Dear backers,

As in the first note, we apologize here again to those for whom this lab note is too technical. Our first note detailed why we were delaying RNA sequencing: we wanted to add a third intron to the yeast rDNA to increase desiccation resistance and the chances of success of the RNA sequencing experiment.

This second note details the mixed results of adding a third intron to yeast rDNA (ribosomal DNA): the good news is that we were able to add the intron at a third location in the yeast rDNA; the bad news is that the sequence of the resulting rDNA is complex and unclear at the moment. This complexity is unexpected and is absent from the original one- and two-intron strains. The time needed to understand this complexity and bypass the problems it creates would delay the project for too long. *Therefore, we will revert to the original RNA sequencing plan using the intron strains we already have.* (We will not give up on understanding the "third-intron problem", but that will not be part of the project you backed).

For those interested in the details of the "third-intron problem", results are in the next page. For you to understand them however, we first need to clarify how we find out whether our 57 basepair-long intron is present or absent at a particular site in the rDNA: we use PCR (Polymerase Chain Reaction). If you don't know how PCR works, just trust us😇! 1) We isolate whole genomic DNA from the yeast strain to be analyzed. 2) On this DNA, PCR is directed to the intron insertion site by primers designed to amplify a short DNA fragment spanning the insertion site. 3) The size of this fragment is short if the site lacks the intron, but longer by 57 basepairs (abbreviation: bp) if the intron is present. 4) We determine the size of the PCR fragment by gel electrophoresis, where DNA fragments appear as light "bands" over a dark background. Fig. 1 shows the band pattern obtained with primers specific for insertion site "A". (You may remember from lab note 1 that our two original insertion sites were designated "A" and "B".)

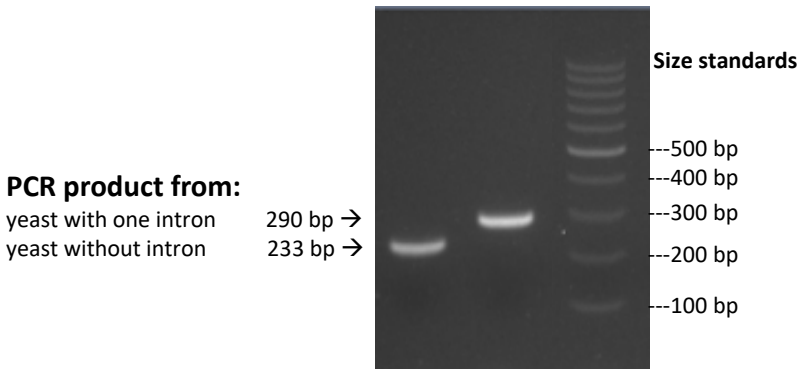

Figure 1: Gel picture illustrating how PCR is used to detect intron presence or absence

CRISPR-mediated intron insertion at sites A and B was fine and gave us no problems, as shown in Fig. 1 for site A. Site B gave us equally clean and unequivocal results. Not so for the third insertion site, "site C", were the resulting gel pattern is problematic. Instead of a single, clean band, indicating *either* presence *or* absence of the intron at this site, each one of the strains into which we inserted the intron at site C shows three bands! Fig. 2 shows the PCR gel from one such strain, but all site C strains give the same three-band pattern.

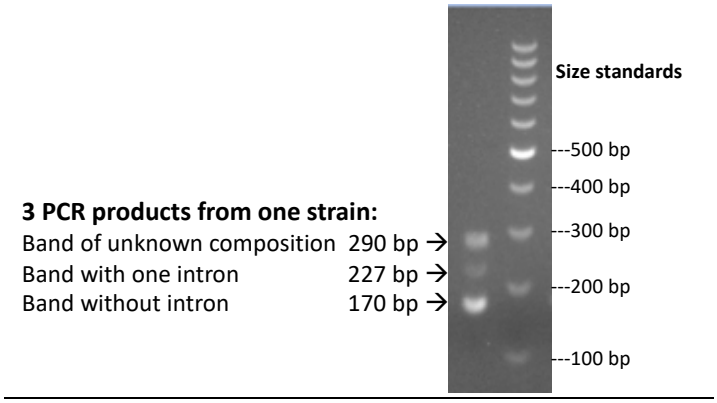

Figure 2: Gel picture illustrating PCR bands obtained by amplifying the "site C" insertion site in one strain

The three bands we get here and the fact that site C is the "third" insertion site are a completely unrelated numerical coincidence! Don't get confused by this coincidence: the primers used for the PCR illustrated in Fig. 2 are specific only for site C and the status of sites A and B does not affect this PCR. Another unrelated and irrelevant coincidence is the presence of a 290 basepair band in both Fig. 1 (site A) and Fig. 2 (site C). Band sizes are determined by PCR primer position and sequence, which are completely different for sites A and C. In fact, the nature of the 290 band from site C (Fig. 2) remains unknown (until we sequence it), whereas we already know that the 290 band from site A (Fig.1) represents the intron-bearing fragment since it has the right size and we also sequenced it.

Trivial coincidences aside, we cannot explain at this point why insertion of an intron at site C generates yeast whith three types of rDNA coexisting probably within the same cell: one type in which site C lacks the intron (170 bp band), one in which it has one intron (227 bp band), and one whose nature needs to be determined by sequencing (290 bp band): strangely, the size of the 290 bp band is compatible with it containing two 57-bp introns! We'll find out whether or not this is true.

Bottom line: the unexpected and so far unexplained complexity of the C strains relative to the A and B strains prevents adding the C strains to the RNA sequencing experiment. Therefore, we are returning to the original plan involving only sites A and B, after a six-month detour! Our apologies.