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Research review paper

Detection of on-target and off-target mutations generated by CRISPR/Cas9 and other sequence-specific nucleases

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ABSTRACT

The development of customizable sequence-specific nucleases such as TALENs, ZFNs and the powerful CRISPR/Cas9 system has revolutionized the field of genome editing. The CRISPR/Cas9 system is particularly versatile and has been applied in numerous species representing all branches of life. Regardless of the target organism, all researchers using sequence-specific nucleases face similar challenges: confirmation of the desired on-target mutation and the detection of off-target events. Here, we evaluate the most widely-used methods for the detection of on-target and off-target mutations in terms of workflow, sensitivity, strengths and weaknesses.

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1. Introduction

The use of sequence-specific nucleases (SSNs) for genome editing has become routine in many laboratories. Genome editing tools such as zinc finger nucleases (ZFNs) (Kim, Cha, & Chandrasegaran, 1996), transcription activator-like effector nucleases (TALENs) (Christian et al., 2010) and especially the more recent clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) system (Jinek et al., 2012), have provided researchers with the ability to create double-strand breaks (DSBs) at any desired position in the genome. In higher eukaryotes, DSBs are usually resolved by the endogenous DNA repair mechanism of non-homologous end-joining (NHEJ) which is intrinsically error-prone, typically resulting in small insertions and/or deletions (indels) at the site of the break. If the indels cause a frameshift mutation, they can knock out the function of the gene due to the production of truncated polypeptides and/or nonsense-mediated mRNA decay (Perez et al., 2008; Ramlee, Yan, Cheung, Chuah, & Li, 2015; Santiago et al., 2008; Sung et al., 2013).

The target sequence of the CRISPR/Cas9 system can be changed simply by altering the 20-nt sequence of the single guide RNA (gRNA), so the generation and testing of multiple targeting constructs has become straightforward. However, once the components of the system have been introduced into the host organism, the next major challenge is to confirm and characterize the resulting mutations. In the relatively simple case of targeting a single diploid cell, there are four potential outcomes: no mutation, a heterozygous mutation (only one allele is mutated), a biallelic mutation (both alleles are mutated but the sequence of each allele is distinct) or a homozygous mutation (both alleles carry the same mutation). The latter can also occur if one allele is used as a template to repair the break in the other allele. More complex outcomes are possible in polyploid host species, when the mutated organism is a chimera, or when pools of samples are screened. Off-target mutations can further complicate the analysis, but specific methods have been developed to identify such events as discussed later in this article. All methods for the analysis of on-target and off-target mutations have pros and cons and the ideal method in any situation depends on a number of factors, including the type of sample, the anticipated size and frequency of the mutations, and the cost of the method.

2. Detection of on-target mutations

The most widely used methods for the detection of targeted mutations are summarized in Table 1. These are all based on the polymerase chain reaction (PCR) and therefore tend to underestimate the frequency of on-target activity because large deletions that extend beyond the boundaries of the PCR amplicon are not detected, and large insertions are amplified less efficiently than small mutations (if at all) and are therefore less likely to be identified. This tends not to be a critical issue when a single gRNA is used because small indels are much more common than large deletions or insertions, but larger indels arise at higher frequency when two gRNAs are designed to target sites on the same chromosome. In the case of mutants present at a very low frequency in an otherwise wild-type background (such as chimeras or pooled clones), the PCR step is often biased towards the more abundant template, and the small number of mutated sequences may not be detected. One way to reduce this problem is to pre-digest the genomic DNA with a restriction enzyme recognizing the wild-type sequence, thus eliminating the wild-type template before the amplification step, although this depends on the availability of restriction sites overlapping the nuclease target sequence. An alternative is "co-amplification at lower denaturation temperature" (ice-COLD-PCR), which improves the detection of rare mutant sequences in chimeric clones because it does not favor the amplification of the proportionally dominant wild-type sequence (Milbury, Li, & Makrigiorgos, 2011). Of course, if the mutated sequences are intentionally enriched, the results cannot be considered quantitative. Regardless of the detection method, it is always

Table 1
Overview of methods for the detection of on-target mutations induced by SSNs.

Methods	Type of mutations preferentially detected	Reported sensitivity	Determination of mutation type?	Cost[a]	Throughput	Limitations	References
Mismatch cleavage assay	Small indels	0.5–3%	No	$	Moderate	T7E1 can overlook single nucleotide changes; Surveyor less sensitive than T7E1	Kim et al., 2013; Qiu et al., 2004; Ran et al., 2013; Vouillot, Thélie, & Pollet, 2015; Zhu et al., 2014
HRMA	Small indels	2%	If insertion or deletion	$ (+ equipment)	High	Misses large indels	Dahlem et al., 2012; Wang et al., 2015
Heteroduplex mobility assay by PAGE	Small indels	0.5%	No	$	Moderate	Misses large indels	Zhu et al., 2014
CAPS	All		No	$	Moderate	Availability of restriction site	Ran et al., 2013
Loss of primer binding site	Indels	10%	Yes	$	High	Misses substitutions	Yu, Zhang, Yao, & Wei, 2014
Sanger sequencing	All	1–2%	Yes	$$/$$$[b]	Low	Costly, labor intensive	Brinkman, Chen, Amendola, & van Steensel, 2014; Liu et al., 2015
NGS	All	0.01%	If insertion or deletion	$$$$	High	Misses large indels	Güell, Yang, & Church, 2014
AFLP	Large indels, also Mb		If insertion or deletion	$	Moderate	Misses small indels	Bauer, Canver, & Orkin, 2015
Fluorescent PCR-capillary gel electrophoresis	Small indels	1%	Number of bp	$$	High	Misses substitutions	Ramlee et al., 2015; Yang et al., 2015

[a] Estimated cost per assay. $: <1 US$; $$: <5 US$, $$$: >100 US$; $$$$: >500 US$.
[b] Sequencing of bulk/cloned PCR products.

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