CRISPR/Cas9 streamlining and avoiding pitfalls

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BACKGROUND/OVERVIEW The process of using CRISPR/Cas9 has been being utilized for genetic manipulations for over a decade. There is no shortage of papers on the topic, but very few describe the actual planning of the new CRISPR/Cas9 project. There are countless details that should be considered during design that can make a huge difference to the success of a project and how easily it can be detected. A few refinements to both design and diagnosis can not only save time and money but also increase the chance of success. Some reagents can even eliminate steps from the diagnostic process as well as allowing pre-screening of candidates prior to sequencing.

INTRODUCTION The CRISPR/Cas9 approach has been used for over 10 years with great success. A lot of this success has come about through not just the fundamental usefulness of the technique but also good design. It isn't even a question of selecting gRNA or paired gRNAs with the least undesirable features it also requires good design of the repair template and therefore how successful changes can be diagnosed.

METHODS Eliminating a restriction site is the most obvious change to bring about in a repair template **(fig 1)**. gRNA sequences need to be eliminated to protect the repair template and to bias conditions to favour integration. Many programs exist to codon optimize sequences and to diagnose restriction patterns. The site "DNA Wobbler" accepts a sequence of interest then explores all silent mutations and not just elimination but introduction of restriction sites **(fig 2)**. A deletion could cause a false positive result for site removal, but site introduction is immune to this danger and provides an extra level of reassurance.

In addition to this problem, some genetic manipulations can prove lethal when introduced. This complicates troubleshooting as there are countless potential causes of experimental failure of CRISPR/Cas9 manipulations. An approach to shed light on this situation is to produce a second repair template that only confers immunity to recognition by Cas9. This "Fake Wild Type", when used in parallel with the intended mutation, will provide a clean confirmation that the Cas9 reagents are targettting successfully in a non-lethal manner **(fig 3)**.

The diagnosis of successful clones by restriction digest is a robust technique but obtaining a PCR product to screen traditionally requires multiple steps. A large number of candidate cells need to be lysed, genomic DNA isolated and then used for PCR. This PCR product is then cleaned and finally digested. For every candidate this results in a lot of time and plastic.

Using **Terra Red Direct PCR mixture** from Takara only a single ul of cultured cells (still in media) can be added directly to a PCR reaction. A sample of the PCR reaction can be added directly to a FastDigest reaction and run on a gel with zero purification steps **(fig 4).** The starting material can also be a fruit fly wing, a blood sample or a tissue sample.

Removing all these steps from the workflow greatly speeds up diagnosis and reduces the chance of errors. In some cases simply diluting a sample of the PCR product that gave a positive RFLP result is enough for submission for sequencing – entirely eliminating purification from the workflow.

If, alternatively, you are just wanting to knock out a gene, rather than introduce precise mutations, then there are ways to enhance your screening efficiency. Selecting gRNAs with a unique restriction site straddling the Cas9 cut site ensures that any gain or loss of nt will eliminate the restriction site and guarantee detection. I call this the "hair trigger" knockout **(fig 5).**

Sequencing of potential knockouts can be challenging due to two (or more) copies of a gene. The "drumbeat method" allows you to decipher overlapping chromas by focusing on a single dNTP rather than all four **(fig 6).** The same approach can be used to analyse knockouts in cell lines, in which breeding is not possible. This is done by observing the absence of the wild type "drumbeat" and comparing the two distinct changes in "rhythm".

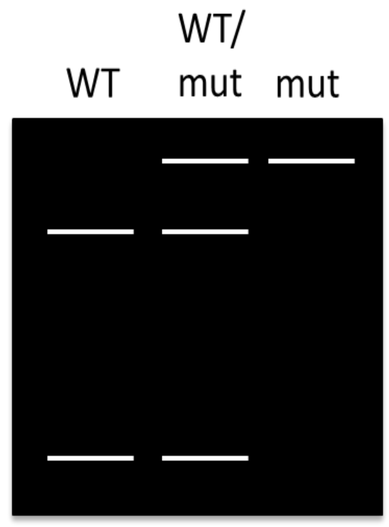


FIGURE 1 DNA Diagnostic digest allows easy distinguishing of mutants – both heterozygote and homozygote

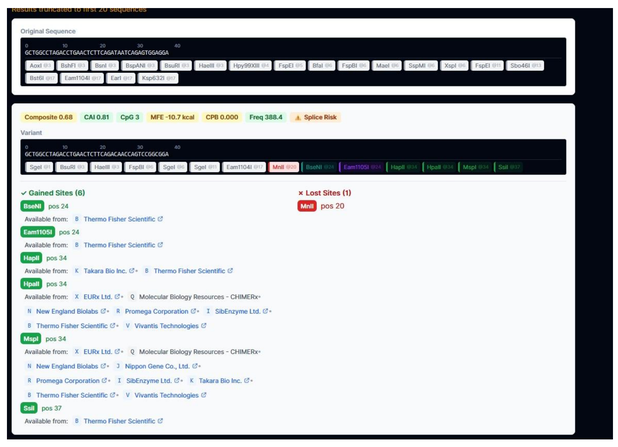
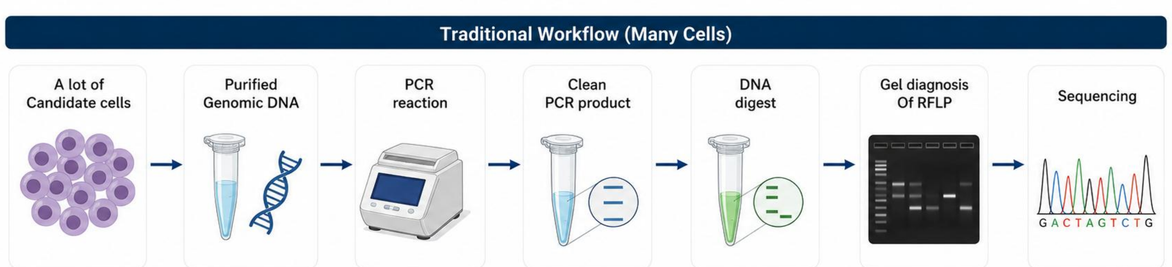


FIGURE 2 DNA Wobbler output example – both gained and lost restriction sites sorted by enzyme supplier.
https://tinyurl.com/dnawobbler



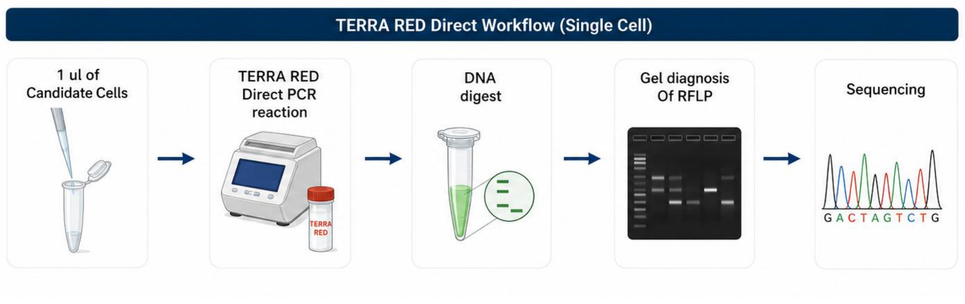


FIGURE 4 Comparing traditional workflow in candidate diagnosis to use of Terra Red Direct PCR mix and FastDigest enzymes on unpurified PCR products

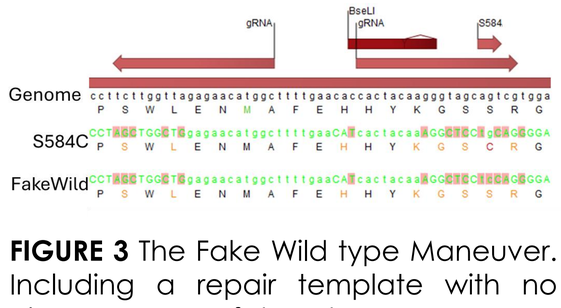


FIGURE 3 The Fake Wild type Maneuver. Including a repair template with no changes as a safety net

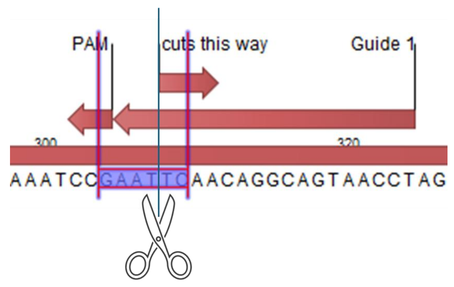


FIGURE 5 The "Hair Trigger" knockout gRNA site. EcoRI site is lost if any bases are added or removed. Base loss usually takes place in the direction of "cuts this way"

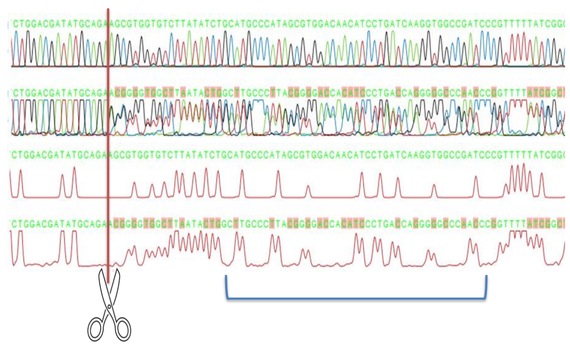


FIGURE 6 The Drumbeat diagnosis of deletions. Isolating a single base allows the size of the deletion to be analysed

DISCUSSION & CONCLUSION Like with many processes you get out what you put in. If you spend some extra time and attention at the outset of a CRISPR/Cas9 project then it will save you considerably down the line. Anticipating problems before they take place will make troubleshooting easier, and hopefully unnecessary.

QR CODES OF USEFUL LINKS

If you want to read more about some of the approaches that I use for cloning and CRISPR/Cas9 manipulations check out the first QR code below. If you want to reward me for my help you can buy me a coffee (see second QR code) and to use the DNA Wobbler site check out the third QR code. For a link to the Takara Terra Red Direct PCR reagents see the QR code on the far right.

TaKaRa
Terra Red Direct PCR reagents

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