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CRISPR SCREENING SERVICES

CRISPR SCREENING METHODS

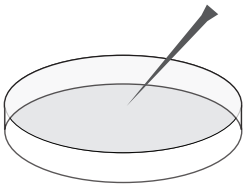
Introduction

The CRISPR-Cas9 gene editing approach has generated much excitement as a powerful investigative tool in molecular biology and as a driver of precision medicine. Its surging popularity can be attributed to its relative simplicity, superior targeting efficiency and improvements to reduce off-target incidences.

However, researchers have to grapple with challenges in the CRISPR-Cas9 experimental process, particularly in the confirmation of desired on-target mutation events. This article presents the commonly used approaches for indel detection and highlights the advantages and limitations of each method.

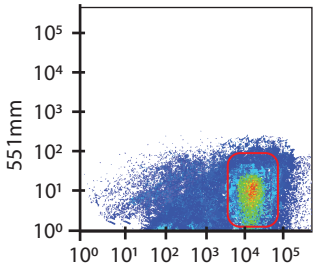
CRISPR Workflow Example

TRANSFECT/TRANSDUCT



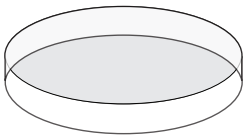
- Transfect/Transduct cells with CRISPR reagents (e.g. Cas9-eGFP/sgRNA ribonucleoprotein complex).

ENRICHMENT



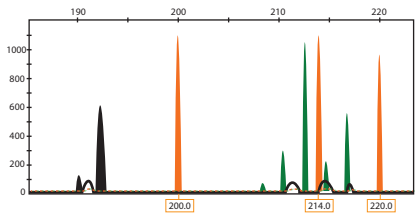
- Isolate marker positive cells by FACs or antibiotic selection.

EXPANSION



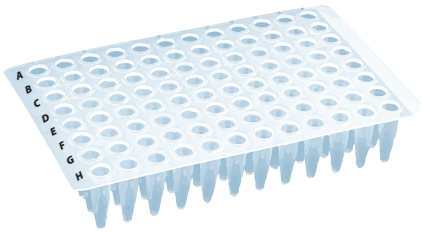
- Plate marker positive cells to grow.

SCREENING



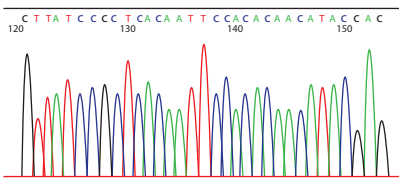
- Perform screening assay (e.g. FA-PCR) on mixed clone population to determine if a significant proportion of cells have the desired genome edit.

EXPANSION



- Expand single cell clones on 96-well plate.

SCREENING



- Perform screening on clonal cell lines to confirm and validate desired genome edit.
  Note: No. of clones to screen is informed by mixed clone population screening analysis.

1.SANGER SEQUENCING

The gold standard method to identify induced mutations at the target locus entails amplifying the target region by PCR, then cloning the amplicon into a vector, followed by Sanger sequencing of the amplicon directly. Each vector should carry only one gene copy, which generates a clean trace in the chromatogram after sequencing. This method is preferably used to investigate individual clonal cell lines. The advantage of sequencing methods is the information on the type and frequency of mutations at the target locus.

Limitations
In order to capture all gene copies, many colonies have to be sequenced. This process is laborious and time-consuming. Moreover, while direct Sanger sequencing of mixed populations/pooled clones is also possible, it is not recommended because the resulting chromatogram contains multiple overlapping traces that are difficult to differentiate (Bell et al. 2014). This is also observed for polyploid or diploid organisms with heterozygous or biallelic mutations. Overlapping traces can be automatically decoded by bioinformatics tools such as DSDecode or TIDE. The latter is able to detect indels with a sensitivity of 1-2% across various target regions in a pool of cells (Brinkman et al., 2014).

2.NEXT GENERATION SEQUENCING

Next Generation Sequencing (NGS) enables indel detection in both mixed populations and clonal cell lines. Its ability to process a large number of samples (samples are pooled together for a high-throughput sequencing run) and simultaneously screen off-target changes makes it a popular choice among researchers.

The data obtained from targeted amplicon sequencing is highly sensitive with detection levels as low as 0.01% (Hendel et al. 2015). This means that the researcher can be relatively certain that their samples do not contain off-target mutations if they are undetectable using this technique.

Besides being able to detect if all alleles of a gene were correctly edited, information on indel location and whether a cell population is truly monoclonal can be derived from NGS data.

Limitations
The NGS method is cost-effective only for larger sample numbers. Furthermore, NGS data typically requires analysis by a bioinformatician. Also, because of its relatively short reads, the method misses larger indels.

3.MISMATCH CLEAVAGE ASSAYS

Mismatch cleavage assays such as the T7E1 mismatch cleavage assays have been widely adopted due to their relative simplicity, speed and cost-effectiveness. They can be used on single clones or pooled samples. They are typically used to preliminarily screen and identiy clones for more detailed analysis such as by sequencing.

These assays rely on pairing or hybridisation between the edited and wild-type strands of the host DNA. The nuclease detects mismatches on the hybridised strands and cleave them. The resulting fragments are dissimilar in size and can be visualised using conventional gel electrophoresis.

Limitations
One limitation of these assays is the lack of sequence-level information . In addition, the T7E1 assay misses SNPs and small indels (Vouillot et al., 2015). Furthermore, the assay may require optimisation of various experimental parameters due to its sensitivity to reaction conditions. One should note that to detect homozygous mutations, wild-type DNA has to be added to the PCR step for the formation of heteroduplexes.

4.FRAGMENT ANALYSIS

Fragment analysis is a capillary electrophoresis (CE) based method for AFLP, MLPA & SNP detection. CE is a proven sensitive, high-throughput and high-resolution system for nucleic acid analysis.

Recently multiple fragment analysis methods such as IDAA, Fluorescent PCR and CRISPR-STAT have been developed in CRISPR/Cas9 genome editing studies. These are reported to be fast, sensitive, precise and cost-effective methods for mutation detection. Moreover, CE instrumentation with Sanger sequencing capability, e.g. Thermo Fisher 3130 and 3730 series, enables single base resolution.

Fragment analysis assays have a simple two-step protocol (PCR followed by CE) independent of enzymatic cleavage. It has been reported that their sensitivity and resolution are comparable to NGS with an indel detection sensitivity of about 0.1% (Lonowski et al., 2017). Hence they can be used in both basic research and more challenging genome editing applications such as therapeutic indel profiling.

Fragment analysis assays are promising for various applications. It has been reported by Gardner et al. (2016) as “an analytical tool [which] fundamentally changes the scale and complexity of experimental design”. According to them, high-throughput CE assays based on fluorescently labeled oligonucleotides can be designed to characterise, discover, engineer, or screen nucleic acid metabolic enzymes. Such studies include that on DNA repair, recombination, restriction, modification and RNA metabolism. Furthermore, it has been applied as reagent quality control assays.

Limitations

The bottleneck for fragment analysis methods lie in secondary data analysis. While there is no limiting factor for primary data processing (baselining, peak detection and sizing), current software such as GeneMapper and Peak Scanner (both from Thermo Fisher) do not fully satisfy data analysis requirements for new applications such as fragment labeling and quantification, and particularly targeting efficiency calculations for genome editing studies.

5.HIGH RESOLUTION MELTING (HRM)

High Resolution Melting (HRM) analysis is a post-PCR analysis method which enables identification of variations in nucleic acid sequences. HRM involves analysis of melting curves generated from performing real-time PCR in the presence of a dsDNA binding fluorescent dye. During melting, the dye is released and emits fluorescence as the dsDNA denature. The fluorescent intensity is plotted against the melting temperature to give the raw melt curve data. Each type of genome edits (wild-type, heterozygous mutation, biallelic mutation, or homozygous mutation) will generate a distinct amplicon-specific melting curve. By analysing the melting curve, it is possible to distinguish between different mutant alleles. The sensitivity and accuracy of the HRM-based method enables detection of CRISPR/Cas9-induced mutations from as early as a 2-cell stage zebrafish embryo (Samurat et al., 2016). HRM shows good detection reliability even for single-base pair indels; results show that less than 5% of mutated DNA containing one bp indel can be detected using HRM (Denbow et al., 2017).

HRM analysis requires a simple set-up and involves minimal pipetting steps compared to enzymatic or SDS-PAGE methods. Then entire flow from genomic DNA extraction to PCR and finally HRM analysis can be completed in as little as 5 hours. This enables rapid high throughput screening of mutations.

Limitations

One disadvantage of HRM analysis is the requirement of a dedicated software. An alternative is the melting temperature analysis function in a regular real-time-PCR machine. However, it has beenshown that melt curve analysis in regular real-time-PCR has decreased sensitivity and takes a longer time than HRM (Denbow et al., 2017).

OVERVIEW OF DETECTION METHODS FOR ON-TARGET MUTATIONS INDUCED BY CRISPR/ CAS9

	Mismatch Cleavage	HRM Analysis	FA-PCR	Sanger Sequencing	NGS
Cost	+	+	++	++	+++
Throughput	++	+++	+++	+	+++
Reported Sensitivity	++	++	++	+	+++
Mutation Sequence?	No	No	No	Yes	Yes
Mixed population or clonal cell line screening?	Both	Clonal	Both	Clonal	Both
Differentiates heterozygosity vs. homozygosity	No	Yes	No	Yes	Yes

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