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In vivo CRISPR-Cas9 expression in Candida glabrata , Candida bracarensis and Candida nivariensis : a versatile tool to study chromosomal break repair.

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Abstract

The CRISPR-Cas9 system is extremely useful for genome editing in many species, including the model yeast *Saccharomyces cerevisiae*, and other yeast species. We have previously reported the use of an inducible CRISPR-Cas9 system in *Candida glabrata*, which allows genome editing but also the study of Double Strand Break (DSB) repair. We report, in this study, a comparable system for *C. glabrata*, relying on a new plasmid, which is more stable than the previous one. We also report the use of this plasmid to induce DSBs in two additional human pathogens, *Candida bracarensis* and *Candida nivariensis*. We examine lethality induced by an *in vivo* DSB in the three species and describe the different types of Non-Homologous End-Joining (NHEJ) events detected in these three pathogens.

INTRODUCTION

Non-*albicans* -*Candida* (NAC) species are a rising concern for immunocompromised patients, and *Candida glabrata* is the major NAC species in many developed countries (Rodrigues *et al.* , 2014). The *Candida* genus is not monophyletic, and *C. glabrata* is not part of the major clade that includes *C. albicans* (Dujon *et al* ., 2004). Instead, it is part of the*Nakaseomyces* clade, which includes two other human pathogenic species in addition to *C. glabrata* ; *Candida bracarensis* and *Candida nivariensis* (Gabaldon *et al* ., 2013). The*Nakaseomyces* clade is quite closely related to the model yeast*Saccharomyces cerevisiae* (Dujon *et al.* , 2004), and thus many molecular tools such as marker genes or replication origins, can be transferred from *S. cerevisiae* to *C. glabrata* (Muller *et al.* , 2007).

The CRISPR-Cas9 system (Knott and Doudna, 2018) has been adapted to many species, including yeasts, to induce chromosome Double-Strand Breaks (DSBs) at loci targeted through the guide RNA (gRNA), most often with the aim of editing genomic sequences. CRISPR-Cas9 has indeed been adapted to *C . glabrata* (Cen *et al.* , 2017; Enkler*et al.* , 2016; Maroc et al, 2019; Vyas *et al.* , 2018), creating DSBs that can be repaired by either Non-Homologous End-Joining (NHEJ) or Homologous Recombination (HR) when a template homologous to the cut-site is provided. Using CRISPR-Cas9 for genome editing generally relies on constitutive promoters for the Cas9 gene and the gRNA, but the use of inducible expression allows the study of repair of DSBs, for example in the model yeast *S. cerevisiae* (Lemos *et al.* , 2018). Inducible expression is also particularly useful in species such as the *Nakaseomyces Candida.* Because these are asexual and haploid, the fact that a gene is essential can never be proven by tetrad analysis after sporulation of a heterozygous diploid deletant. It is also difficult, when deletion experiments yield no transformants, to determine whether this is because the gene is essential or because experiments fail for technical reasons. On the other hand, when the creation of a DSB for gene editing is controlled in living cells, a negative result, *ie* when no mutants

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are obtained in the targeted gene, can be interpreted with more confidence as proving the essential nature of the gene.

We have developed an inducible system CRISPR-Cas9 (Maroc and Fairhead, 2019) based on the *MET3* promoter, and with the *URA3* gene as marker, both sequences coming from *C. glabrata* (Zordan*et al.* , 2013). We have previously shown that this can induce DSBs in this species, by targeting the *ADE2* gene. This gene is involved in the *de novo* purine nucleotide synthesis pathway, and its inactivation causes the accumulation of a red pigment in cells, leading to a red colony color in *S. cerevisiae* (Dorfman, 1969) and the *Candida* tested here (Maroc and Fairhead, 2019; and this work). We have since constructed a new version of the expression plasmid, because the original one contained sequence repeats that led to frequent rearrangements when cloned in *E. coli* (see Materials and Methods).

We report here the use of this new inducible CRISPR-Cas9 system in the pathogens *C. glabrata, C. bracarensis,* and *C. nivariensis* . Cas9 creates a DSB at the *ADE2* target locus, that is repaired by NHEJ. We confirm that expression of a CRISPR-Cas9 targeting *ADE2* does not affect viability on plates in the *C. glabrata* , although we observe a decrease in viability in the first hours of induction in liquid medium. For the other two *Candida* species, survival is lower. We also report that repair of the DSB by unfaithful NHEJ results in different sequence modifications in *C. glabrata* as compared to the two other species.

MATERIALS AND METHODS

Strains and cultures

Yeast strains are shown on table 1. Strains BR450 and NI150 are spontaneous *ura3* - mutants of the respective type strains obtained by plating strains on 5-FOA medium (Synthetic Complete Medium lacking uracil, see below, to which is added a small quantity of uracil; 50mg/L, and 5-fluoro-orotic acid, 1g/L). These alleles of *ura3* were sequenced by Eurofin Genomics^{TM} France. In *C. bracarensis* , the *ura3* gene, *CABR0s13e0133* has a deletion encompassing nt 240-657 with respect to the first nucleotide (nt) of the start codon, and in *C. nivariensis* , the *ura3* gene,*CANI0s15e01067* has a deletion encompassing nt 488-610 with respect to the first nt of the start codon. (Gene names from http://www-archbac.u-psud.fr/genomes/nakaseomycetes/nakaseomycetes.html).

Yeast strains are grown in broth or on plates at 30°C, in YPD (non-selective, 1 % Yeast Extract, 1 % Peptone, 2 % glucose), in Synthetic Complete medium lacking uracil, methionine, and cysteine (induction conditions for the *MET3* promoter, SC-Ind, 1.67 % Yeast Nitrogen Base without amino acids, 0.5 % ammonium sulfate, 2 % glucose, supplemented with adenine and all amino acids except methionine and cysteine), and SC-Ind supplemented with 2 mM each of methionine and cysteine (repression conditions for the *MET3* promoter, SC-Rep). For growth of cells without the plasmid as controls in the induction experiments, 2 mM uracil is added to the SC-Ind and to the SC-Rep medium. When repression, but no selection is needed, cells are grown in YPD supplemented with 2 mM each of methionine and cysteine (YPD-Rep). For Synthetic Complete media, pH is brought to 5.8 by addition of drops of 5M NaOH until correct pH is reached.

PCR, DNA preparation, cloning

For analysis, PCR was performed directly on colonies, as described for*C. glabrata* in Boisnard et al. (2015), using the DreamTaq from ThermoFisher Scientific. For constructions, PCR was performed on DNA prepared with Qiagen Genomic DNA kits, according to manufacturer's conditions. Some constructs were cloned into pBlueScript into *E. coli* DH5, with standard protocols.

Yeast transformation

For selection of transformants of the Cas9 plasmid, and maintenance in SC-Rep. Transformation is done according to a modified one-step lithium acetate transformation protocol from (Gietz *et al.* , 1995). The duration of the heat shock at 42°C differs according to the species; 25 min for *C. glabrata* , 20 min for *C. bracarensis* , 15 min for*C. nivariensis* .

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For induction of DSBs, all species were transformed with the pCFYF plasmid into which is cloned the gRNA specific for each species, but also with the plasmid that does not express any gRNA, to control for medium/plasmid/Cas9 expression effects (see Results).

Plasmid construction and molecular biology techniques

The pCGLM1 plasmid we described in (Maroc and Fairhead, 2019) proved unstable when cloned in *E. coli* , probably because of the presence of the f1 ori region, repeated at two different positions in the plasmid. We decided to construct a new version, pCFYF, shown on Figure 1. We cloned the p*MET3* promoter from plasmid pCU-*MET3* from (Zordan et al., 2013), and introduced it in place of the *PGK* promoter in front of the Cas9 gene in plasmid pJH-2972 from J. E. Haber (https://protocolexchange.researchsquare.com/article/nprot-5791/v1). For this, the p*MET3* promoter was amplified by PCR with primers shown in table 2). These primers contain sequences identical to the borders of the PGK promoter in pJH-2972. *Saccharomyces cerevisiae* cells from strain W303 (Ralser et al., 2012) were transformed with the p*MET3* promoter PCR fragment along with plasmid pJH-2972, linearized at the *Cla* I site, inside the *PGK* promoter. Ura+ transformants contained a circular plasmid, where homologous recombination has resulted in the replacement of the *PGK* promoter by the p*MET3* promoter. The plasmid was extracted from *S. cerevisiae* and transformed into *E. coli* cells. The new construct pCFYF (p*MET* -cas9) was checked by sequencing of the junctions on both sides of the *MET3* promoter by Eurofin Genomics^{TM} France.

Targeting the ADE2 gene in different species

The systematic names of orthologs of the *ADE2* gene, *YOR128C* in *S. cerevisiae,* are shown in table 3 and were extracted from: http://www-archbac.u-psud.fr/genomes/nakaseomycetes/nakaseomycetes.html. Cut sites were chosen at a similar location in the gene (see table 3). Individual gRNAs were cloned into pCFYF for targeting the *ADE2* gene in each species.

Inducing the CRISPR-Cas9 expression in solid medium

For continuous induction on solid medium, transformants carrying the Cas9 plasmid are grown overnight in liquid SC-Rep medium. The optical density at 600nm of the culture is determined, and aliquots are plated on SC-Ind plates as well as on repressive SC-Rep medium. Colonies are grown, counted and plates are compared in order to estimate the survival rate (number of colonies obtained on induction medium divided by number of colonies obtained on repression medium, expressed as a percentage). Induction plates are replicated on YDP-Rep, grown and examined for colony color; both sectored and completely red colonies being scored together. The rate of red colonies is expressed as a percentage of the total number of colonies. In experiments that were used to compute the survival and *ade-* rates, the minimum number of colonies counted on SC-Rep is 50, and usually around 500. All experiments were performed on at least two independent transformants and repeated at least twice per transformant. Controls without the gRNA (transformation with pCFYF) and controls without plasmid (with uracil added to media) were prepared and analyzed in the same way. Standard deviations are calculated and shown as error bars in graphs.

Time-course of CRISPR-Cas9 expression in liquid medium followed by cell rescue

Flasks containing 10mL of induction medium are inoculated with transformants grown overnight in liquid SC-Rep medium (as above) at OD = 0.1. Growth is followed by measuring optical density at 600nm. Rescue is performed at different time points by plating an aliquot on repressive medium. All experiments were performed on at least two independent transformants and were normalized with reference to transformants without the gRNA (transformation with pCFYF).

Sequencing of NHEJ events

Red colonies from different species were picked on induction plates. The junction was amplified by PCR using primers shown in table 2 and sent for Sanger sequencing at Eurofin Genomics^{TM} France.

RESULTS

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Rationale

Since our previous plasmid for *in vivo* expression of CRISPR-Cas9 in *C. glabrata* was genetically unstable because of the presence of sequence repeats (see Materials and Methods), we decided to construct a new plasmid, pCFYF, as described in Materials and Methods, and shown on Figure 1. The *MET3* promoter for inducible Cas9 expression, the *SNR52* promoter for constitutive gRNA expression, and the *URA3* marker are the same as in the previous version.

We decided to test our new expression system on the three pathogenic *Candida* species of the *Nakaseomyces* clade, targeting the *ADE2* gene, since it provides a phenotypic screen of red/white colony color, allowing the detection of inactivating mutations in the gene. All species contain a homolog of the *ADE2* gene (table 3). We chose the cut site within the gene so as that it is always situated in the same region of the gene, at a distance of 464 nucleotides from the first nucleotide of the ATG. Even though the gene sequence is diverged and individual gRNAs had to be designed, the environment of the cut sequence is rather well conserved, as observed in the gRNAs sequences (table 3).

Once the DSB has been created, since the *ADE2* gene is in single copy in the haploid genome of the strains considered, and since no homologous template for repair is transformed into cells, the DSB cannot be repaired by Homologous Recombination, but only by NHEJ. If NHEJ is faithful and does not introduce a sequence modification, the site can be recut, as long as Cas9 and the gRNA are expressed, i.e. during continuous growth of cells in induction medium and in absence of genetic modification of the plasmid. Cells may then undergo multiple rounds of cut-ligate-recut events, in a futile cycle (Maroc and Fairhead, 2019). If repair occurs by unfaithful NHEJ, the sequence will be modified and the gRNA may not recognize it anymore as a cut-site. Furthermore, most unfaithful NHEJ should result in a non-functional gene, so that cells will become Ade- and the red pigment should accumulate.

Continuous induction on plates

For these experiments, cells are plated in parallel on induction medium (SC-Ind, without methionine and cysteine) and repression medium (SC-Rep, with methionine and cysteine), both of which select for the presence of the *URA3* plasmid. Counting colonies on both types of plates allows estimation of the lethality caused by the CRISPR-Cas9-induced DSB.

As shown on figure 2, controls without the plasmid and with the plasmid that does not express the gRNA, show that there is no major deleterious effect of the induction medium, nor of the expression of Cas9, since survival ranges between 84 and 100%. for all species When the gRNA is expressed in addition to Cas9, survival drops to approximately 17% for *C. nivariensis* and 34% for *C. bracarensis* , showing that the continuous induction of a DSB is lethal for most cells of these two species. Survival is much higher, almost 90%, in *C. glabrata* . This could be due to inefficient cutting, or to a high level of unfaithful NHEJ (see below and discussion).

One way of testing the efficiency of the cut is to observe how many are repaired by unfaithful NHEJ, which we expect to result in a non-functional *ADE2* gene, and therefore in a red colony phenotype. We observe that in *C. glabrata* , 100% of colonies are red, and in the two other *Candida* species, more than 96% of colonies are red (not shown). This shows that the large majority of surviving cells in all species has repaired the cut by unfaithful NHEJ, thus inactivating the *ADE2* gene. This is much higher than what we reported with our previous system in *C. glabrata* (Maroc and Fairhead, 2019), and probably reflects a more efficient cutting because of a different choice of gRNA, or because the plasmid is more stable in this yeast.

Induction followed by "rescue" of cells

In order to follow death/survival of cells through time, we performed induction in liquid medium, followed by "rescue" at different time-points, by spreading aliquots on repressive medium, as we had done previously (Maroc and Fairhead 2019). As shown on figure 3, survival rate drops in the first hours of induction, and then stabilizes at values that are similar to survival on plates. Thus the cut decides the fate of cells, and stopping the induction does not «rescue» cells. The percentage of red colonies rises from zero to 100% in 24

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hrs for *C. glabrata* and *C. nivariensis* . For *C. bracarensis* , there is already a large number of red colonies at T0 and the percentage of red colonies does not reach above 80%. These observations may be explained by promoter leakage before the induction, that would promote the appearance of cells resistant to cutting through both unfaithful NHEJ and plasmid rearrangement.

Sequence of cut-sites repaired by unfaithful NHEJ

We amplified the cut region by PCR in 19-20 red colonies per species, from the continuous induction experiments. As seen on figure 4, the large majority, 15/20, of unfaithful NHEJ events in *C. glabrata* create frameshift mutations by deletion of a single base pair. The remaining five cases consist of a frameshift mutation by deletion of 4 base pairs (one occurrence), addition of one base pair (three occurrences) and addition of two base pairs (one occurrence).

In the other two species, both insertions and deletions are found, that either create frameshifts or delete and/or modify a few codons at the cut site. The most frequent repaired sequence is found 6 times in each species, and corresponds to a single bp deletion in *C. nivariensis* , and to a single bp insertion in *C. bracarensis* .

DISCUSSION

Our results show that the CRISPR-Cas9 system can be used to induce DSBs in all three species studied here, when desired, after transformation of cells with the same plasmid in which the appropriate gRNA sequence is cloned. All species here are haploid, and since we did not provide an intact template for homologous repair of the cut-site in the *ADE2* gene, repair must occur through NHEJ. Our red/white screening strategy, in parallel with monitoring of cell death on plates and in time-course experiments, allows us to estimate the efficiency of cut and repair in each species. Repair by faithful NHEJ results in a regenerated, intact, cut-site, and thus cutting by CRISPR-Cas9 can occur once more. If cells do not die from this, through cell-cycle checkpoint arrest, or through degradation of chromosomal DNA at some point, they can undergo a futile cut-ligate-recut cycle (Maroc and Fairhead, 2019). Escaping this potential futile cycle can occur through plasmid mutation/rearrangement, or through plasmid loss, accompanied by integration of the *URA3* gene (whose presence is selected in the medium), and finally through events of repair by unfaithful NHEJ that modify the cut-site recognition sequence. In our experiments targeting the *ADE2* gene, we observe a very high percentage of red colonies in survivors, indicating a very efficient chromosomal cutting followed by unfaithful NHEJ.

In *S. cerevisiae* , a CRISPR-Cas9-created chromosomal cut, in a haploid strain in a unique region is lethal for most cells (Yarrington et al., 2018). In *C. bracarensis* and *C. nivariensis* , even though survival is higher than in the model yeast, only 17%-34% of cells survive continuous induction on plates. In temporary liquid medium induction, we observe that survival drops with increasing exposure and that long exposure results in survival rates similar to induction on plates. The percentage of cells repaired by unfaithful NHEJ (red colonies) increases with exposure time, reflecting either the time course of cutting by CRISPR-Cas9, or the prevalence of faithful NHEJ at the beginning of exposure, gradually replaced by unfaithful NHEJ events which are resistant to further cutting.

It is remarkable that *C. glabrata* has a much higher survival rate than the other two *Candida* . We have previously determined that cells of *C. glabrata* undergo futile cycles of cut-religation and that the cell-cycle chekpoint must be “leaky” (Maroc and Fairhead, 2019). The situation may well be different in the other two other *Candida* studied here. Another, non exclusive, reason for lower survival may be that unfaithful NHEJ is much less efficient in the other two species than in *C. glabrata* , preventing the creation of non-cuttable *ade2* - alleles in the former. On the other hand, perhaps the cell cycle checkpoints are less ”leaky” in *C. bracarensis* and *C. nivariensis* , and cells with unrepaired DSBs die more frequently.

Sequencing of the new junctions created by this type of NHEJ, i.e. from red colonies, shows that cells have repaired the DSB with varying amounts of rearrangements, that differ between species. There is more variation in the types of unfaithful NHEJ repair events in *C. bracarensis* and *C. nivariensis* than in *C. glabrata*

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, where survival is also much higher. Perhaps an efficient and reproducible unfaithful NHEJ mechanism in *C. glabrata* allows this survival rate, accompanied by leakiness of checkpoints. This may signify different adaptation mechanisms, and remains to be investigated thoroughly.

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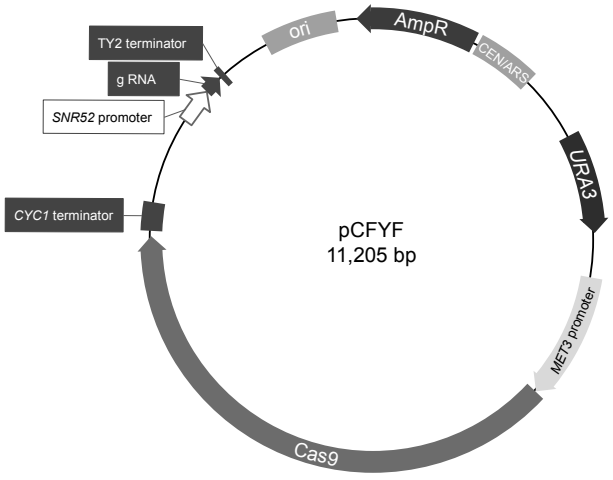
Legends to figures:

Figure 1: Schematic representation of plasmid pCFYF. Elements shown not to scale. The plasmid contains origins and selection markers for *E. coli* : ori and AmpR, as well as for yeast: ARS-CEN and *URA3* . The Cas9 gene is under the control of the inducible *MET3* promoter. The gRNA gene can be cloned under the control of the constitutive *SNR52* promoter.

Figure 2: Survival on solid induction medium. Survival is calculated as the ratio of the number of colonies that grow on plates with induction medium (SC-Ind) over the the number of colonies that grow on plates with repression medium (SC-Rep). Black bars: untransformed yeast (with Uracil added to the media); dark gray bars: yeast transformed with the plasmid expressing Cas9 but where no gRNA is cloned; light gray bars: yeast transformed with the plasmid expressing both Cas9 and the gRNA. Error bars represent standard deviation.

Figure 3: Survival to limited induction time and efficiency of induction. Cells are placed in liquid SC-Ind medium for the time, in hours, shown on the graph, followed by plating on repression medium (YPD-Rep). The upper survival curve, starting at 100 %, is the ratio of colonies on YPD-Rep at a given time-point over the number of colonies on YPD-Rep at T0. This is normalized by division with the survival rate in the same conditions, of cells transformed with pCFYF (expressing Cas9 but no gRNA). The lower curve starting at 0 % for *C. glabrata* and *C. nivariensis* , and at 40 % for *C. bracarensis* is the percentage of red colonies on total colonies on SC-Rep medium. Error bars represent standard deviation.

Figure 4: Sequence of NHEJ junction in Ade- cells of the three species. First line shows sequence before the DSB in all three species, with double arrow indicating cut site, and letters in italics, the PAM sequence. Coordinates relative to the first base of coding gene is shown. Following lines show sequences after unfaithful NHEJ events, with letters in bold indicating insertions and dashes indicating deletions, the (-1) indicating deletion of the nt at position 471. The frequency of each repair event is indicated on the right.



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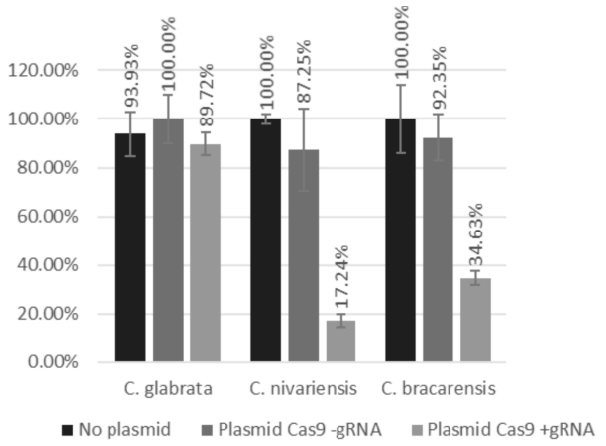

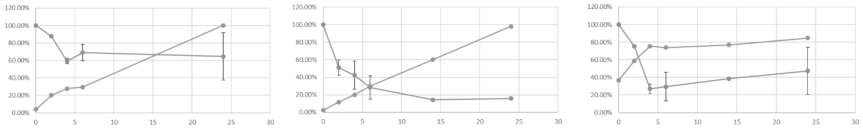

C. glabrata *C. nivariensis* *C. bracarensis*

C. glabrata

447-ACATTAGCCTACGATGG|AAG*CGG*-**471**	**WT**
447-ACATTAGCCTACGATG- AAG*CGG*-**471**	**15/20**
447-ACATTAGCCTACGAT- - - - G*CGG*-**471**	**1/20**
447-ACATTAGCCTACGATGG**G**AAG*CGG*-**471**	**2/20**
447-ACATTAGCCTACGATGG**T**AAG*CGG*-**471**	**1/20**
447-ACATTAGCCTACGATGG**TA**AAG*CGG*-**471**	**1/20**

C. nivariensis

447-ACACTGGCTTATGATGG|TAG*AGG*-**471**	**WT**
447-ACACTGGCTTATGATG - TAG*AGG*-**471**	**6/19**
447-ACACTGGCTTATG- - - - TAG*AGG*-**471**	**1/19**
447-ACACTGGCTTATG- - - - - - - - - -**471**	**1/19**
447-ACACTGGCTTATGAT - - - - - - -(-1)-**470**	**1/19**
447-ACACTGGCTTATGATG - **T** - - - - - -**471**	**1/19**
447-ACACTGGCTTATGATGG**A**TAG*AGG*-**471**	**4/19**
447-ACACTGGCTTATGATGG**G**TAG*AGG*-**471**	**3/19**
447-ACACTGGCTTATGATGG**TA**TAG*AGG*-**471**	**1/19**
447-ACACTGGCTTATGATGG**TT**TAG*AGG*-**471**	**1/19**

C. bracarensis

447-ACTCTTGCCTATGATGG|TAG*AGG*-**471**	**WT**
447-ACTCTTGCCTATGATG - TAG*AGG*-**471**	**2/20**
447-ACTCTTGCCTATGAT - - -AG*AGG*-**471**	**1/20**
447-ACTCTTGCCTATGA- - - -AG*AGG*-**471**	**1/20**
447-ACTCTTGCCTATG- - - - - - - -*AGG*-**471**	**1/20**
447-ACTCTTGCCTAT - - - - - TAG*AGG*-**471**	**1/20**
447-ACTCTTGCCTAT - - - - - -AG*AGG*-**471**	**1/20**
447-ACTCT- - - - - - - - - - - -AG*AGG*-**471**	**1/20**
447-ACTCTTGCCTATGATGG**A**TAG*AGG*-**471**	**6/20**
447-ACTCTTGCCTATGATGG**T**TAG*AGG*-**471**	**2/20**
447-ACTCTTGCCTATGATGG**G**TAG*AGG*-**471**	**1/20**
447-ACTCTTGCCTATGATGG**TG**TAG*AGG*-**471**	**1/20**
447-ACTCTTGCCTATGATGG**AA**TAG*AGG*-**471**	**1/20**
447-ACTCTTGCCTATGATGG**AAA**TAG*AGG*-**471**	**1/20**

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