Optimization of a genomic editing system using CRISPR/Cas9-induced site-specific gene integration

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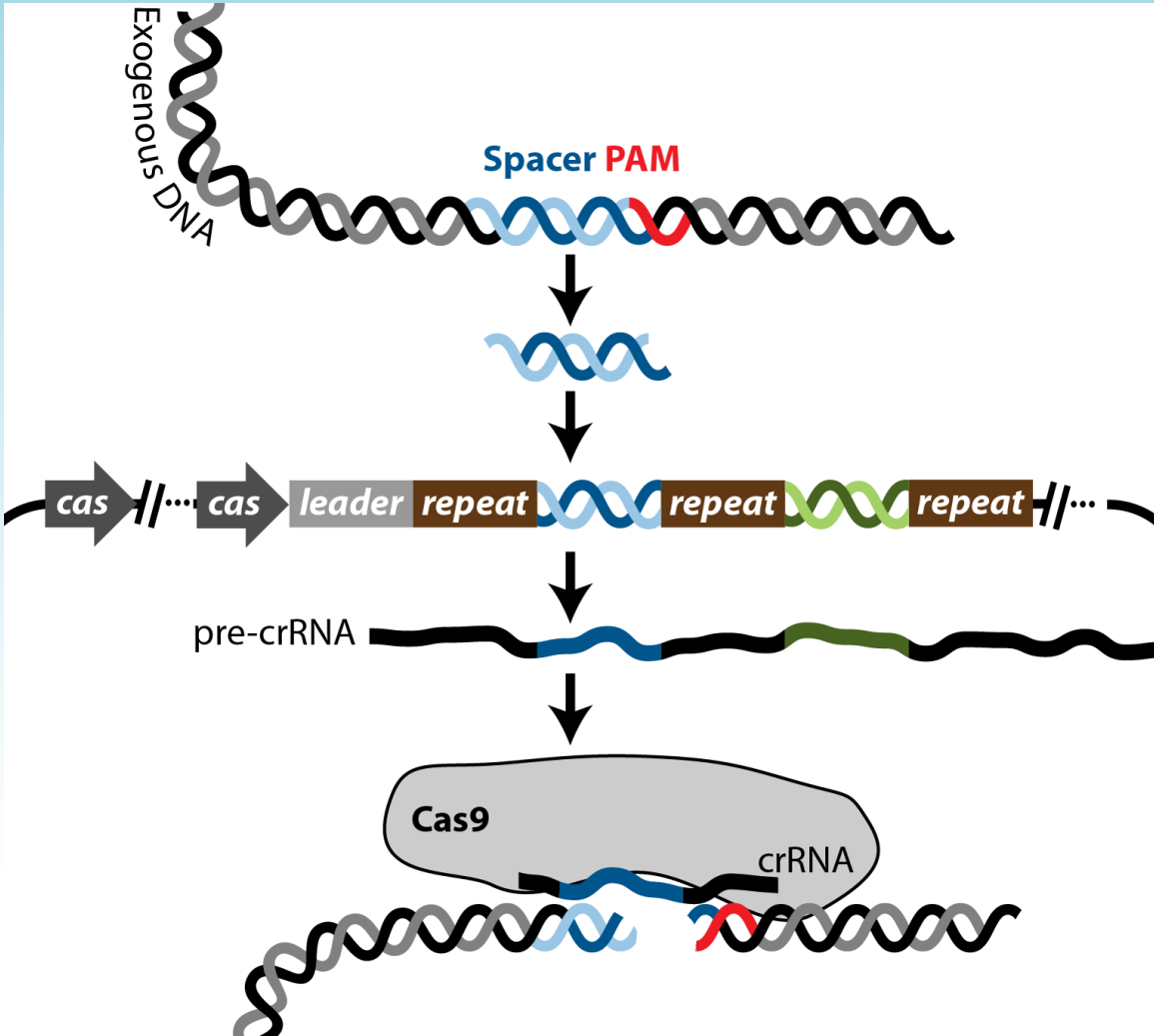
What is the CRISPR-Cas System?

- Bacterial defense and adaptive immunity against foreign genetic material
- **C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats
- **C**RISPR-**AS**sociates Proteins
- Many CRISPR pathway types
  - Type 1- Cleave/Degrade DNA
  - **Type 2- Cleave DNA**
    - **Acquisition**
    - **Expression**
    - **Interference**
  - Type 3- Cleave DNA or RNA
- CRISPRs are transcribed into short RNAs, and guide Cas-protein to cleave genomic material

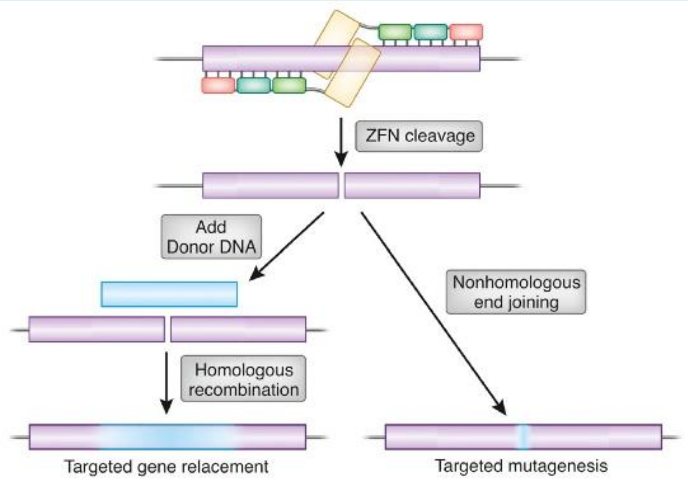


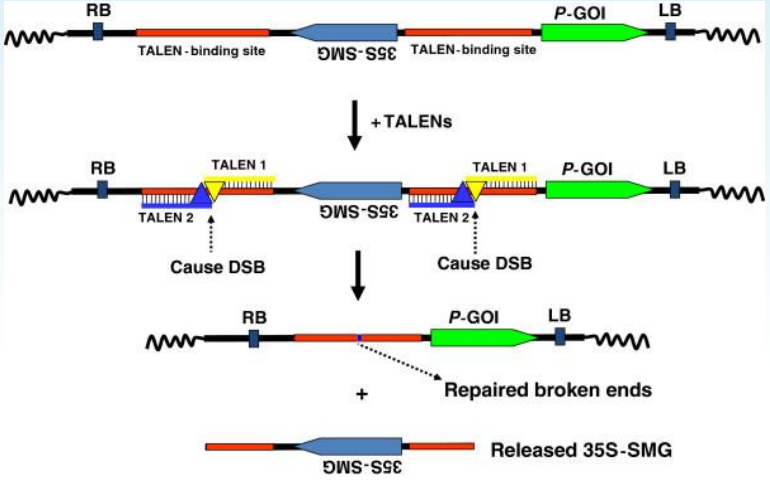
Traditional creation of transgenic lines

- Techniques
  - microinjecting the transgenic construct into a fertilized egg
  - Retrovirus or bacterial vector
  - transfect a transgenic construct into mouse embryonic stem (ES) cells and then mouse blastocysts

- Limitations
  - Variations in the mouse responses
  - Injections and zygote harvesting is a skill
  - Consistency
  - Generational systems
  - Poor transgene incorporation
  - Transgene size

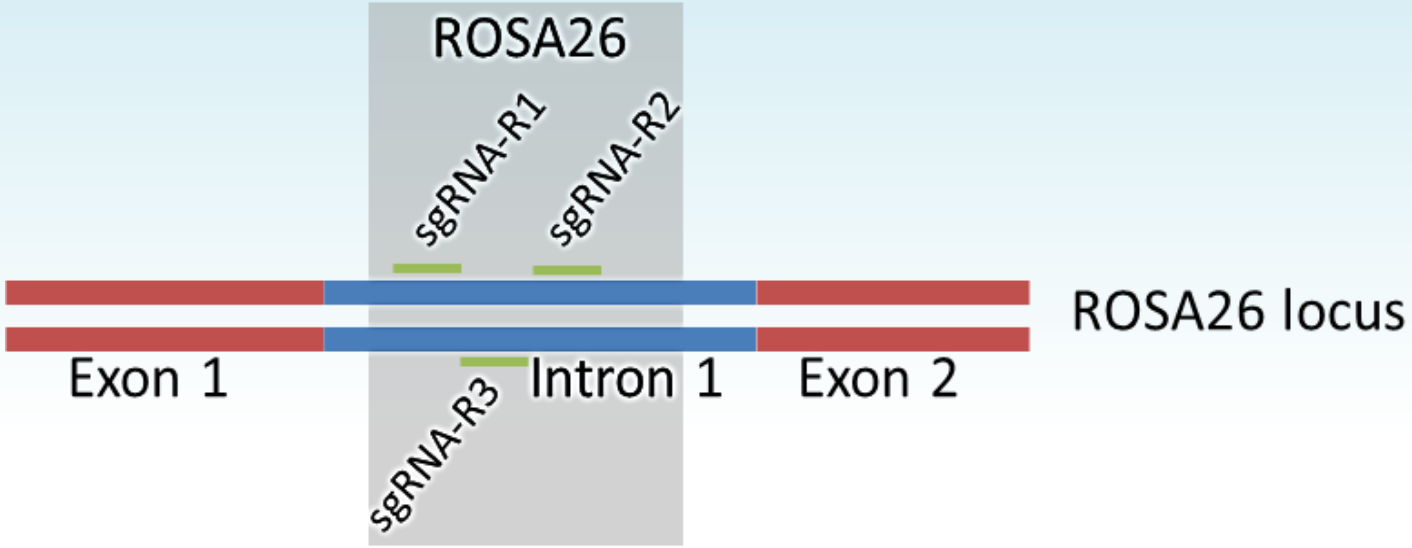
- ZF (zinc finger) and TALEN (transcription activator-like effector nuclease)
  - Repetitive nature this timely construct development
  - Expensive and difficult to compare to or alter multiple genes,





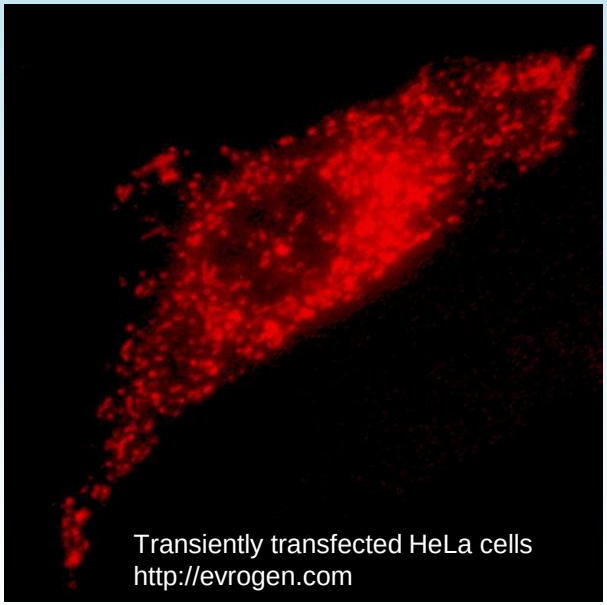
ROSAβ26

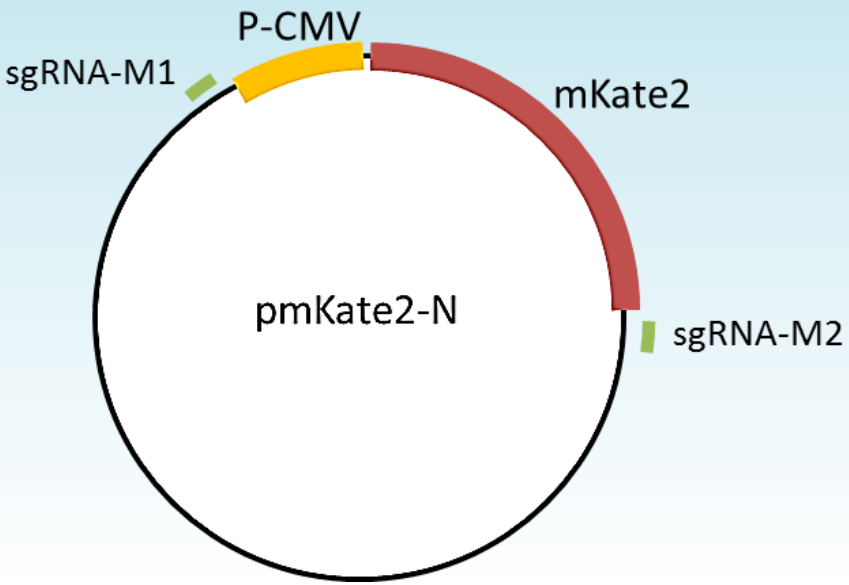
- ROSAβ26 - used as a target for reporter genes through genomic insertions
- "Safe harbor" locus which allows for
  - transgene integration
  - single copy insertion
  - cis-regulatory elements
- Not prone to gene-silencing effects or decreases in cell viability.
- Completely sequenced so easy to target
- Non-fluorescent so screening involves a different process



pmKate2-N

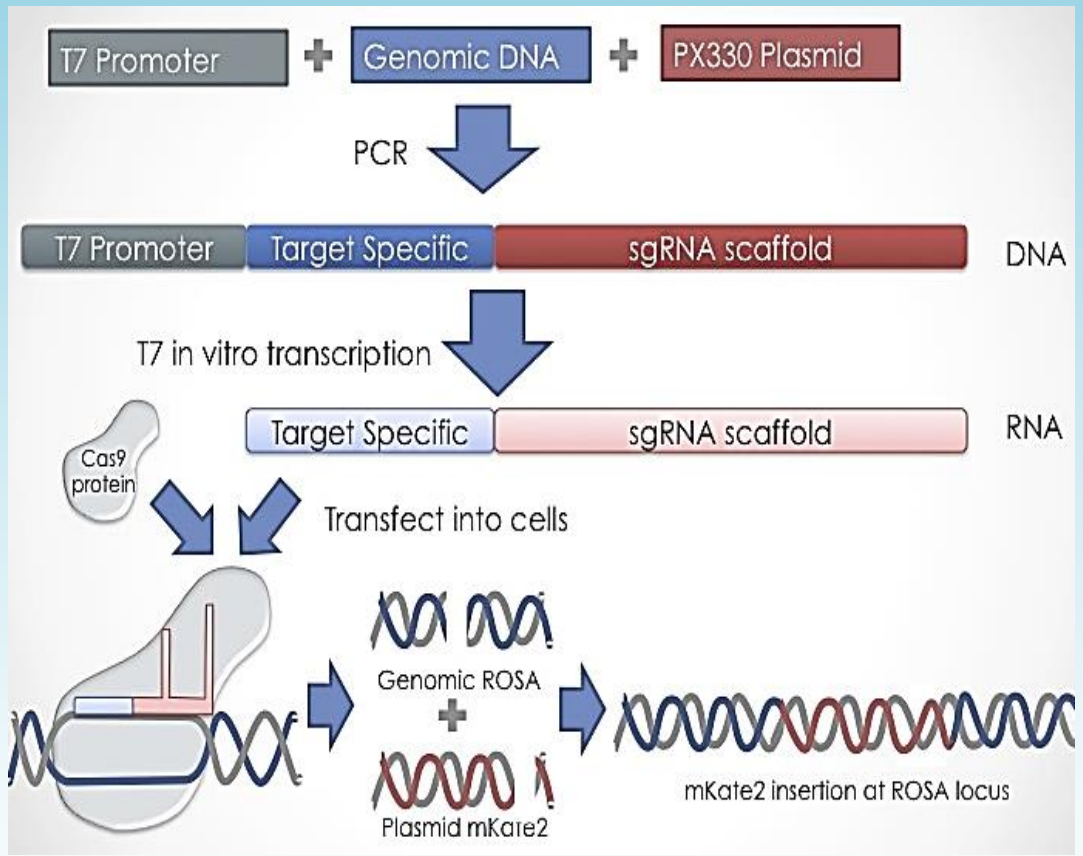
- pmKate2-N - expression vector encoding for far-red fluorescent protein
  - mKate2 is a monomeric protein
  - Highly stable
  - Fluoresces between 588 and 633 nm



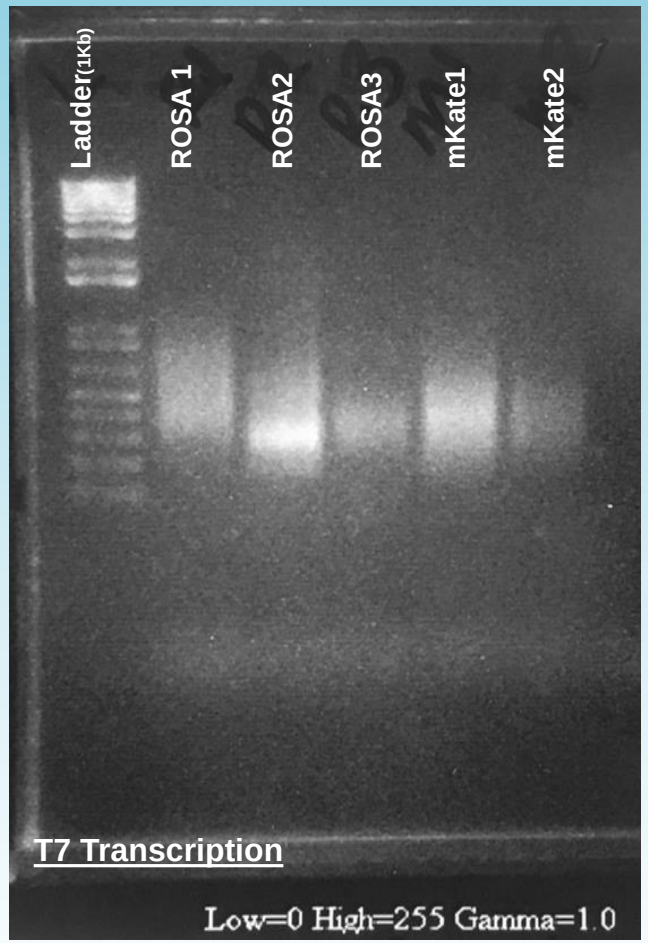


Optimized CRISPR Protocol:

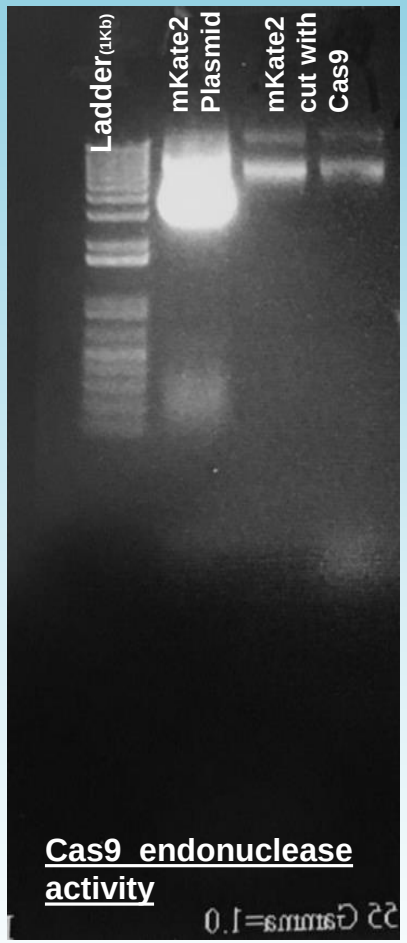
1. Design sgRNAs targeting the ROSA26 and mKate2
2. Construction of sgRNA-scaffold-target via PCR
3. *In vitro* Transcription
4. RNA Recovery via EtOH precipitation
5. *In vitro* digestion with Cas9 nuclease



Gel Validations of Transcription and Cas9 Digest

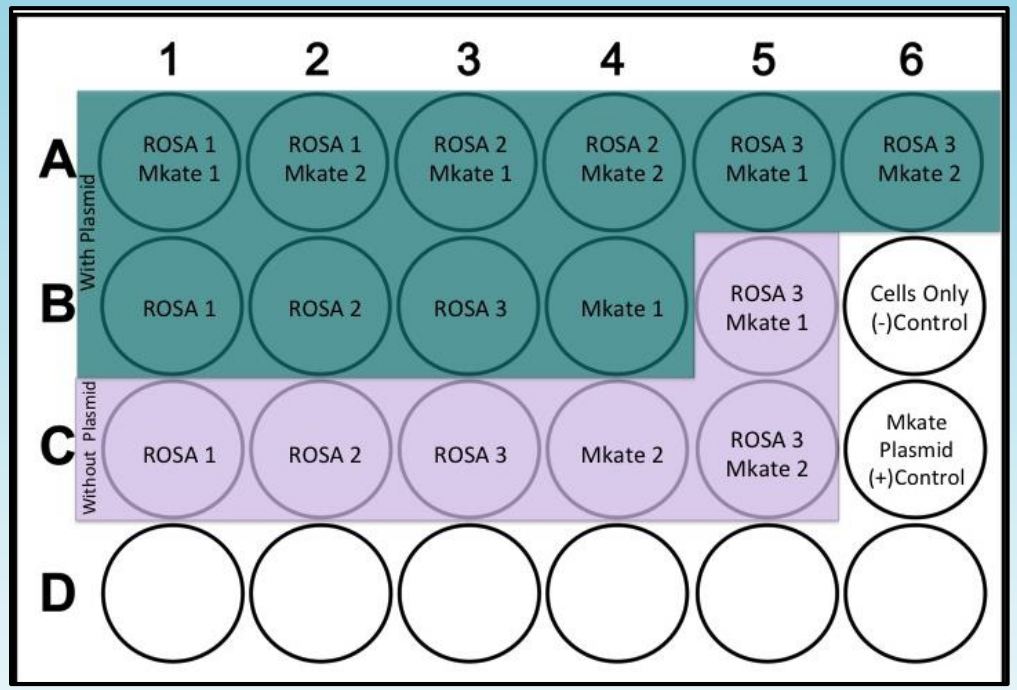


*Bands between 250 and 350 indicate successful transcription of the gRNAs



*Uncut mKate2 plasmid and ROSA locus compared with the the Cas9 digested mKate and ROSA

Optimized CRISPR Protocol Continued:



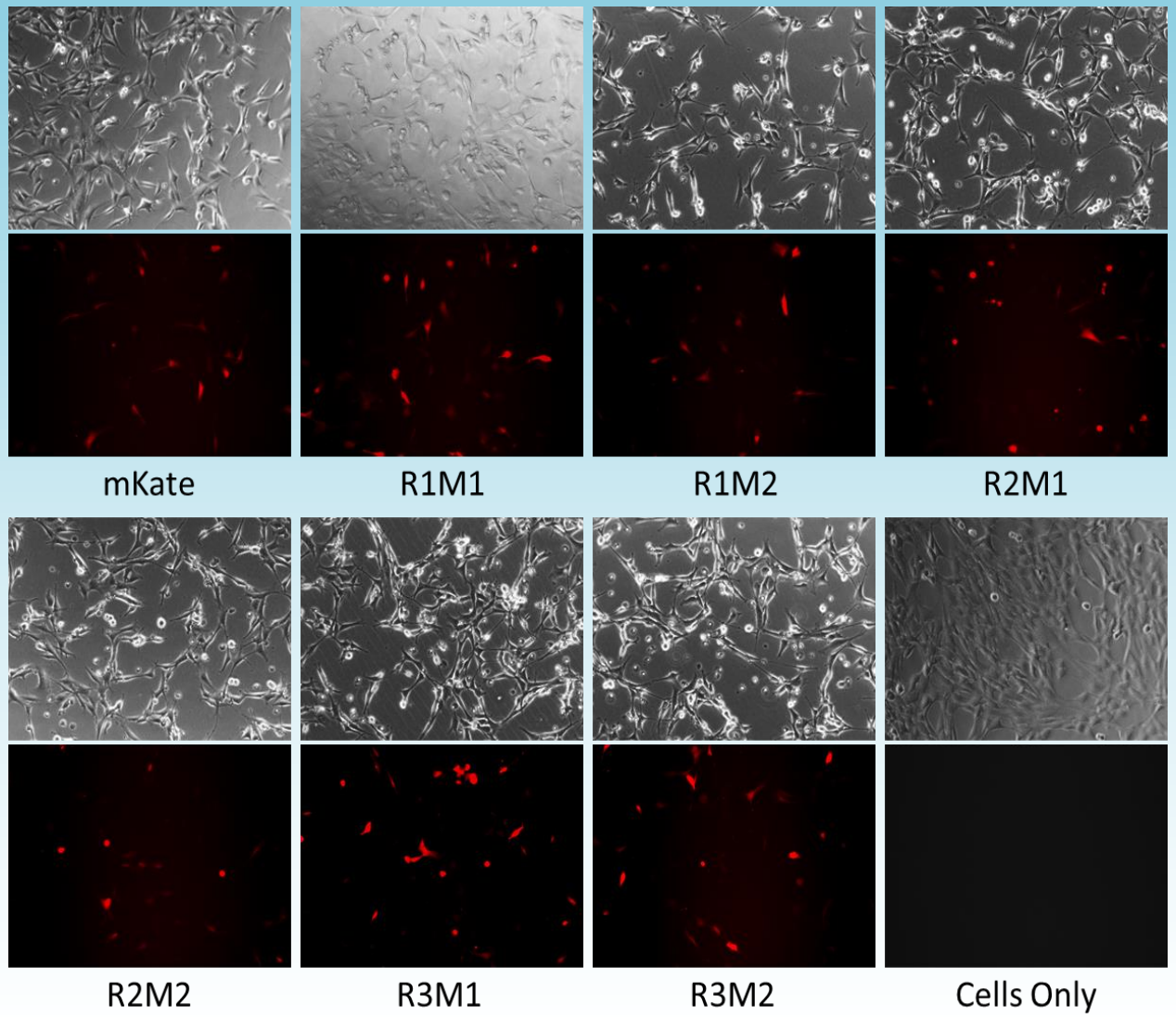
*Schematic of the 24-well plate set up for transfections into MC3T3 cells.

- Green=Plasmid in solution
- Purple= No plasmid in solution
- ROSA and mKate refer to sgRNA type added in transfection of MC3T3 cells.

6. Transfection of gRNA, Cas9, and mKate2 plasmid into mouse osteoblast (MC3T3) cells and incubate.

7. Flow Cytometry for validation of integration of mKate2 into ROSA26 through fluorescence.

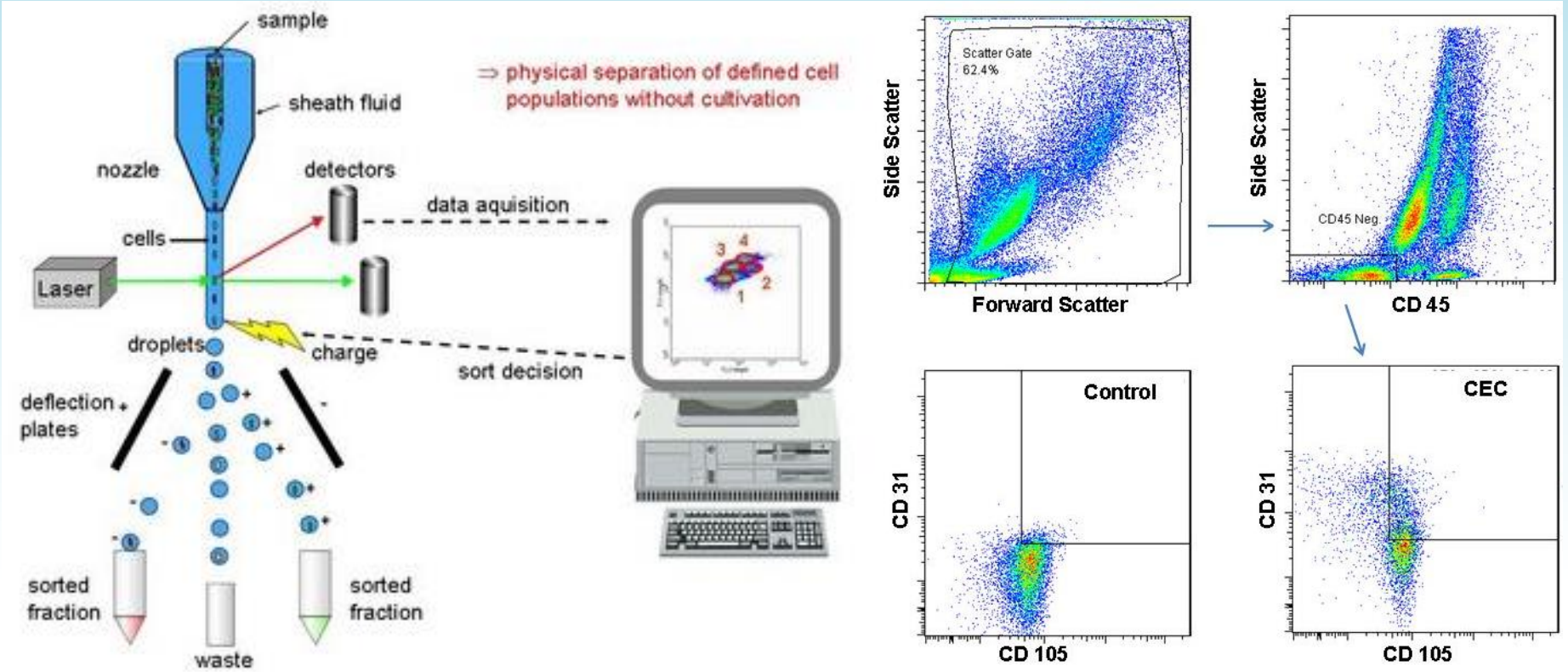
CRISPR/Cas9 Results: Fluorescent Imaging



*Cells imaged 48 hours post-transfection under brightfield (top rows) and fluorescence (bottom rows)

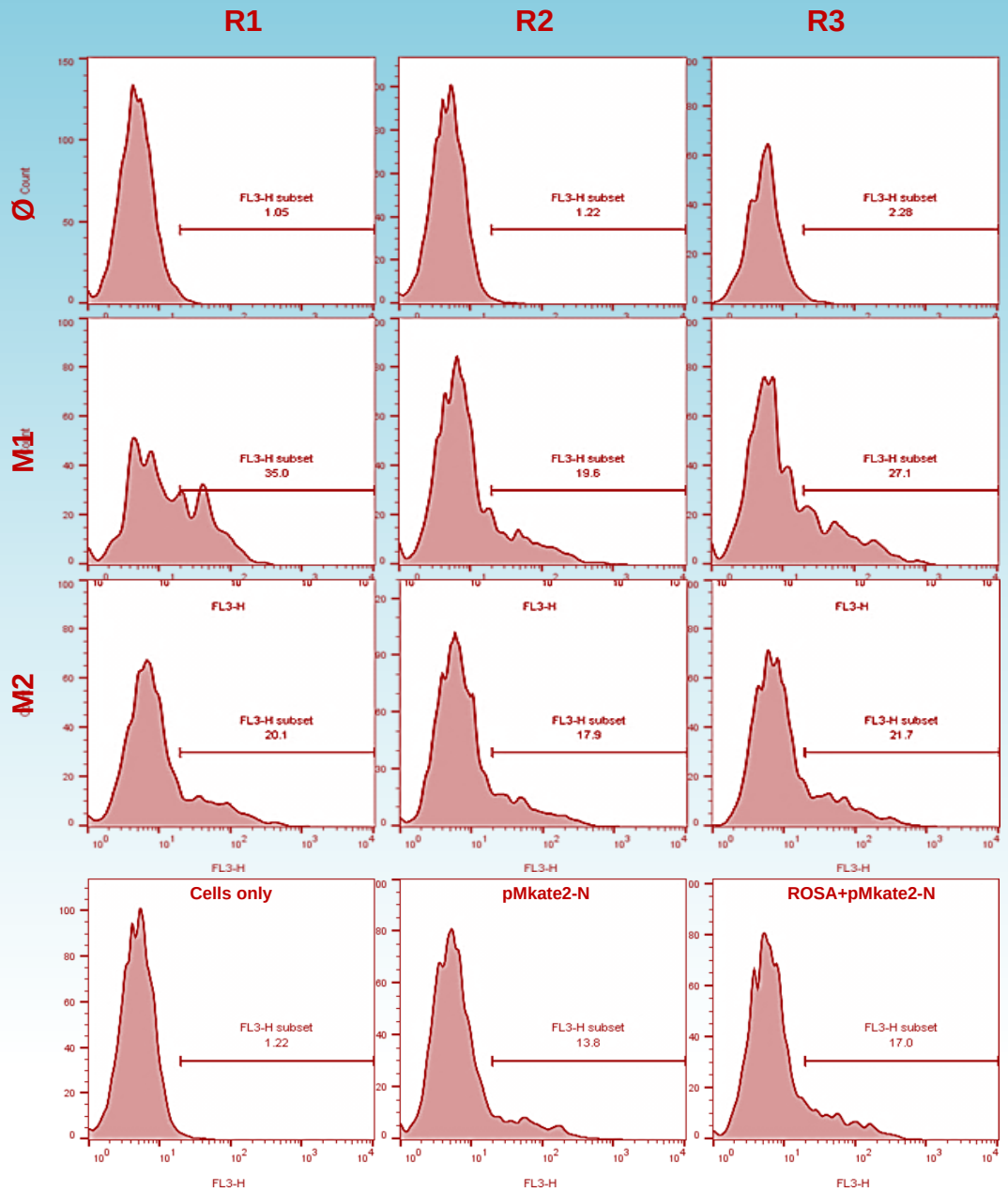
What is Flow Cytometry?

- Laser-based, biophysical technology
- Cell counting, sorting and biomarker detection
- Cells are suspended in fluid and passed by electronic detection apparatus.
- Allows for analysis of physical and chemical characteristics of cells.



CRISPR/Cas9 Results: Flow Cytometry

* Flow Cytometry conducted 96 hours post-transfection



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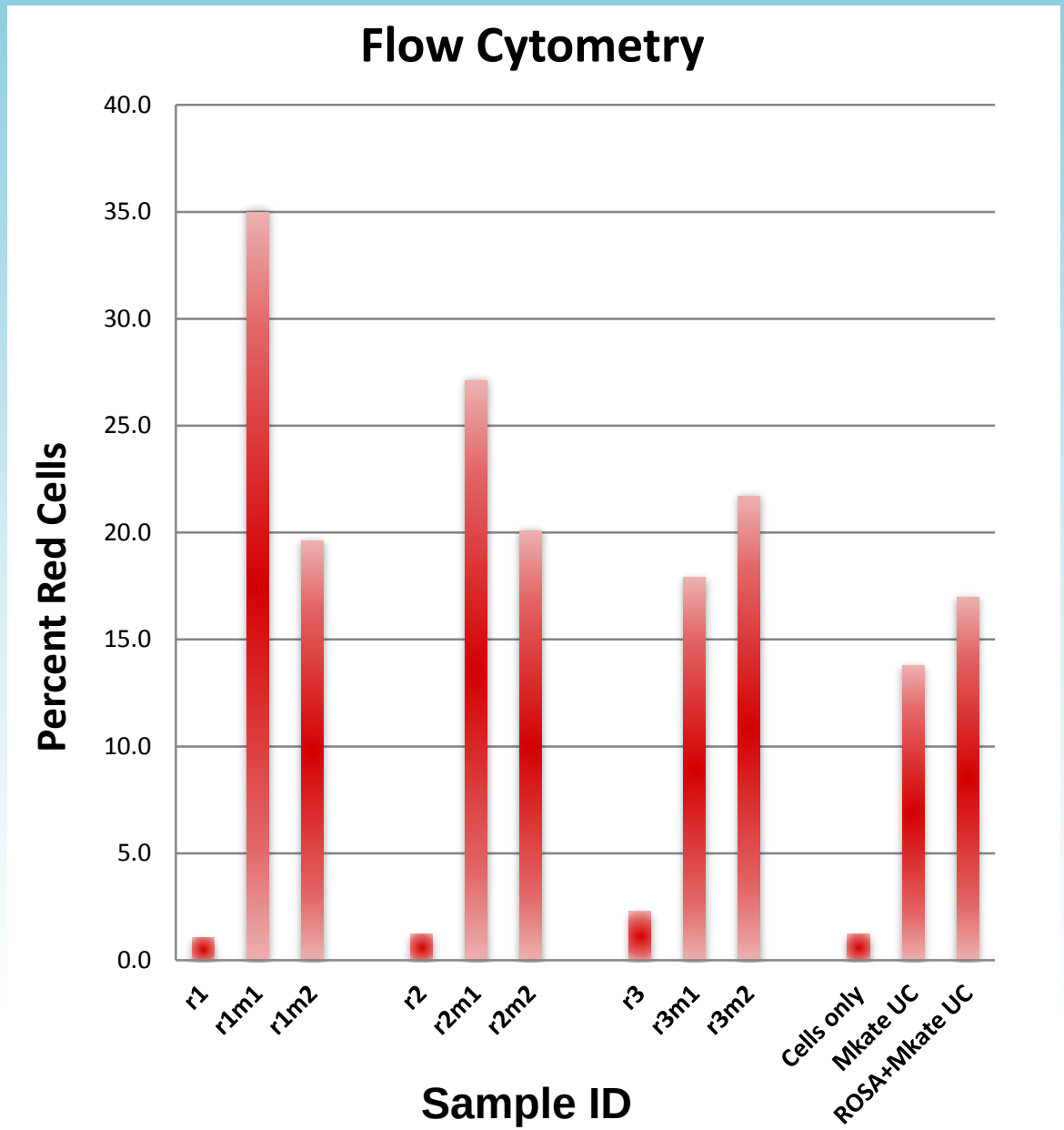

Conclusion

- <2.5% positive red cells in all control samples
- mKate plasmid without CRISPR activity produced 13.8% positive red cells
- 17% of cells were positive in the presence of the ROSA locus and UC mKate plasmid
- An increase in fluorescence by 10%-30% was found in cells transfected with ROSA sg-RNA **and** mKate sg-RNA compared to those cells with only a single sg-RNA
- Cells transfected with a sg-RNA had a 5%-10% increase compared to the fluorescence levels of the mKate plasmid with no sg-RNAs.
- The most efficient integration was found in samples containing sg-RNA targeting both the genomic locus and plasmid, specifically the R1 and M1 combination
    - suggests linearized mKate fluorescent plasmid improves integration

Future Work….

- T7 endonuclease screening
    - Cleavage of NHEJ from CRISPR/Cas9 Activity
- Check for off target effects
    - Southern Blot/Sequencing
- Expression vs. integration
- Guide efficiency: ROSA vs. mKate
    - Which works better and why?
- New transgene incorporation into the ROSA locus
- Additional cell lines and altered sgRNA loci for knocking out genes
- Final Goal: *In vivo* experimentation to create new transgenic lines through embryonic injections

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