microinjection

The sequences of oligonucleotides, protospacers and donor DNAs used within the examples presented in this study are shown in Supplemental Table 1.

2.1.1. Design tool and choice of guide

Single guide RNA (sgRNA) sequence selection is carried out using any of the following online tools:

- http://tefor.net/crispor/crispor.cgi [14]
- http://www.sanger.ac.uk/hgtg/wge/ [16]

SgRNA sequences are selected with as few predicted off-target events as possible, particularly on the same chromosome as the intended modification. When selecting sgRNAs to introduce indels or point mutations, the region for guide selection is more limited as the cut site is to be within a given sequence or as close to the point at which the single base change is to be introduced. For exon deletion projects, where the intention is to generate a null allele, the exon chosen should be represented in all transcripts and its removal should result in a frame shift. The number of guide sequences selected is dependent upon the intention of each project i.e. to introduce point mutations and indels into genomic sequence, a single sgRNA is required, whereas in our high-throughput pipeline, exon deletion projects generally employ four sgRNAs with two each side of the targeted deletion.

2.1.2. Donor oligonucleotides

Sequences for donor templates are designed with homology arms at least 60 nt in size flanking the intended point mutation. These are generally centred on the cutting site but might be offset towards a point mutation to favour a recombination that includes the desired change. Wherever possible, silent point mutations are introduced to the ssODN sequence to disrupt the PAM sequence and prevent re-cutting of the modified allele by Cas9. If altering the PAM sequence in the donor is not possible, further changes in the seed sequence to which the sgRNA will bind will be introduced to the donor. Modifications to introduce restriction digest sites to aid with subsequent animal genotyping can also be incorporated into the ssODN. Single-stranded oligo-deoxynucleotide donor sequences were ordered as Ultramer™ DNA oligonucleotides.
2.4. Genotype screening and characterisation

2.4.1. Genomic DNA extraction from ear biopsies

Genomic DNA from F₀ and F₁ animals was extracted from ear clip biopsies using DNA Extract All Reagents Kit (Applied Biosystems) according to manufacturer's instructions. The crude lysate was stored at −20 °C.

2.4.2. Primer design

Primer sequences were selected using NCBI Primer-BlAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Optimal primers anneal at least 200 nt away from the intended sgRNAs cutting sites to ensure them not to be included within large deletion events and to allow for the full characterisation of nucleotide changes induced by the CRISPR/Cas9 system. Care was taken to avoid amplicons containing repeat sequence wherever possible as these would potentially affect subsequent sequence analysis.

2.4.3. Optimisation of the assay conditions

On reception, primer pairs were set up in a PCR reaction containing 500 ng genomic DNA extracted from a wild type mouse, 1× Expand Long Range Buffer with 12.5 mM MgCl₂ (Roche), 500 μM PCR Nucleotide Mix (dATP, dCTP, dGTP, dTTP at 10 mM, Roche), 0.3 μM of each primer, 3% DMSO, and 1.8 U Expand Long Range Enzyme mix (Roche) in a total volume of 25 μL. Using a T100 thermocycler (Bio-Rad), PCRs were subject to the following thermal conditions: 94 °C for 2 min followed by 40 cycles of 94 °C for 20 s, a gradient of annealing temperatures between 55–65 °C for 30 s and 68 °C for 1 min/kb and a final elongation step for 10 min at 68 °C. PCR outcome was analysed on a 1.5 to 2% agarose gel, depending on the amplicon size and the highest efficient annealing temperature was identified for the primer pair. If no temperature allows for an efficient and/or specific PCR amplification the assay was repeated with an increased DMSO concentration (up to 12%). On rare occasions when initial primer sequences could not be optimised to obtain specific PCR products, new oligonucleotides were designed and optimised.

2.4.4. PCR amplification and sequencing

Using optimised conditions, as defined above, PCRs for each project were run and an aliquot analysed on an agarose gel. PCR products were purified employing QIAquick Gel extraction kit (Qiagen) or Illu presenta ExoProStar™ (GE Healthcare Life Sciences) and sent for Sanger sequencing (Source Bioscience, Oxford).

2.4.5. Sub-cloning PCR products

For point mutation projects, PCR products amplified from F₀ DNA showing mixed sequencing traces and/or possible integration of the ssODN, were then sub-cloned using Zero-Blunt PCR cloning Kit (Invitrogen). The appropriate number of clones (usually twelve to twenty-four) per founder were picked and grown overnight in accordance with the complexity of the traces observed prior to sub-cloning. Plasmids were mini-prepped (Qiaprep Miniprep Kit, Qiagen) and analysed by Sanger sequencing (Source Bioscience, Oxford) using the M13R oligonucleotide.

2.4.6. Sequencing data analysis

Sequencing data were analysed differently depending on whether they are obtained from F₀ or F₁s. At the F₀ stage, animals are screened for evidence of the expected change i.e. mixed sequence traces or difference from wild type reference for indel projects; sequencing showing deletion of the targeted sequence for deletion projects; and evidence of expected base change for point mutation projects. F₀ animals can be mosaic, heterozygous, homozygous or trans-heterozygous. All F₁s are heterozygous animals containing one wild type allele and one allele to be deter-
Table 1 Screening and genotyping techniques for the analysis of CRISPR-generated mutants.

<table>
<thead>
<tr>
<th>Mutation type</th>
<th>Molecular assay</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indels</td>
<td>PCR amplification analysed by Sanger</td>
<td>[2]</td>
</tr>
<tr>
<td></td>
<td>sequencing</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Surveyor®-type assay</td>
<td>[2]</td>
</tr>
<tr>
<td></td>
<td>Indel detection by amplicon analysis</td>
<td>[9]</td>
</tr>
<tr>
<td></td>
<td>Next generation sequencing</td>
<td>[10]</td>
</tr>
<tr>
<td></td>
<td>High resolution melt analysis</td>
<td>[11]</td>
</tr>
<tr>
<td>Deletions</td>
<td>PCR amplification analysed by restriction</td>
<td>[3]</td>
</tr>
<tr>
<td></td>
<td>digests</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PCR amplification analysed by Sanger</td>
<td>[3]</td>
</tr>
<tr>
<td></td>
<td>sequencing</td>
<td></td>
</tr>
</tbody>
</table>

Different techniques that can be employed to characterise mouse mutants depending on the type of mutation to be introduced through microinjection of CRISPR/Cas9 reagents are summarised here, together with examples of references that employ them. Only PCR amplification analysed by Sanger sequencing and next generation sequencing allow for the full characterisation of the alleles detected in DNA extracted from a founder animal (i.e. total number of alleles and composition of the alleles). Neither the number, nor the composition of the alleles present can be specified by Surveyor®-type assays, indel detection by amplicon analysis or high resolution melt analysis. These techniques also require the samples to be mixed and analysed with a WT DNA sample in order to differentiate homozygous mutants from WT. Next generation sequencing allows for an in-depth characterisation of the genetic makeup of mutants, but remains an expensive solution in terms of financial cost, time and expertise for data analysis.

Table 2 Summary of 74 CRISPR-aided mutagenesis projects.

<table>
<thead>
<tr>
<th>Mutation type</th>
<th>Indel</th>
<th>PM</th>
<th>Deletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of projects</td>
<td>16</td>
<td>16</td>
<td>42</td>
</tr>
<tr>
<td>Total number of founders F0</td>
<td>181</td>
<td>844</td>
<td>639</td>
</tr>
<tr>
<td>Total number of mutants</td>
<td>68</td>
<td>235</td>
<td>ND</td>
</tr>
<tr>
<td>Total number of animals bearing the required mutation</td>
<td>55</td>
<td>42</td>
<td>114</td>
</tr>
<tr>
<td>Percentage of mutated founders/F0 born</td>
<td>37.6</td>
<td>26.6</td>
<td>ND</td>
</tr>
<tr>
<td>Percentage of correct mutants/total mutants born</td>
<td>80.9</td>
<td>17.9</td>
<td>ND</td>
</tr>
<tr>
<td>Percentage of correct mutants/F0 born</td>
<td>30.4</td>
<td>4.8</td>
<td>17.8</td>
</tr>
</tbody>
</table>

The table shows a summary of projects aimed at the introduction of indels, point mutations (PM) or deletion of a segment of up to 4 kb. Total numbers of founders born, F0 mutants and animals carrying the required mutation are shown. The percentage of expected mutants over the total number of pups born describes the complexity at which each type of mutation can be obtained. Thirty percent of pups born from indel projects contain a correct modification. However, this percentage drops to eighteen percent and five percent of the total number of F0 for deletion and point mutation projects respectively. For deletion projects, only animals showing smaller amplicon after electrophoresis were analysed, not permitting the assessment of the global mutation rate from these experiments.

Table 1). Their range varies greatly in sophistication from simple restriction digest of a PCR amplicon, to the building of a barcoded library and next generation sequencing. Method selection should take in consideration both the stage at which the animals are within the process i.e. F0 or subsequent generation, and the kind of allele to be generated (indel, point mutation or deletion).

In our experience, F0 animals (born from microinjection of 1-cell embryos) and F1 animals (obtained by mating a F0 and a wild type mouse) represent very different challenges in terms of their genotyping [8]. As F0 animals are often mosaic, we propose to approach their molecular characterisation as a screen for the presence of an allele of interest. On the other hand, alleles can be definitively characterised at the F1 generation and their quality controlled in order to establish a new mutant mouse line.

The strategy for allele characterisation is directed by not only the mutagenesis aim, but the likelihood of achieving the intended gene edit. Table 2 summarises a total of 74 CRISPR-aided mutagenesis projects we have conducted. This survey shows that frequency of mutagenesis success, and therefore the number of animals involved, varies greatly according to the experimental aim. These numbers illustrate that significantly more animals are usually generated and analysed to obtain a targeted point mutations than to produce an indel. The deletion of a short segment of sequence represents an intermediate level of challenge and thus, animal numbers. Those projects involving larger numbers of animals should use a high-throughput and low cost initial screen before implementing more in depth characterisation of the allele. We employ kits for a number of the processes presented for convenience and gain of time. Many of these could be replaced by independently bought components to further reduce the cost of screening and genotyping.

The key to an efficient genotyping screen is choosing the appropriate assay, depending on the stage of the process (F0 or F1 animals) specific to each type of attempted mutation. This enables researchers to reach an unequivocal conclusion as to the outcome of the experiment, whilst minimising what can be a substantial amount of investment in molecular assays and associated optimisation. Full characterisation of F1 animals is essential because the outcome of CRISPR induced mutagenesis is variable and can not be predicted by initial characterisation of F0s. Below, we describe methods for the screening of founders for three broad categories of alleles: indels, point mutations and deletions of segments such as exons, and for the characterisation their progeny.

3. Protocol implementation

3.1. Considerations for choosing a genotyping strategy for animals resulting from CRISPR microinjection

Many molecular techniques have been described for the analysis of CRISPR-aided mutagenesis outcomes (summarised in

3.2. Screening for indels

Introduction of indels to disrupt a sequence of interest (i.e. coding sequence or a transcription factor binding site) can be obtained
with a high efficiency by co-injecting one sgRNA with the Cas9 mRNA or protein. While the introduction of an indel is targeted via the selection of the sgRNA used, the resulting sequence alterations on target are variable. Thus, we prefer screening founder F0 animals for the introduction of an indel through generation of a PCR amplicon and direct Sanger sequencing of the targeted region. Fig. 1 illustrates the outcome of such screening of an experiment aimed at introducing an indel in the Kcnk13 gene: the target sequenced is amplified by PCR (Fig. 1A) and sequenced (Fig. 1B). The unpredictability of the changes produced via the error-prone non-homologous end joining (NHEJ) pathway yields alleles containing a wide range of nucleotide changes at the site of repair (Fig. 1B). These changes include deletions or insertions varying in size from one to hundreds of nucleotides (Fig. 1, founder 1 and 5), combinations of nucleotide deletions and insertions (Fig. 1, founder 4), small inversions, small duplication events and/or a combination of these local rearrangements. The chosen genotyping method must be able to identify these, sometimes very small or large sequence changes. Accordingly, when designing genotyping primers, it is essential to span sufficient flanking regions as deletions can extend hundreds of base pairs away from the sgRNA target sequence (Fig. 1, founder 5) and be missed during PCR amplification.

While electrophoretic analysis of PCR products, HRMA analysis or Surveyor™-type assay allow the characterisation of indels, they do not permit identification of all sequence alterations nor inform on composition of the new alleles, including whether they are producing a frame-shift. These limitations can result in an increase of assays to be performed (i.e. run the sample alone and the sample mixed with WT control to screen for homozygous mutant for HRMA and Surveyor™-type assay). The increase in number of assays to be performed makes these genotyping strategies more time consuming and less cost effective. Moreover, samples positive after a first step of screening via these methods remain to be sequenced in order to confirm for the presence of mutations and assessment of the exact sequences variations. Conversely, screening F0 animals via Sanger sequencing allows the characterisation of any outcome of mutagenesis event (i.e. WT (0 altered alleles)/ heterozygous mutant (1)/trans-heterozygous mutant (2)/homozygous mutant (2)/mosaic mutant (>2)) and details the exact sequence composition for heterozygous and homozygous mutant animals. Assessing the exact sequence composition in trans-heterozygous and mosaic mutants can be complex or impossible without an additional sub-cloning step. However, while trying to disrupt a sequence, the presence of multiple mutated alleles is often a sufficient characteristic to select founders for mating as

Fig. 1. Ear biopsy DNA analysis of Kcnk13 mice reveals a wide range of alleles and genotypes. (A) Gel electrophoresis analysis of PCR amplification of Kcnk13 target using Geno_Kcnk13_F3/R3 oligonucleotides. Presence of products differing from wild type (495 bp) confirms mutagenesis on target. However, small nucleotide changes cannot be detected (see animal 2 containing an allele with 3 nucleotide deletion). (B) Allele sequences characterised through Sanger sequencing using the Geno_Kcnk13_F2 oligonucleotide, for each of the five founders obtained from the micro-injection. Sequencing analysis confirms mutagenesis on-target with alleles containing NHEJ repair events with indel mutations ranging from a one nucleotide insertion to deletion of 270 nucleotides. The target sequence of the sgRNA is shown in blue. PAM sequence is shown in green. Inserted nucleotides are shown in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
these are most likely containing one or several correctly mutated alleles (i.e. 2 chances out of 3 to induce a frameshift).

3.3. Screening for point mutations

Introducing targeted point mutations within the mouse genome can be achieved by co-delivering sgRNA and Cas9 mRNA or protein together with a DNA template (generally a single stranded oligodeoxynucleotide (ssODN); [3]). Although double stranded breaks (DSBs) are mostly repaired through the NHEJ pathway, a smaller percentage of repairs can be generated through the homology directed repair (HDR) pathway (Table 2), allowing the introduction of defined nucleotide changes in the genome. Whilst the creation of an indel results in sgRNA recognition site disruption, introducing a targeted point mutation is often insufficient to fully disrupt the recognition site of the sgRNA used, potentially allowing for further rounds of cutting and repair of the target site. In order to prevent re-processing of the correctly modified alleles by the CRISPR system, additional “silent” mutation(s) (synonymous in coding sequences or phenotypically inactive in non-coding region) are included within the donor oligo so that the protoscaler seed or PAM sequences recognised by the sgRNA used are disrupted in the engineered allele. The desired and “silent” mutations can be used in such a way to create new restriction sites to facilitate genotyping at later stages (i.e. F2 generation and onward).

Fig. 2 illustrates a typical outcome of an experiment aimed at introducing a point mutation in the Ptc1 gene: the designed “silent” mutations and the desired mutations (Fig. 2, stars) can often be accompanied with undesired sequence modifications in close proximity on the same allele (Fig. 2, allele 2). Furthermore, partial incorporation of the donor ssODN at the target site can also occur (Fig. 2, allele 1). We have named these alleles obtained through imperfect or incomplete HDR events “illegitimate repairs” [8]. For this reason, the chosen genotyping method must be able to identify discrete sequence variations, as well as ascertain that no other sequence changes have occurred in the vicinity. Therefore, we recommend screening founder F0 animals by Sanger sequencing of PCR amplicons of the targeted locus. In the case of F0 animals showing evidence of the desired mutations, an additional sub-cloning and sequencing step must be performed to ascertain that the intended mutation is not accompanied by additional undesired sequence changes on the same allele. This is required to identify, without ambiguity, positive founder F0 animals to establish a new mutant line.

3.4. Screening for deletions

Generating tailored deletions is achieved through the co-injection of sgRNAs targeting the sequences flanking the segment to delete. Simultaneous activity of the sgRNAs at each side of the target is required to obtain deletions. Using one sgRNA each side of the region to delete is often sufficient to obtain the expected modification. However, as the efficiency of sgRNA/Cas9 complexes and the kinetics of cutting are difficult to predict, we currently prefer employing two sgRNAs on each side of the target to increase the likelihood of success. The range of alleles that can be obtained whilst aiming to delete a sequence is broad and includes: indels in 5′, indels in 3′, indels in both 5′ and 3′, deletion, inversion, duplication, insertion and combinations of the above (Fig. 3).

Fig. 4 illustrate the outcome of an experiment aimed at introducing a deletion in the Slc22a17 gene. Despite the genotype complexity that can be obtained, screening for deletion of a DNA fragment in founder F0s, can efficiently be achieved through amplification of the targeted locus and analysis by electrophoresis (Fig. 4, animal 3, 4, 12 and 13). Subsequent Sanger sequencing of PCR reactions that produce smaller amplicons is performed, enabling the boundaries of the deletion allele generated to be defined and selection of positive founder F0s to mate for the establishment of the mutant line.

Whilst most of the deletions generated will occur between the cutting sites of the sgRNAs used, partial deletions or larger deletions (extending further than the cutting sites of the sgRNAs used) may also be obtained, as represented by the different size of deletion products seen in Fig. 4 (animals 3, 4, 12 and 13). The potential for genotype complexity is even further increased by the use of four sgRNAs that can generate alternative breakpoints (two on either side) and work in all possible combinations.

3.5. Germ line transmission: establishing a new mutant line for the introduction of indel, point mutation or deletion

At this stage, and for all mutation types (indels, point mutations or deletions), all founder F0 animals should be considered mosaic. In fact, even if only two alleles are identified during analysis of the founder ear biopsy DNA, the possibility of additional undetected alleles in these animals cannot be dismissed. These alleles may be present in another part of the body including the germ line, or poorly represented in the ear clip, potentially resulting in the transmission of novel alleles not previously characterised at the
screening step. Reciprocally, alleles characterised in the ear biopsies, might be absent or poorly represented in the germ line of these F₀ animals, potentially resulting in the non-transmission of alleles characterised at the founder F₀ screening step (Fig. 5). Thus, the precise sequence of alleles generated is only confirmed in alleles transmitted to F₁ offspring. We therefore recommend genotyping all F₁ animals for indels and point mutations, and only those showing a smaller amplicon after electrophoresis analysis for deletions, through Sanger sequencing of the targeted locus.

While mosaicism in founder F₀s results in complex and unpredictable genotypes, F₁ animals all have defined heterozygous genotypes with one known WT allele and one allele to characterise. Therefore, Sanger sequencing of F₁s animals will result in a chromatogram containing two traces, with the exception of animals for which the transmitted allele to be characterised correspond to a WT allele or a deletion preventing PCR amplification. Then, by deduction of the known WT sequence, analysis of the chromatogram allows for the final characterisation of alleles transmitted establishing the genetic makeup of new mutant lines.

3.6. Further analysis and considerations

3.6.1. Off-target mutations

As early as 2013, Fu and colleagues [19] raised questions over the specificity of a CRISPR-aided mutagenesis approach, based on a study led with a cell culture model. However, there is growing evidence that off-target mutations are rare when CRISPR is applied to one-cell mouse embryos [10,20,8]. Furthermore, those rare events will most likely be bred out while mouse colonies are amplified. Therefore, although whole genome sequencing does not represent a QC requirement for new lines, it is prudent to avoid using sgRNAs that have a higher likelihood of off target sites on the same chromosome as the targeted allele. If sgRNAs are selected with potential off-target sites on the targeted chromosome with three or fewer mismatches, then PCR amplification and Sanger sequencing of these sites should be performed to confirm their integrity.

3.6.2. Random insertion of the DNA donor

Co-microinjection of genome editing reagents and a ssODN donor has been shown to possibly result in additional random insertions of ssODN-derived sequences [21]. As part of the quality control of new mutant lines, it is wise to ascertain that random insertion of the ssODN sequence has not occurred when generating a designated point mutation. We propose that copy counting of the sequence of the donor in the F₁ offspring by droplet digital PCR (ddPCR) is an accurate, practical and affordable alternative to a whole genome sequencing of positive founders [22].

Taqman assays are designed to detect the ssODN (mutated) sequence (Fig. 6.a). Genomic DNA extracted from a wild-type (WT) mouse is run in parallel to that extracted from mutants (see Fig. 6.b). If the assay also recognizes WT sequence, the copy number should remain unchanged between WT and mutant. Random integration of the donor oligo will be identified by a copy number of 3 and over. If the WT sequence is not recognized by the assay, the copy number should be 1 in correct mutants. It is possible that truncated donors randomly integrate in the genome. It is for the operator to decide the extent to which assays are multiplied along the donor sequence to check for such events.

3.6.3. Phenotyping

Using data from a large number of projects, we commonly found analysis of F₀ animals revealed mosaicism. The extent of this mosaicism is often not revealed until the F₁ stage. This should be kept in mind when considering phenotyping data derived using...
F0 animals. This genetic complexity is further compounded when experiment aims for the simultaneous alteration of several targets. Thus, while phenotype traits of interest might be seen in founder animals, we recommend that phenotype data should be acquired from the F1 generation and onward.

4. Summary

In summary, the genetic complexity of the founders generated by microinjection is common to the three types of CRISPR-aided modification and that the genotype of new mutants can only be ascertained in the subsequent generation. The complexity of outcomes can be further enhanced by choices made at the point of sgRNA design (i.e. multiple sgRNAs). Appropriate and efficient strategies according to the desired allele type are summarised in Table 3.

Careful sequencing of the targeted locus is required for all CRISPR/Cas9-aided mutagenesis projects. For projects aiming to introduce an indel, PCR amplification and Sanger sequencing of the target region is required for both the F0 (screening) and F1 (genotyping) generations. Screening of F0 animals for point mutation projects has to be more stringent. We propose to PCR amplify and sequence all founders. Sub-cloning of PCR products and sequencing is used to disentangle the alleles present in those founders where there is evidence that the intended point mutation has been introduced. Once the founder alleles have been characterised, F1 animals can be genotyped by PCR amplification and Sanger sequencing. When deleting segments of sequence from the

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**Table 3**

<table>
<thead>
<tr>
<th></th>
<th>F0s</th>
<th>F1s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indels</td>
<td>PCR amplification analysed by Sanger sequencing</td>
<td>PCR amplification analysed by Sanger sequencing</td>
</tr>
<tr>
<td>Point mutations</td>
<td>PCR amplification analysed by Sanger sequencing, sub-cloning of amplicons and Sanger sequencing</td>
<td>PCR amplification analysed by Sanger sequencing</td>
</tr>
<tr>
<td>Deletions</td>
<td>PCR amplification and electrophoresis, Sanger sequencing of samples showing deletion</td>
<td>PCR amplification and electrophoresis, Sanger sequencing of samples showing deletion</td>
</tr>
</tbody>
</table>

The table shows appropriate and efficient screening (F0) and genotyping (F1) strategies according to the desired allele type.
genome, PCR amplification and electrophoresis will enable identification of animals at the F₀ stage that harbour deletions. Sequencing of PCR products containing the smaller PCR amplicons will enable characterisation of the deletion found in these F₀ animals. In subsequent generations, PCR amplification and sequencing of those animals amplifying a smaller product is used to confirm which deletion alleles have been transmitted.

In conclusion, the outcome of CRISPR-aided mutagenesis is unpredictable and it is essential that mutants are characterised to sufficient standards. Here we have proposed a framework for the analysis of F₀ and F₁ animals.

Author’s contributions
JM, GC, AC, RF, RK and LT designed protocols, sgRNA and oligonucleotides, synthesised reagents and genotyped progeny mice. MH, MES and SW undertook mouse colony management. JM, GC, AC and LT conceived the study. All authors contributed to the writing of the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare no competing interest.

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Appendix A. Supplementary data
Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jymeth.2017.03.016.

References