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Using CRISPR/Cas platform for Genetic Modification of Commercial *Saccharomyces cerevisiae* strains

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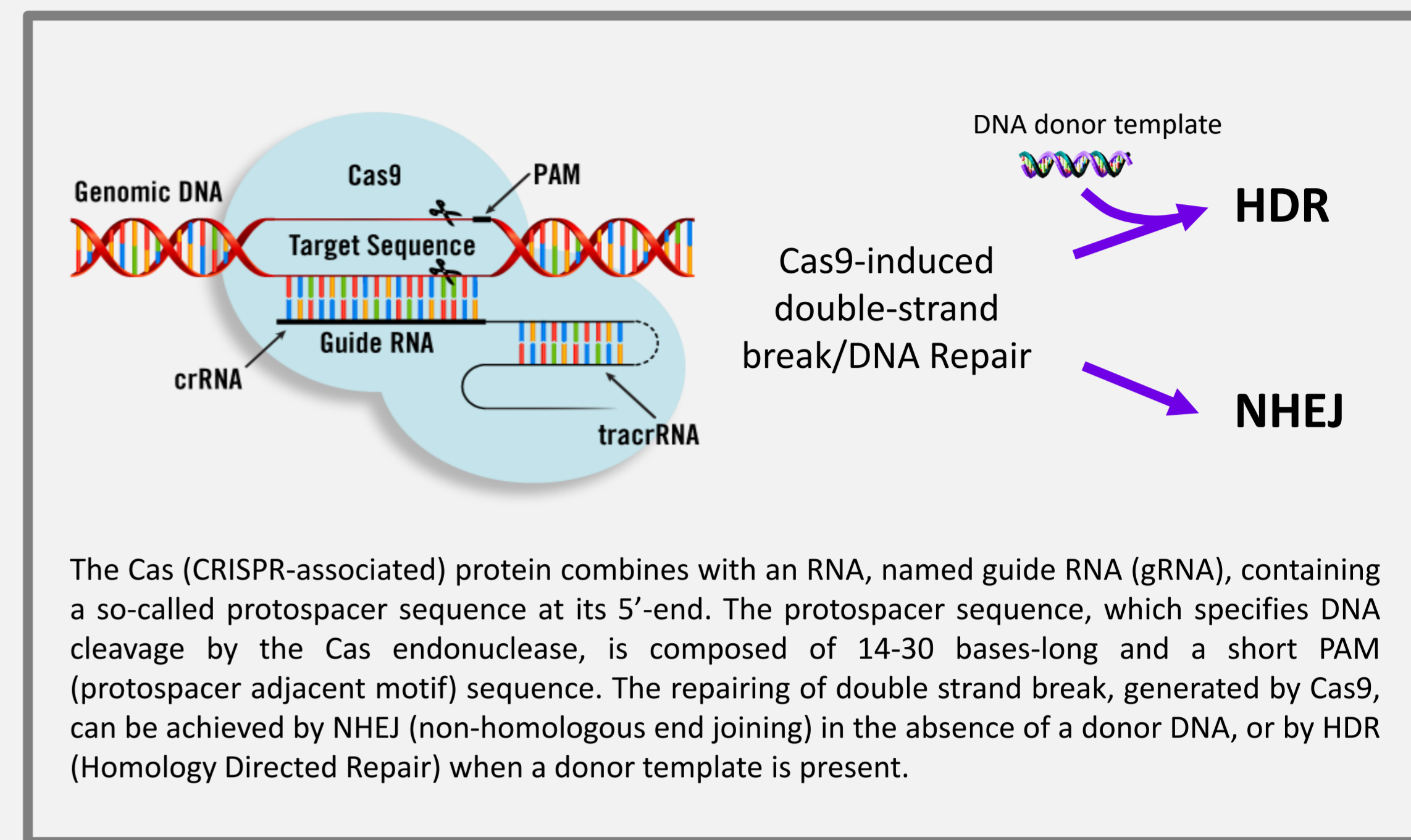
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Background: Traditional wine fermentation is a complex microbial process initiated by various yeast species classified as *Saccharomyces* and non-*Saccharomyces* species. To better understand the different interactions occurring within wine fermentations and track a specific yeast population, we wish to obtain GFP tagged yeast cells that stably express fluorescence signal without compromising the fermentative capability of the strain. To this end, the CRISPR/Cas9 system was investigated to genetically modify the commercial *Saccharomyces cerevisiae* diploid strain Lalvin EC1118® (LALLEMAND OENOLOGY).

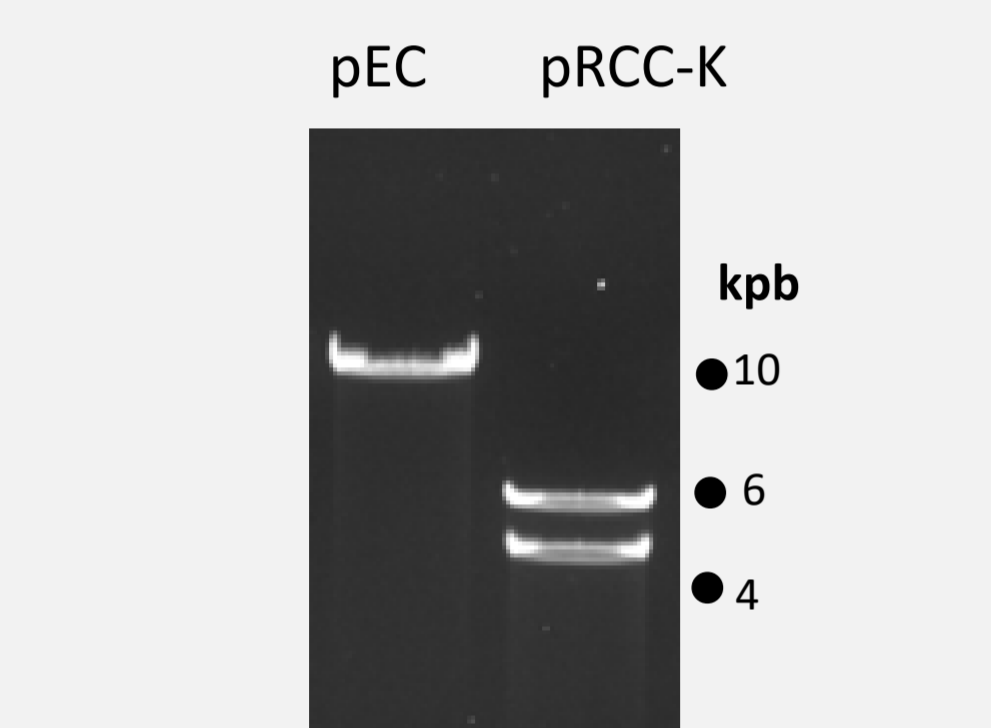
The emergence of the CRISPR/Cas9 system has revolutionized genome-editing technologies. Recently a set of vectors for simultaneous expression of Cas9 with gRNA cassette enabling simple and fast deletions of genes have been developed (Generoso et al., 2016). To modify genetically commercial yeast, the CRISPR/Cas9 machinery, along with a homologous donor sequence, were undertaken to insert *eGFP* gene into the EC1118® *AMN1* gene. The *AMN1* gene, which encodes a protein required for daughter cell separation, has already been deleted in haploid cells without modify fermentation properties (Marsit et al., 2015).



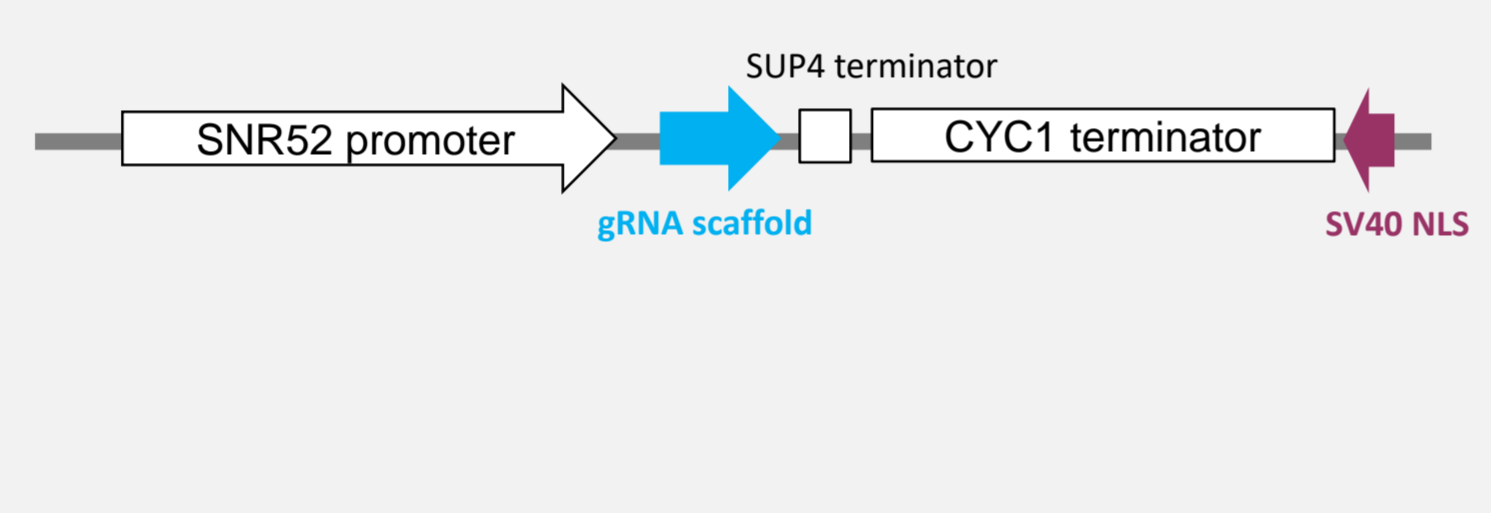
CRISPR/Cas9 construction elements

Targeting CRISPR/Cas9 system to *AMN1* gene

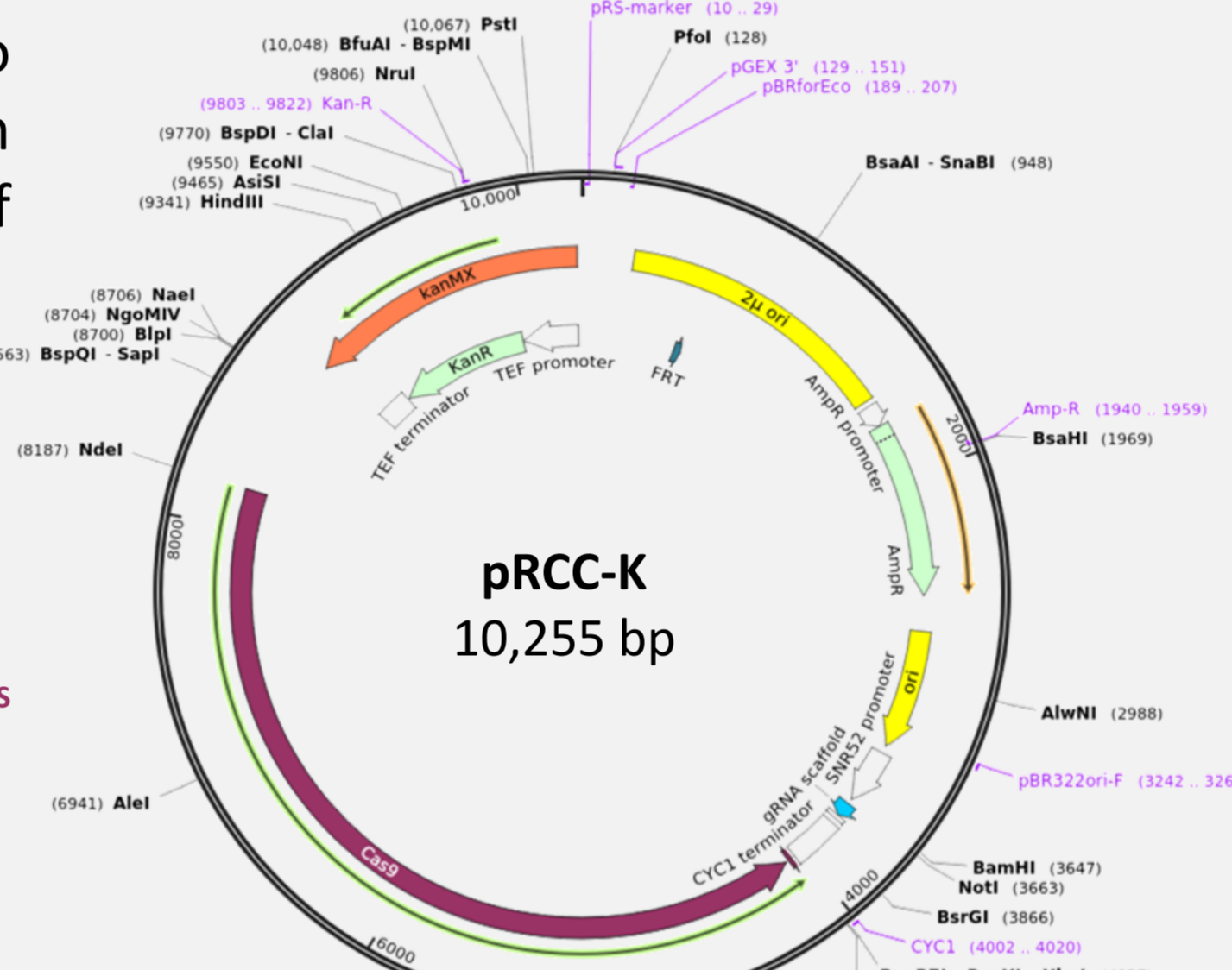
The pRCC-K plasmid was used as vehicle to deliver CRISPR/Cas9 machinery in yeast. To specify the Cas9 break to *AMN1*, a sgRNA was designed using *gRNA Design* from ATUM web site (<https://www.atum.bio/>) and inserted into gRNA scaffold region of pRCC-K using the NEBuilder HiFi DNA Assembly Cloning kit (NEB).



*Bam*HI/*Hind*III restriction analysis. The loosening of *Bam*HI site verified the presence of gRNA in pEC vector derived from pRCC-K.

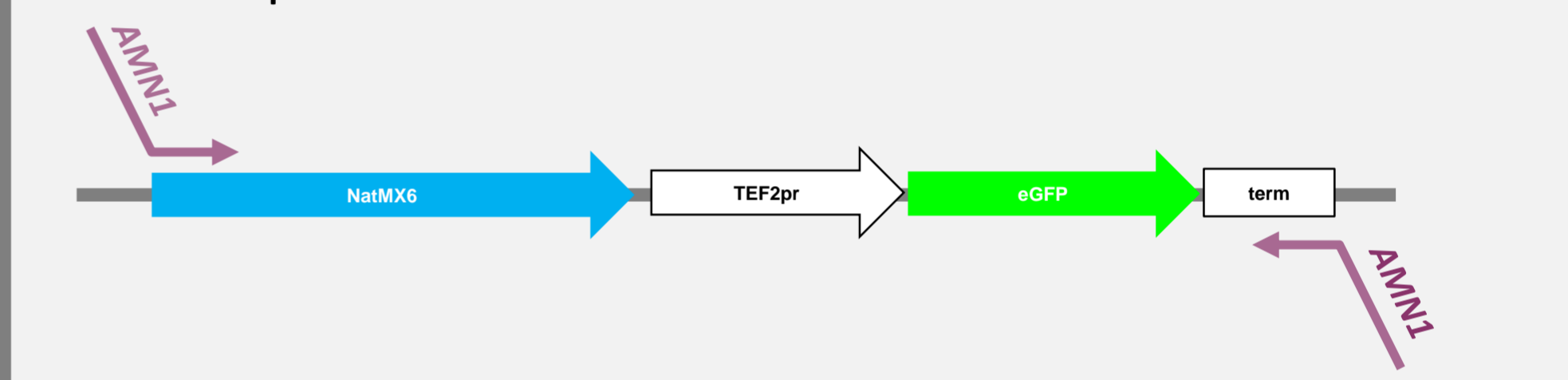


The vector-based delivered of CRISPR/Cas9 pRCC-K (Addgen) developed by Generoso et al. (2016) and elements of gRNA region.



Donor DNA for Homology-Directed Repair (HDR) to *AMN1* locus

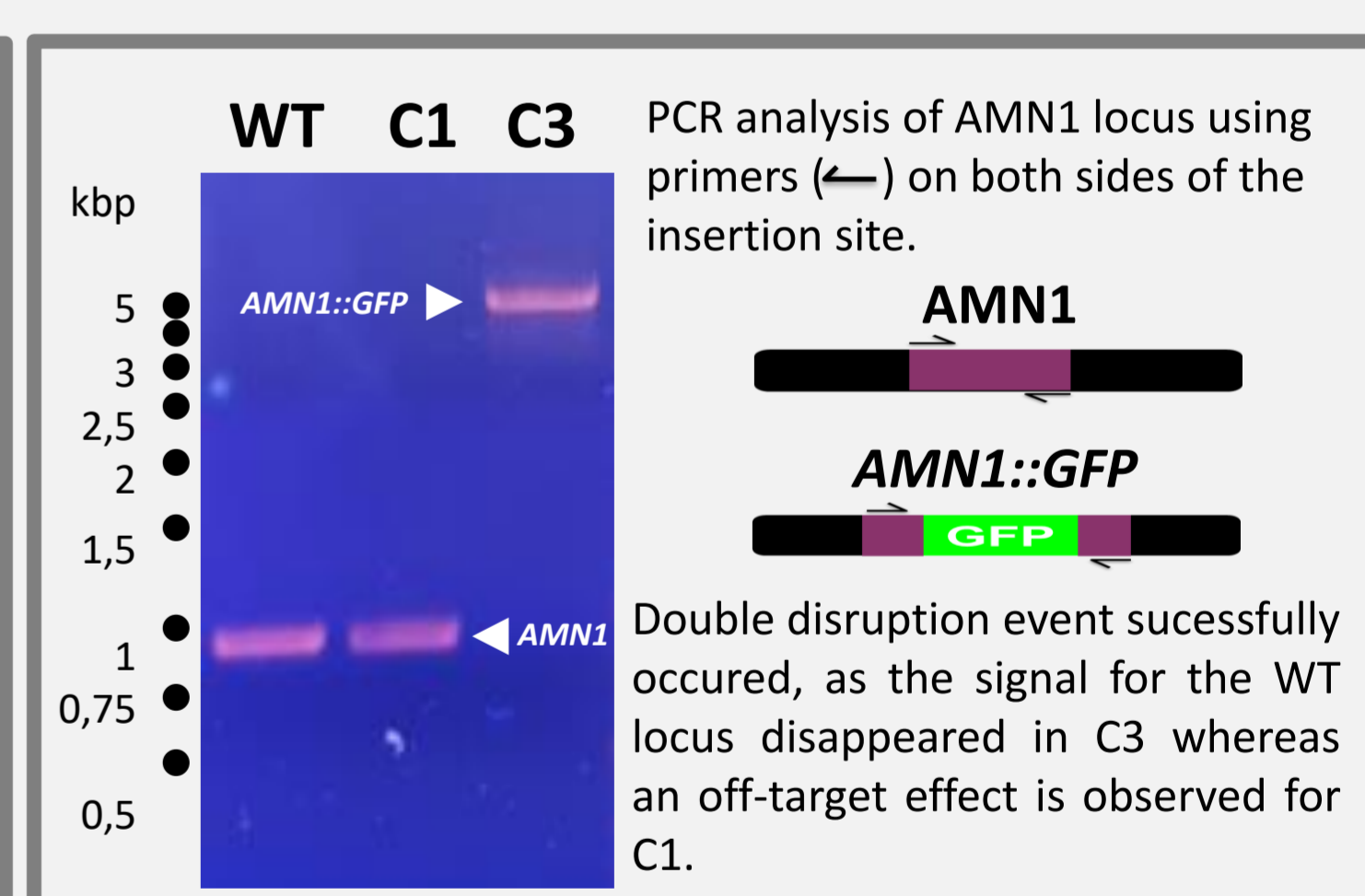
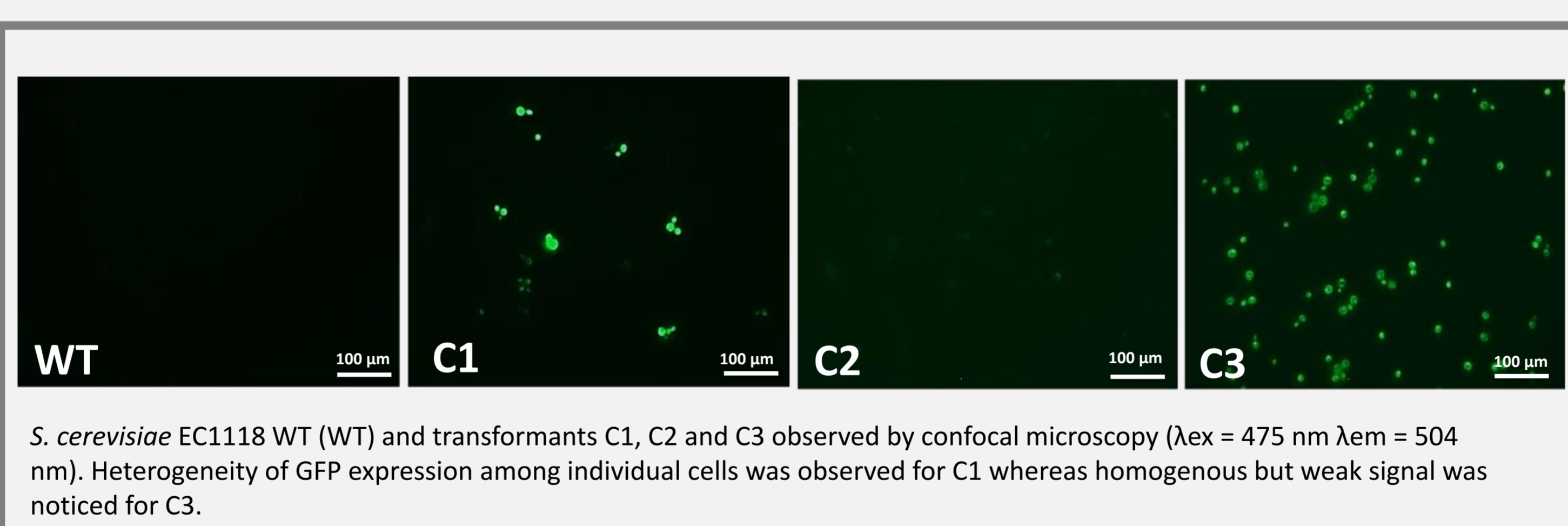
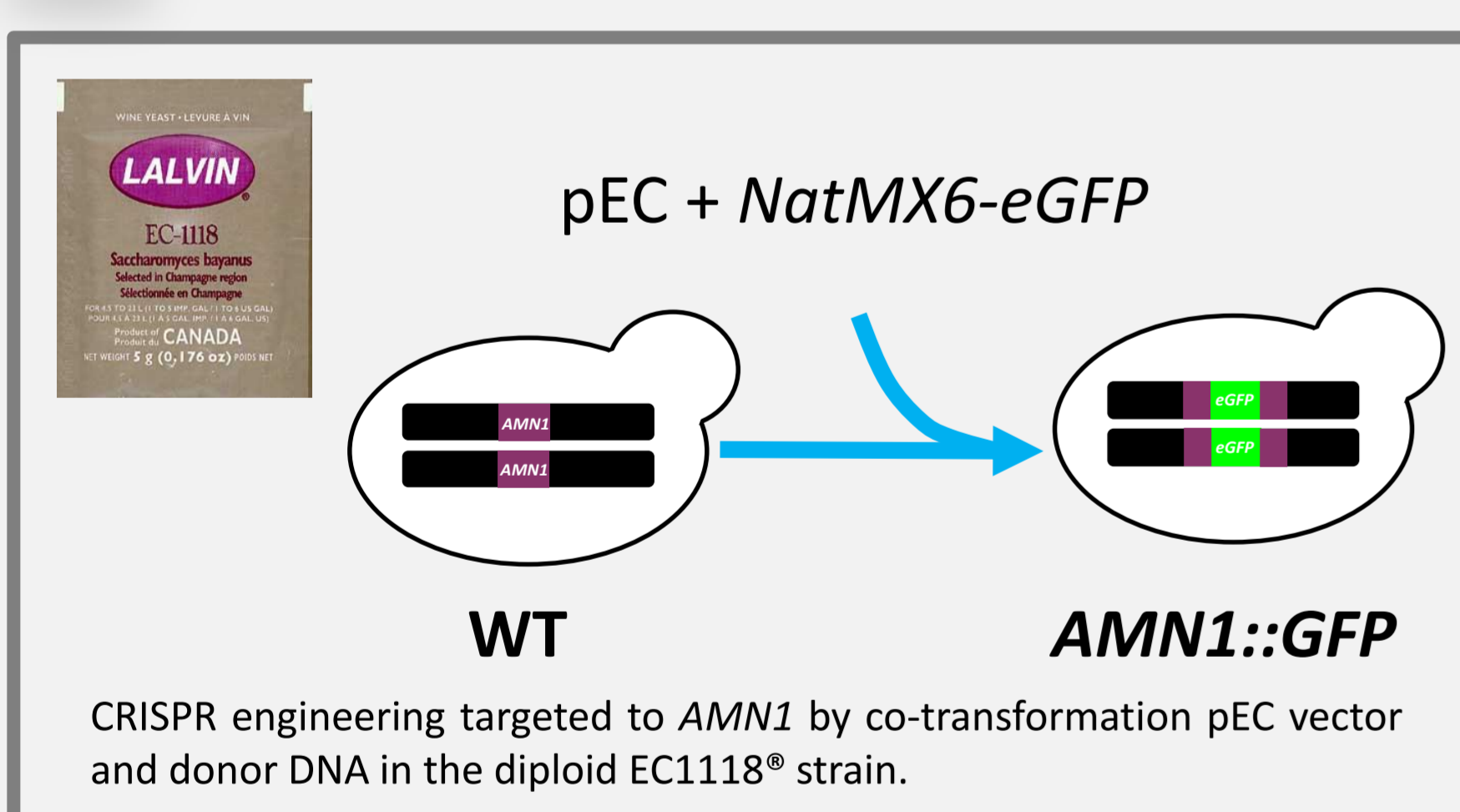
To ensure the repairing of the Cas9 DNA double strand break by homologous recombination, a double strand DNA (donor) was co-transformed with the CRISPR-Cas9 plasmid in yeast cells. The donor DNA was composed of the resistance gene for nourseothricin (*NatMX6*) and the *eGFP* gene amplified with chimeric primers.



NatMX6-eGFP donor DNA amplified by PCR from pFA6a-TEF2p-eGFP-ADH1-NATMX6 plasmid (Marsit et al., 2015) using 60bp long chimeric primers carrying 40 bp sequence homologous to *AMN1* gene targeted by CRISPR/Ca9 system.

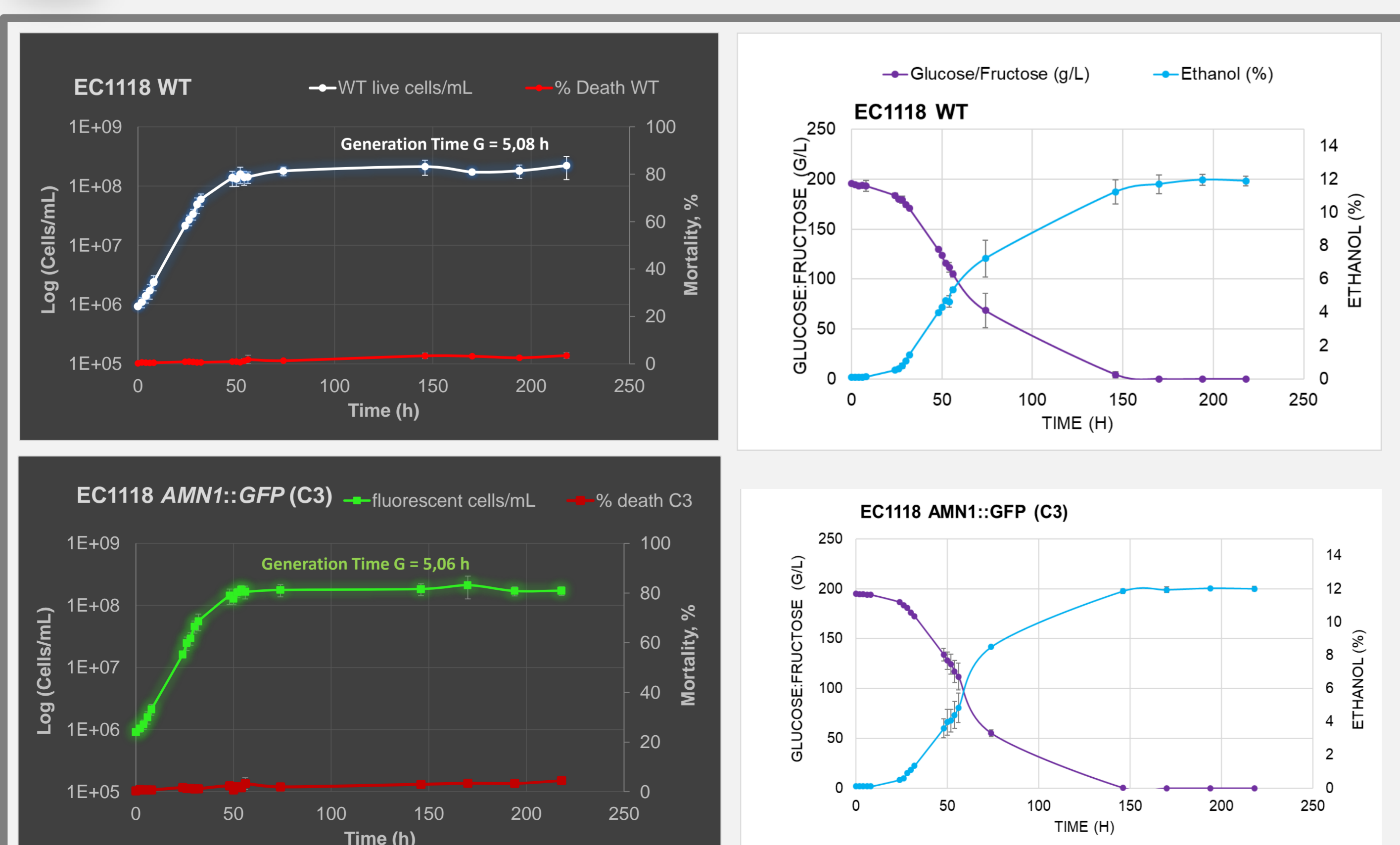
★ Cloning of sgRNA in pRCC-K lead the new pCE plasmid which is able to target a DNA cleavage on *AMN1* by the Cas9 endonuclease

CRISPR engineering of *Saccharomyces cerevisiae* Lalvin EC1118® strain



★ The CRISPR/Cas9 system successfully GFP-tagged the commercial EC1118® strain by insertion of a GFP gene into *AMN1* (C3). Nevertheless off-target effect was detected (C1) suggesting a non-optimal sgRNA design to target *AMN1*.

Oenological characterization of CRISPRed yeast



Growth of *S. cerevisiae* EC1118 WT (up) and CRISPRed strain C3 (bottom) in synthetic medium which mimics grape juice. Total cells and cell viability were determined by flow cytometry and Propidium iodide (PI) labelling. Monitoring of glucose/fructose consumed and ethanol produced was carried out using OenoFoss™.

★ No modification of oenological capability was detected between CRISPRed strain and parental strain EC1118. CRISPRed yeast C3 presents a stable GFP signal during fermentation.

Conclusions

In this successful experiment, we used the single plasmid pRCC-K, expressing Cas9 and guide-RNA (Generoso et al., 2016) to insert GFP gene into the Lalvin EC1118® commercial yeast. CRISPRed yeast didn't present any modification of oenological characteristics (i.e. fermentation rate, total alcohol production) compare to parental strain, as expected from this chosen target gene. More optimization, however, is required to obtain better transformation frequencies particularly if this system has to be used with industrial *Saccharomyces* and non-*Saccharomyces* strains. Nevertheless, the EC1118 *AMN1::GFP* C3 strain could be used in future experiments to test yeast-yeast interactions in oenological conditions

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