Development of Efficient CRISPR-Cas9 Precision Editing for *Leishmania* to Investigate Protein Kinase Function

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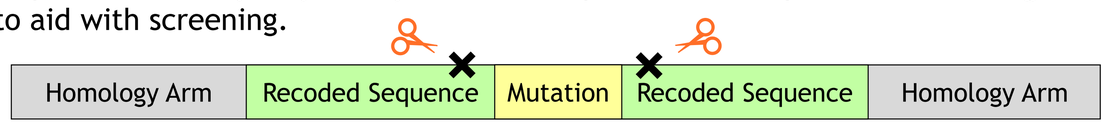
1. Background

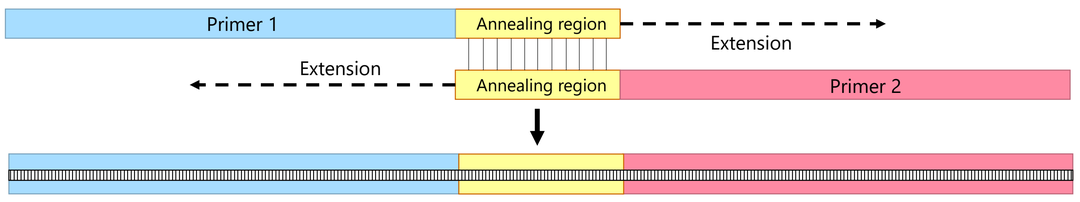
- *Leishmania mexicana* is a single celled protozoan parasitic organism that causes Cutaneous Leishmaniasis (CL). The World Health Organisation estimates CL causes 30,000 new cases per year [1].
- Study of essential genes is challenging as *L. mexicana* lack RNAi machinery[2] and there are limited inducible gene-deletion options.
- CRISPR-Cas9 system allows precise double-stranded DNA (dsDNA) breaks at specific sequences, directed by a single-guide RNA (sgRNA)[3,4].
- Precision editing is the process of making small scale genetic mutations without the inclusion of selection markers.
- Precision editing can be used to investigate the function and regulation of essential protein-coding genes, with a reduced chance of generating a lethal phenotype. Additionally, it can be used to investigate the role of specific amino acids of a protein *in vivo*.

2. Precision Editing Methodology

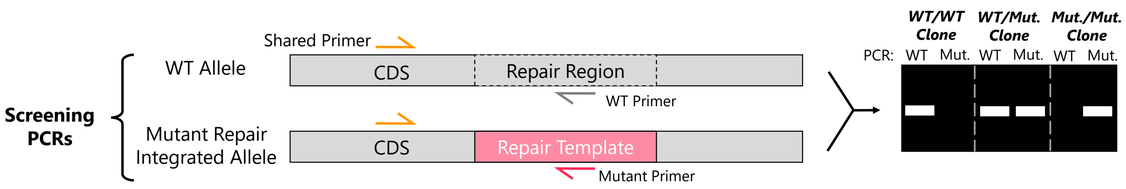
160 bp repair templates were designed as below. Each template had 50 bp homology arms and two sgRNA targeting sites. The PAM and sgRNA-recognition sequences were synonymously recoded to prevent subsequent cas9 cleavage and to aid with screening.



Repair templates were generated through a PCR-based approach, using two oligonucleotides that anneal to form a 160 bp dsDNA template[4].



L. mexicana cells expressing T7 RNA polymerase and cas9[4] were transfected with the repair template and two sgRNAs. Up to 24 clones from each transfection were screened by PCR to detect integration of the repair template. Sanger sequencing was used to confirm the genotype of mutant PCR-positive clones.

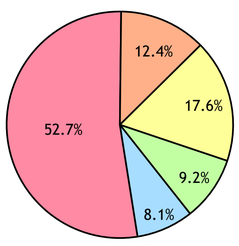


3. 29% of Clones Screened Integrated the Repair Template

Homozygous mutants were identified in all 35 different mutations within 24 clones. Efficiency varied between transfections (data not shown).

Sanger sequencing confirmed 23.1% of clones were homozygous mutants, 4.1% heterozygous mutants, and 1.9% "complex" mutants. Complex mutants were any other integration event such as partial integration or unexpected deletions etc.

PCR Screen



Sequenced Clones

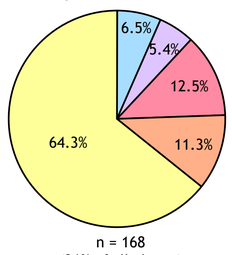


Table 1. Target sites and mutations.[5, 6]

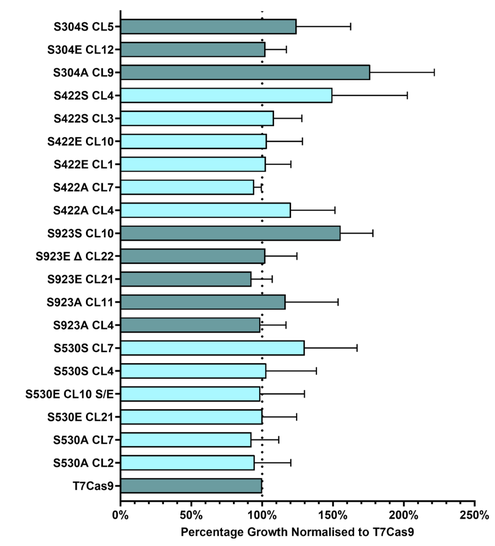
	Gene	Target Site	Replacement Residues	Target type
Non-Essential Genes	LmxM.29.3580 (SRPK)	F234	Alanine (A) Glycine (G) Synonymous (F)	Gatekeeper
	LmxM.31.0120 (MRK1)	M346	Alanine (A) Glycine (G) Synonymous (M)	Gatekeeper
	LmxM.33.3020	M404	Alanine (A) Glycine (G) Synonymous (M)	Gatekeeper
	LmxM.36.0720 (MPK2)	F91	Alanine (A) Glycine (G) Synonymous (F)	Gatekeeper
	LmxM.18.0640 (HDK1)	L86	Alanine (A) Glycine (G) Synonymous (L)	Gatekeeper
		C166	Alanine (A) Synonymous (C)	Catalytic residue
Essential Genes	LmxM.36.5350 (KKT2)	S25	Alanine (A) Glutamic Acid (E) Synonymous (S)	Phosphosite
		S493		
		S530		
		S923		
	LmxM.10.0300 (KKT4)	S422	Alanine (A) Glutamic Acid (E) Synonymous (S)	Phosphosite
	LmxM.27.0430 (KKT7)	S307	Alanine (A) Glutamic Acid (E) Synonymous (S)	Phosphosite

4. Kinetochore Phosphosite Mutants Have No Apparent Cell Cycle Defects

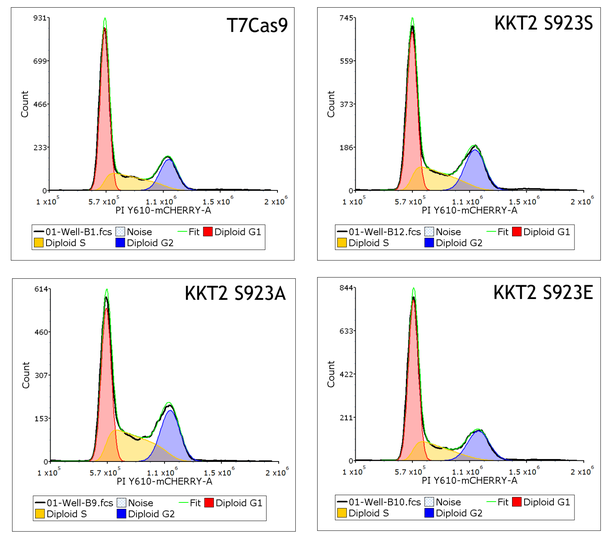
The kinetochore is an essential protein complex that connects microtubules to DNA during chromosomal segregation in mitosis[5]. The kinetochore is highly divergent in kinetoplastids.

KKT2, KKT4 and KKT7 mutants were assessed for growth rate changes by Alamar blue assay and cell cycle defects by propidium iodide flow cytometry.

Alamar Blue Growth Assay

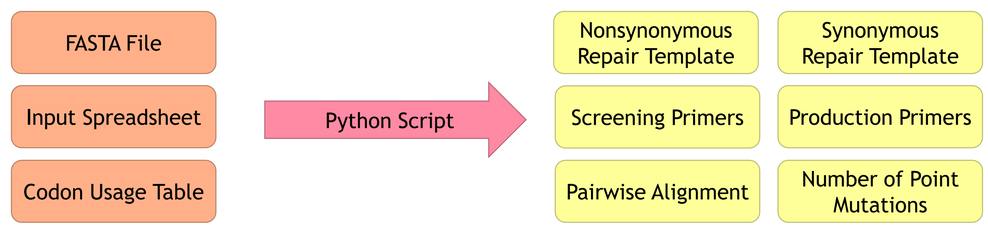


Cell Cycle Analysis by Flow Cytometry

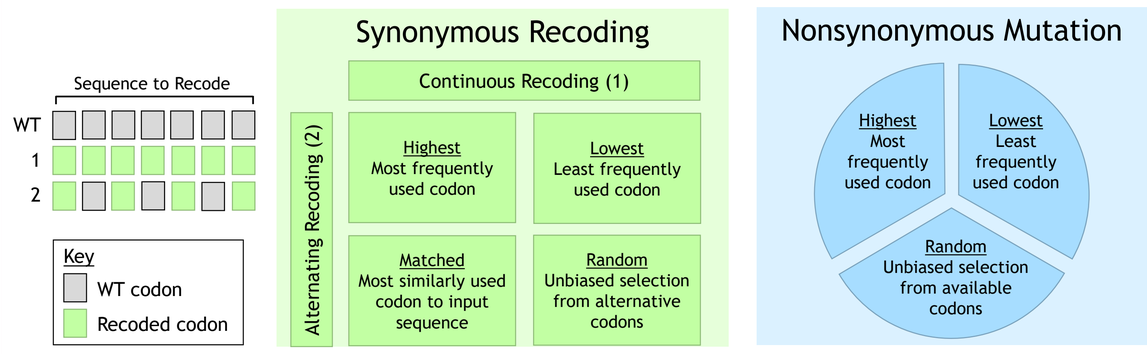


5. Development of a Repair Template Design Tool using Python

A design tool for kinetoplastids has been developed to replicate the recoding process used to create the repair templates. The tool is accessible to non-programmers. It offers rapid generation of a recoded repair sequence, as well as designing primers for both repair template production and screening of clones.



The tool offers several methods of recoding for customisability.



6. Summary

- The precision editing method presented here is efficient at generating homozygous mutants across a range of different genes. It is flexible, affordable, and does not require any laborious plasmid cloning.
- The creation of a computerised design tool allows rapid design, with a consistent recoding strategy, to enable standardisation.
- There was no apparent growth defects nor cell cycle defects seen in the kinetochore phosphosite mutants generated.

Future Plans

- To develop the precision editing method further to enable higher-through put screening approaches.
- To add more features to the design tool to enable CRISPR guide design, batch processing of target sites and web-based access.

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