Received: 23/11/2022 Revised: 14/01/2023 Accepted: 12/03/2023 Published: 09/06/2023

ORIGINAL ARTICLE
DOI 10.58430/jib.v129i2.21

A simple and rapid CRISPR-Cas12a based detection test for diastatic *Saccharomyces cerevisiae*

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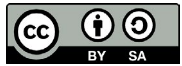

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Abstract

Diastatic *Saccharomyces cerevisiae* is a common microbial contaminant in the brewing industry. Currently available detection methods are either time consuming or require specialised equipment. The aim of this study was to develop a new rapid and simple assay for the detection of diastatic yeast in samples of beer and yeast. More specifically, the aim was to develop a simple and rapid assay that requires minimal laboratory equipment or training, and yields results as accurate as PCR-based methods. The assay consists of three main steps: DNA extraction, pre-amplification of DNA, and CRISPR-Cas12a based detection and visualisation. Different pre-amplification and visualisation techniques were compared, and the final assay involved a one-pot reaction where LAMP and Cas12a were consecutively used to pre-amplify and detect a fragment from the *STA1* gene in a single tube. These reactions required a heat block, a pipette, and a centrifuge with the assay result visualised on a lateral flow strip. The assay was used to monitor an intentionally contaminated brewing fermentation and was shown to yield results as accurate as PCR with previously published primers. Furthermore, the assay yielded results in approximately 75 minutes. The developed assay offers reliable and rapid quality control for breweries of all sizes and can be performed without expensive laboratory equipment. It is suggested that the assay will be particularly useful for smaller breweries without well-equipped laboratories who are looking to implement better quality control.

Keywords:
beer, contamination, diastatic yeast, detection, CRISPR-Cas12a

Introduction

Diastatic *Saccharomyces cerevisiae* (var. *diastaticus*) is a common spoilage microorganism in the brewing industry (Meier-Dörnberg et al. 2017; Powell and Kerruish 2017; Meier-Dörnberg et al. 2018; Krogerus and Gibson 2020). These strains, which are genetically closely related to brewing strains of *S. cerevisiae*, produce an extracellular glucoamylase that can hydrolyse otherwise unfermentable dextrin to fermentable glucose. Contamination by diastatic yeast can lead to extended fermentations and organoleptic faults. In serious cases, the formation of additional carbon dioxide in packaged beers can lead to gushing and exploding packages. Contamination by diastatic *S. cerevisiae* is most common during bottling. Smaller breweries, with less quality control and experimentation with novel yeast strains, are more susceptible to contamination (Meier-Dörnberg et al. 2017). Accordingly, to assure beer quality and to prevent economic and reputational losses, it is vital that diastatic *S. cerevisiae* is rapidly and reliably detected in the brewery.

Various methods for the detection of diastatic yeast are currently available, including culture based methods and more modern molecular methods (Powell and Kerruish 2017; Krogerus et al. 2019; Krogerus and Gibson 2020; Traynor et al. 2021; Burns et al. 2021). However, as diastatic strains are genetically and physiologically similar to brewing strains, it can be difficult to distinguish diastatic contaminants from production strains. The culture-based methods, which rely on growth on selective media, are popular because of their simplicity and low cost, but are time consuming and prone to false positives. The molecular methods typically rely on the detection of the *STA1* gene, which encodes an extracellular glucoamylase responsible for the diastatic phenotype. Detection can be accomplished with the polymerase chain reaction (PCR), either endpoint or quantitative, and several commercial kits are available. A typical workflow includes extraction of DNA from a (potentially pre-enriched) yeast sample, performing PCR using a primer pair for *STA1* (e.g. SD-5A/SD-6B from Yamauchi et al. (1998)), separation and visualisation of PCR products using gel electrophoresis and DNA staining. The advantages of the molecular methods are speed and accuracy, as results can be obtained within hours instead of days. However, these methods require specialised equipment and training, and therefore are less accessible to smaller breweries.

Recently, a number of nucleic acid detection techniques exploiting various CRISPR-Cas nucleases have been described (Gootenberg et al. 2017; Chen et al. 2018; Li et al. 2018; Kellner et al. 2019). The CRISPR-Cas nucleases can be directed to cleave specific nucleic acid sequences using a single guide RNA molecule. The detection techniques exploit a feature of the Cas12a and Cas13 enzymes, where the activated nuclease exhibits non-specific *trans* cleavage activity on any nearby single stranded DNA (ssDNA) or RNA, respectively. For Cas12a, this means ssDNA is cut non-specifically when the nuclease is bound to a double stranded DNA activator with complementary base pairing to the guide crRNA (Chen et al. 2018). This *trans*-activity can be read out, as an increase in fluorescence, if a ssDNA reporter containing a fluorophore and a quencher is used. A further increase in sensitivity can be obtained if the target DNA is pre-amplified prior to the CRISPR-Cas reaction, using PCR or isothermal methods (Li et al. 2018; Wang et al. 2019; Kellner et al. 2019). An overview of the technique is presented in Supplementary Figure 1. These systems have been used for the detection of various human viral diseases, including human papillomavirus and SARS-CoV-2 (Chen et al. 2018; Broughton et al. 2020; Ali et al. 2020), and food contaminants such as *Salmonella* sp. and *Escherichia coli* (Wang et al. 2020; Ma et al. 2021; Liu et al. 2022). Recently, a Cas12a-based assay was also developed for the detection of common beer spoilage lactic acid bacteria (Meng et al. 2021).

The objective of the work reported here was to develop a CRISPR-Cas12a based assay for the detection of diastatic yeast from samples of beer and yeast. More specifically, the aim was to develop a simple and rapid assay that requires minimal laboratory equipment or training, yielding results as accurate as PCR-based methods with a total processing time of approximately one hour. The assay consists of three main steps: DNA extraction, pre-amplification of DNA, and CRISPR-Cas12a based detection and visualisation. Different pre-amplification techniques were compared, including

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PCR and two isothermal techniques (recombinase polymerase amplification (RPA) and loop-mediated isothermal amplification (LAMP)), and two different visualisation techniques (fluorescence and lateral flow strips). The final assay involved a one-pot reaction where LAMP and Cas12a were consecutively used to pre-amplify and detect a fragment from the *STA1* gene in a single tube. The result was then visualised on a lateral flow strip. The assay was used to monitor a contaminated beer fermentation, with results as accurate as PCR using previously published primers. Furthermore, the assay yielded results in approximately 75 minutes. The assay offers a reliable and rapid quality control method for breweries of all sizes, without the requirement for expensive laboratory equipment.

Materials and Methods

Yeast strains

The yeast strains (Table 1) included ale, lager, and diastatic yeast strains. Yeasts were pre-cultured overnight in 25 mL YPD media (1% yeast extract, 2% peptone and 2% glucose) at 25°C and 120 rpm (Minitron incubator, Infors HT, Bottmingen, Switzerland).

Table 1.

Yeasts used in this study.

Commercial name	**Short code**	***STA1* expression**	**Species**	**Source**
VTT-A75060	A60	-	*Saccharomyces cerevisiae*	VTT Culture Collection, Espoo, Finland
VTT-A63015	A15	-	*Saccharomyces pastorianus*	VTT Culture Collection, Espoo, Finland
WLP023 Burton Ale Yeast	WLP023	-	*Saccharomyces cerevisiae*	White Labs Inc., San Diego, CA, USA
3711 French Saison	WY3711	+	*Saccharomyces cerevisiae*	Wyeast Laboratories, Odell, OR, USA
TUM PI BA 109	TPB109	+	*Saccharomyces cerevisiae*	BLQ Weihenstephan, Freising, Germany
TUM 71	TUM71	+	*Saccharomyces cerevisiae*	BLQ Weihenstephan, Freising, Germany
WLP565 Belgian Saison	WLP565	+	*Saccharomyces cerevisiae*	White Labs Inc., San Diego, CA, USA
WLP570 Belgian Golden Ale	WLP570	+	*Saccharomyces cerevisiae*	White Labs Inc., San Diego, CA, USA

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For testing of the protocols, a serial dilution of cultures of *S. cerevisiae* A60 (*STA1*$^{-}$) and WY3711 (*STA1*$^{+}$) was prepared based on optical density (absorbance at 600 nm). Pre-cultures of both strains were diluted to equal optical density with sterile deionised water, after which serial ten-fold dilutions of the two yeast cultures was performed. Stepwise dilution was carried out to achieve 10^{-1} to 10^{-6} aliquots of WY3711 in A60 (containing 7 × 10^{6}-10^{1} cells/mL of WY3711).

DNA extraction

DNA was extracted from yeast cultures with 'GC Prep' (Blount et al. 2016) using 425-600 µm acid-washed glass beads (Sigma-Aldrich, USA) and a 5% suspension of Chelex® 100 sodium form of particle size 50-100 mesh (dry) (Sigma-Aldrich, USA) diluted in sterile Milli-Q water. Yeast was separated by centrifugation (1 min, 5000 × *g*). 50 µL 5% Chelex 100 solution and approximately 50 µL glass beads were added to yeast pellet. The yeast sample was vortexed for 4 minutes, and the mixture incubated for 2 minutes at 98°C. The genomic DNA was dissolved in the liquid phase and was collected from the supernatant after centrifugation (1 min, 15,000 × *g*). The concentration of DNA and A260/280 ratio was measured with a Qubit 2.0 fluorometer and Nanodrop spectrophotometer.

Design of crRNA

Targeting of the Cas12a endonuclease occurs with a guide RNA (gRNA) molecule that is complementary to the target site and requires a 5' TTTV protospacer adjacent motif (PAM). As *STA1* is a chimeric gene of *FLO11* and *SGA1*, a suitable protospacer sequence specifically targeting *STA1*, and not *FLO11* nor *SGA1*, needed to be designed. Potential protospacer sequences were obtained using CCTop (Stemmer et al. 2015), with the *STA1* promoter and open reading frame (ORF) sequence of *S. cerevisiae* WY3711 (Krogerus et al. 2019) being the target, and the *S. cerevisiae* S288C genome (Engel et al. 2014) being used for detection of off-target sites. Any potential protospacer sequences, lacking predicted off-target activity (i.e. more than four mismatches) in *FLO11*, *SGA1* or any other genomic location, were then queried for matches in the genomes of the 157 *S. cerevisiae* strains sequenced by (Gallone et al. 2016) using 'blastn' in the online suite of NCBI-BLAST. 100% matches were only allowed in the 21 strains carrying *STA1* (Krogerus et al. 2019). From this, a single protospacer sequence - (TTTC) AATTAGAACCACAACATGAC - was selected for the trials (Table 2). The sequence is located 40 bp upstream of the STA1 start codon. A crRNA molecule containing the selected protospacer sequence was obtained from IDT (Integrated DNA Technologies, Inc., Coralville, Iowa, USA) (Table 2).

Design of primers

PCR and RPA primers (Table 3 and Supplementary Table 1) were designed using Primer-BLAST (Ye et al. 2012) with default settings. The aim was to produce amplicons containing the above Cas12a protospacer sequence, with amplicon lengths around 200 bp. The primers were designed to be around 20 and 30 bp long for PCR and RPA reactions, respectively.

LAMP primers were designed using GLAPD (Jia et al. 2019) and the NEB LAMP Primer Design online tool version 1.2.0 (https://lamp.neb.com/), with the amplicons containing the Cas12a protospacer sequence. In GLAPD, the *S. cerevisiae* S288C genome was used to test the specificity of the generated primers. The primers were tested for mismatches against the 17 *STA1* ORF and promoter sequences from Krogerus et al (2019) in Geneious 10.2.6 (Biomatters, Auckland, New Zealand). STA1_LAMP_S1_FIP and STA1_LAMP_S1_LB both contained a single mismatch in the sequences from all ten of the strains with a deletion in the *STA1* promoter. Two different LAMP primer sets were obtained and tested (Table 4). All primers were purchased from Thermo Scientific (Finland).

Table 2.

Protospacer and crRNA sequences

Name	Sequence (5' > 3')
Protospacer	(TTTC)**AATTAGAACCACAACATGAC**
crRNA	UAAUUUCUACUAAGUGUAGAU**AAUUAGAACCACAACAUGAC**

Table 3.

Primers designed for PCR reactions.

Name	Sequence (5′ > 3′)
STA1_PCR_F1	AAAAAGCACCCTATTCATCAGTTAT
STA1_PCR_F2	ATAAAAAGCACCCTATTCATCAGTT
STA1_PCR_R1	TATGGATTTTTGAGGCCTACCA
STA1_PCR_R2	AGTGATGTTGCTAGAGGAGGA

Table 4.

Primer set designed for LAMP reaction.

Primer type	Sequence (5′>3′)
Set 1	
STA1_LAMP_S1_F3	TCTTTTGCTTCCTAAACTAAACCTA
STA1_LAMP_S1_B3	AGGAGGATCCTCTAGGAACT
STA1_LAMP_S1_FIP	ATGGATTTTTGAGGCCTACCATAGTAAAAGCACCCTATTCATCAGT
STA1_LAMP_S1_BIP	ATACGCACACTATGCAAAGACCATTGGAAAACCCAAAGCTGAG
STA1_LAMP_S1_LF	AACCACAACATGACAAGAGATTAT
STA1_LAMP_S1_LB	TTCTACTCGCTTATTTGGTCCTTTC
Set 2	
STA1_LAMP_S2_F3	TCATAGACTTACCTGTACAAGTT
STA1_LAMP_S2_B3	AAACCCAAAGCTGAGTTAAAT
STA1_LAMP_S2_FIP	AGAACCACAACATGACAAGAGATTTAAAAAAGGATCTTTTGCTTCCT
STA1_LAMP_S2_BIP	CTATGGTAGGCCTCAAAAATCCATCGAAAGGACCAAATAAGCG
STA1_LAMP_S2_LF	-
STA1_LAMP_S2_LB	ACACTATGCAAAGACCATTTCTACT

PCR amplification

PCR reactions were carried out with Phusion polymerase. Both Phusion High-Fidelity PCR Master Mix with HF Buffer (Thermo Scientific, Vantaa, Finland) and Phusion™ Plus Green PCR Master Mix (Thermo Scientific, Vantaa, Finland) were used. The reactions were performed according to manufacturer's instructions, with reaction volumes adjusted to 14 µL with primer concentrations of 0.5 µM. PCR reactions were performed using a Mastercycler® Nexus (Eppendorf, Hamburg, Germany).

The PCR reactions with the newly designed primers were optimised with increasing annealing temperature from 62.1 to 64°C, decreasing elongation time from 30 to 6 seconds and increasing cycles from 30 to 35. As a control, *STA1* was detected with the primer pair SD-5A 5´-CAACTACGACTTCTGTCATA-3´ and SD-6B 5´-GATGGTGACGCAATCACGA-3´ (Yamauchi et al. 1998) and the PCR programme used with the SD-5A/6B primers was: 98°C for 2 min, 30 cycles of (98°C for 10 s, 57.5°C for 30 s, 72°C for 30 s), 72°C for 10 min and cooling to 8°C. PCR products were separated on 1.5% agarose gels in 0.5 × TBE buffer with gel electrophoresis.

RPA amplification

Pre-amplification of the *STA1* gene was performed with recombinase polymerase amplification (RPA) (Piepenburg et al. 2006). RPA is an isothermal amplification method, performed at a constant temperature. The RPA reactions were carried out with a commercial TwistAmp® Liquid Basic Kit (TALQBAS01, TwistDx, Maidenhead, UK) according to the manufacturer's instructions but with minor modifications to volumes. Reaction volumes were adjusted to 15 µL containing 7.5 µL of 2x Reaction Buffer, 1.8 mM of dNTPs, 1.5 µL 10x Basic E-mix, 0.48 µM of each primer, 0.75 µL 20x Core Reaction Mix, 0.96 µL of 280 mM MgOAc and 1.5 µL of DNA template. Reaction conditions were optimised for RPA with incubation temperatures of 37, 39 and 42°C. Amplicons were cleaned with AMPure XP beads (Beckman Coulter Inc., Brea, CA, USA) prior to separation on 1.5% agarose gels in 0.5× TBE buffer with gel electrophoresis.

LAMP amplification

Pre-amplification of the *STA1* gene was performed with loop-mediated isothermal amplification (LAMP) (Notomi 2000). Like RPA, LAMP is a isothermal amplification method. LAMP reactions were performed using the NEB WarmStart® LAMP Kit (DNA & RNA) (E1700S, New England Biolabs, Ipswich, Massachusetts, USA) with the two designed primer sets. Reactions were carried out in 10 µL reaction volume containing 5 µL WarmStart LAMP 2X Master Mix, 1.6 µM FIP, 1.6 µM BIP, 0.2 µM F3, 0.2 µM B3, 0.4 µM LF, 0.4 µM LB and 1 µL of DNA template. Primer set 2 lacked the LF primer and was replaced with water. Reaction conditions were optimised by testing different incubation temperatures (65, 67 and 69 °C) and dimethyl sulfoxide (DMSO) concentration (0, 5 and 7.5%) (Wang et al. 2015). The incubation time was 30 minutes according to the kit protocol.

CRISPR-Cas12a based nucleic acid detection

CRISPR-Cas12a reactions were carried out in 20 µL reaction volumes with 2 µL NEBuffer™ r2.1 (New England Biolabs, Ipswich, Massachusetts, USA), 500 nM crRNA, 500 nM Cas12a enzyme, varying ssDNA oligonucleotide concentrations, 0.5 µL RNase inhibitor (TaKaRa Bio Inc., Japan) and 1 µL sample. Two different Cas12a enzymes were used: EnGen® Lba Cas12a (Cpf1) (New England Biolabs, Ipswich, Massachusetts, USA) and Alt-R™ L.b. Cas12a (Cpf1) Ultra (Integrated DNA Technologies, Inc., Coralville, Iowa, USA). Reactions were performed in RNase free tubes with RNase- and DNase-free pipette tips to avoid any RNase or DNase contamination. Different ssDNA oligonucleotide reporters were used depending on desired read-out (described below in 2.8.1 and 2.8.2).

Fluorescent read-out

A fluorophore quencher (FQ)-labelled ssDNA oligonucleotide reporter was used in order to detect Cas12a activity through fluorescence (Chen et al. 2018). The reporter was HPLC-purified 5' / 6-FAM / TTATT / Iowa Black® FQ / 3' purchased from IDT (Integrated DNA Technologies, Inc., Coralville, Iowa, USA). CRISPR-Cas12a reactions were prepared on

Microfluor 2 Black 'U' Bottom Microtiter Plates (Thermo Scientific, Rochester NY, USA). The reactions contained 500 nM of FQ-labelled reporter, and were incubated at 37°C for 30 minutes during which the change in fluorescence was monitored. The intensity of fluorescence was measured with excitation at 495 nm and emission at 520 nm with a VarioSkan (Thermo Scientific) using SkanIt™ Software version 2.3.4.

Lateral flow read-out

A biotin labelled ssDNA oligonucleotide reporter - HPLC-purified 5' / 6-FAM / TTATT / Biotin / 3' purchased from IDT - was used to detect Cas12a activity with lateral flow strips (Broughton et al. 2020). Different reporter concentrations were tested, with reactions performed at a concentration of 400 nM. CRISPR-Cas12a reactions were incubated at 37°C for 30 minutes and the assay result was read out with lateral flow strips from a HybriDetect - Universal Lateral Flow Assay Kit (MGHD 1, Milenia Biotec GmbH, Gießen, Germany). The CRISPR-Cas12a reaction product (5 µL) was added to the application area of lateral flow dipstick and strips were incubated for 1 min in 100 µL of HybriDetect assay buffer in an upright position and results were observed immediately and photographed with a camera phone.

One-Pot LAMP-Cas12a reaction

A one-pot reaction, combining the LAMP and CRISPR-Cas12a reactions in a single vessel (to reduce operating steps and cross-contamination risks), was designed based on previous studies (Wang et al. 2019; Wang et al. 2021). As the LAMP reaction is incubated at 65°C - the temperature at which the EnGen® Lba Cas12a enzyme is inactivated - the LAMP reaction had to be physically separated from the Cas12a reaction mix with mineral oil and an air phase. LAMP reaction (10 µL) was set up in the bottom of a 1.5 mL tube and sealed with 25 µL of mineral oil, with 20 µL CRISPR-Cas12a reaction mix assembled in the screw cap. The reagent concentrations of CRISPR-Cas12a reaction mix were increased 1.5-fold to take into account the increased total volume. Reactions were performed with both the FQ reporter and biotin-labelled reporter. Reaction tubes were incubated for 30 minutes at 65°C, during which the LAMP reaction occurs, after which tubes were cooled for 1 minute at room temperature followed by mixing with five inversions. The liquid was briefly centrifuged, after which tubes were incubated at 37°C for 30 minutes. Results were read depending on the reporter used in the reaction. When the FQ reporter was used, fluorescence was observed under UV light. With the biotin-labelled reporter, lateral flow read-out was performed as above (5 uL of sample was taken under the oil phase).

Wort fermentation

Small-scale wort fermentations were performed to test the developed method on wort and beer samples. Fermentations were inoculated with (i) diastatic yeast (TPB109), (ii) non-diastatic yeast (WLP023) and (iii) non-diastatic yeast (WLP023) contaminated with diastatic yeast (TPB109). Overnight cultures were prepared in 50 mL YPM media (1% yeast extract, 2% peptone and 2% maltose) at 25°C with shaking at 120 rpm. Yeasts were pelleted and resuspended in deionised water to 20% (w/v) slurries. The viability and cell concentration of the slurries was measured with a NucleoCounter® YC-100™ (Chemometec A/S, Allerod, Denmark). Wort was prepared from Spraymalt Extra Light extract (Muntos, Suffolk, UK) to 12°P with 10 g/L Cascade hop pellets which was boiled for 30 minutes. The wort was filtered and pasteurised at 80°C for 30 minutes. Yeast was pitched at a rate of 1.2×10^7 cells/mL into 80 mL of wort. The contaminated fermentations (iii) were spiked with 8.4×10^3 cells of diastatic yeast TBP109 to a final level of 120 cells/mL. Fermentations were performed in duplicate in 100 mL sterile Schott bottles capped with glycerol-filled airlocks at 20°C for 11 days.

Samples were drawn regularly during the fermentation. Prior to sampling, the bottles were carefully agitated, and 1 mL of fermenting wort pipetted to an Eppendorf tube and centrifuged (3 min, 4000 × *g*). The supernatant was analysed for fermentable sugars as described below. The yeast pellets were washed twice, by suspending in 1 mL of sterile Milli-Q water and centrifugation (3 min, 4000 × *g*). Yeast pellets were stored at -20 °C until DNA extraction with 'GC Prep', for the genomic DNA

to test the developed LAMP-Cas12a protocol. The sugar and ethanol concentration in samples were analysed with high-performance liquid chromatography (HPLC) using a Waters Alliance® HPLC system consisting of a Waters 2695 Separation Module, Waters System Interphase Module and Waters 2414 differential refractometer (Waters Co., Milford, MA, USA). A Bio-Rad Aminex HPX-87H HPLC column for organic acid analysis (300 × 7.8 mm; Bio-Rad Hercules, CA, USA) was equilibrated with 5 mM H_2SO_4 (Titrisol, Merck, Germany) in water at ambient temperature 55°C and analysed samples were eluted with 5 mM H_2SO_4 in water at flow rate of 0.3 mL/min.

Results and discussion

The aim of this work was to develop a simple and rapid assay for the detection of diastatic *S. cerevisiae*, and the workflow is shown in Figure 1. The developed assay consisted of three steps: DNA extraction, pre-amplification of DNA, and CRISPR-Cas12a based detection and visualisation. The final step involved a one-pot reaction where LAMP and Cas12a were consecutively used to pre-amplify and detect a fragment from the *STA1* gene in a single tube. The assay result was then visualised on a lateral flow strip.

DNA extraction and testing of pre-amplification strategies

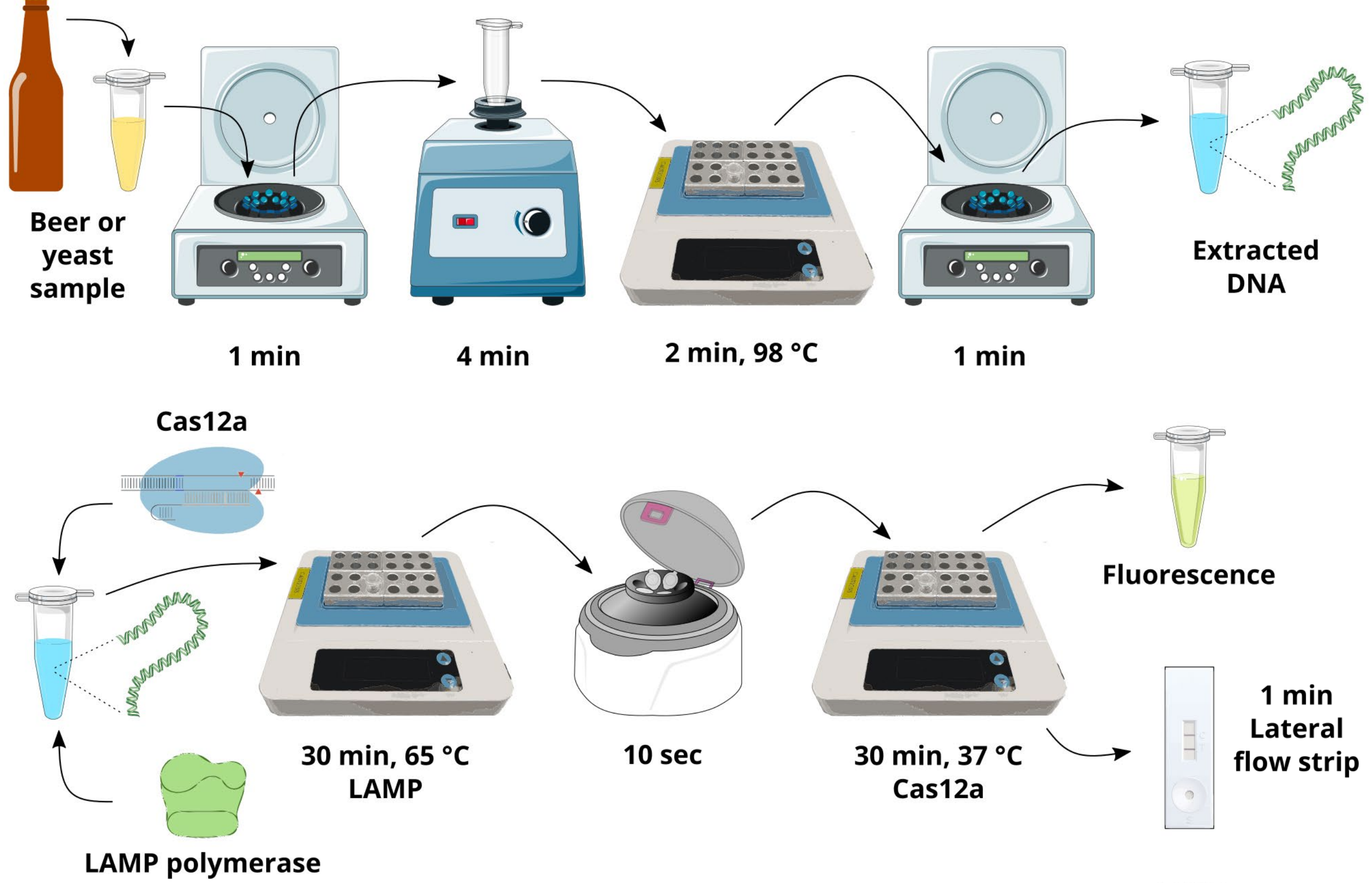
The first step of the assay involved DNA extraction with 'GC Prep' was selected for speed, simplicity and resulting DNA quality (Blount et al. 2016). DNA was extracted from the brewing yeast strains ranging from 2.6 to 6.8 ng/µL, while A260/280 ratios varied from 2.1 to 2.4. The sample processing took approximately 10 minutes. The extracted DNA was used to compare the different pre-amplification strategies.

Pre-amplification by PCR

Four different primer combinations (F1-R1, F1-R2, F2-R1 and F2-R2) were designed to target the *STA1* gene of diastatic *S. cerevisiae* and with PCR

Figure 1.

Detection assay for *STA1* from beer or yeast samples.



produced approximately 200 bp amplicons containing the selected Cas12a protospacer sequence. Initial trials used a PCR programme of 98°C for 2 min, 30 cycles of (98°C 10 sec, 62.1°C 20 sec and 72°C for 30 sec), and 72°C for 10 minutes. With this programme, all four primer combinations produced an amplicon with the positive control (Saison yeast WY3711) (Figure 2A). However, the negative control (A60) also produced target amplicons for two of the pairs (F1-R1 and F2-R1), and larger unwanted by-products (~1000 and 1200 bp) with the other two primer pairs. To remove these unwanted by-products, the PCR programme was optimised for the F2-R2 primer pair. By increasing the annealing temperature from 62.1 to 64°C, decreasing elongation time from 30 to 6 seconds and increasing amplification cycles from 30 to 35, the formation of the unwanted by-product in the negative control was eliminated (Figure 2B). These modifications also decreased the overall run time of the PCR programme. Two different Phusion master mixes, Phusion High-Fidelity PCR Master Mix with HF Buffer and Phusion Plus Green PCR Master Mix, were compared and these yielded identical results. Pre-amplification with PCR using primer pair F2-R2 was therefore successful, however the step would require a thermal cycler taking approximately 45-60 minutes. However, using PCR as a pre-amplification method would yield few advantages compared to using PCR and gel electrophoresis directly for *STA1* detection.

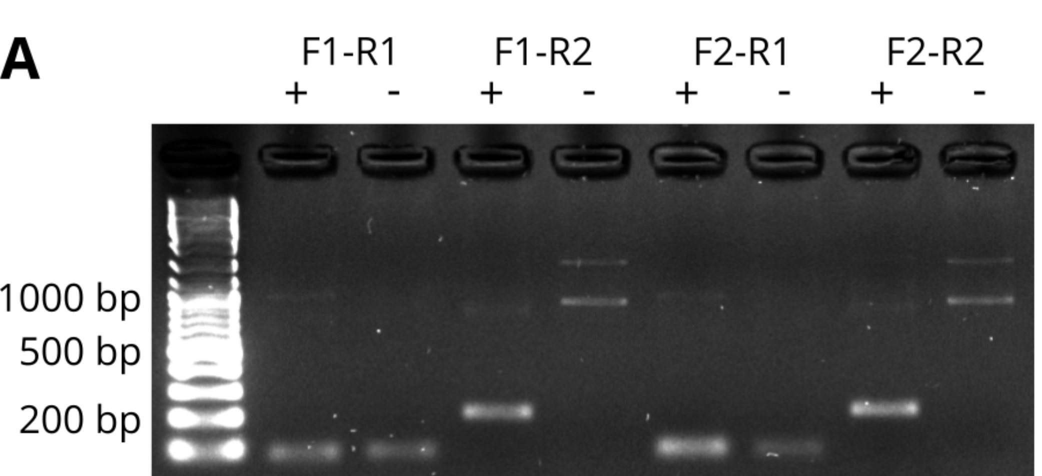


Figure 2(A)

PCR reactions with four different primer pairs targeting *STA1* using DNA from the *STA*+ control *S. cerevisiae* WY3711 and *STA*- control *S. cerevisiae* A60.

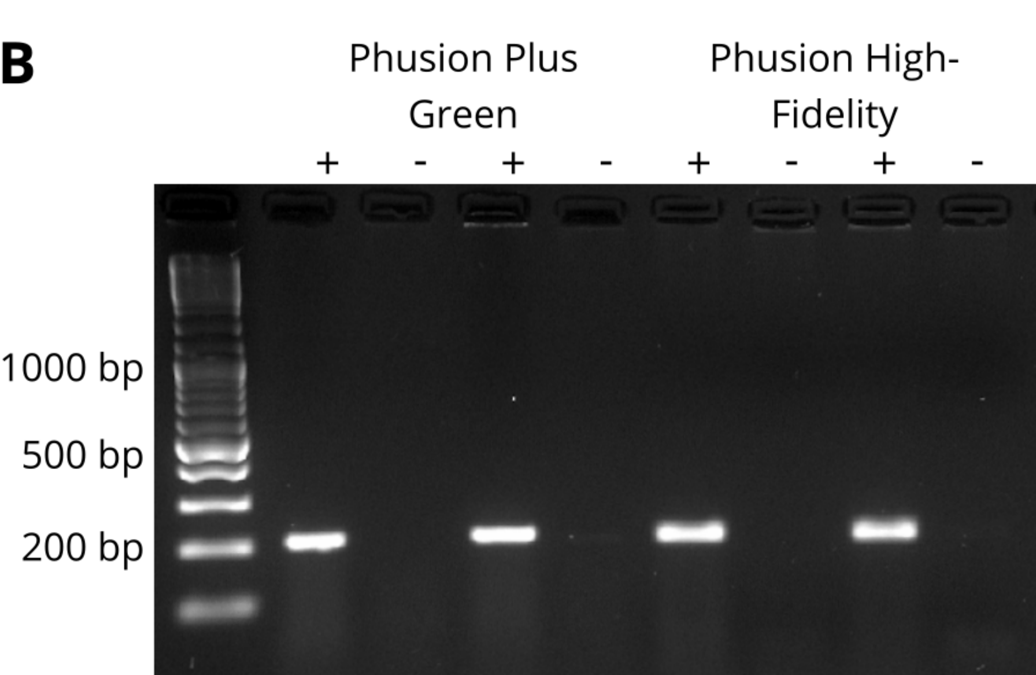


Figure 2(B)

Optimised PCR reactions with the F2/R2 primer pair using DNA from the same strains as in (A). The first four reactions were carried out with Phusion™ Plus Green PCR Master Mix and the last four were carried out with Phusion High-Fidelity PCR Master Mix with HF Buffer.

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Isothermal pre-amplification by RPA and LAMP

To simplify and speed up the pre-amplification step, two different isothermal DNA amplification methods were explored: recombinase polymerase amplification (RPA) and loop-mediated isothermal amplification (LAMP). For RPA, a total of twelve primer combinations were tested on a *STA*$^{+}$ (WY3711) and *STA*$^{-}$ strain (A60) with a commercial TwistAmp® Liquid Basic Kit. The four most promising primer combinations (which produced the most and least products for the positive and negative sample) were optimised by evaluating different incubation temperatures between 37 and 42°C.

Overall, RPA was unable to differentiate the positive and negative samples as clearly as PCR (Supplementary Figure 2). Poor amplification was observed with all primer pairs at 42°C. For primer pairs F3-R3 and F3-R4, amplicons of equal intensity were produced in both samples at all temperatures. With primer pairs F1-R3 and F2-R2, a clearer difference between the positive and negative samples was observed, with the most intense bands produced at an incubation temperature of 39°C. However, both primer combinations still produced faint bands with the negative control (*S. cerevisiae* A60). An incubation temperature of 40°C was tested, but negative samples still produced faint bands (Supplementary Figure 2B). The F2-R2 primer pair was chosen for further experiments.

LAMP reactions are carried out with four to six primers, and two different designed primer sets were tested on a *STA*$^{+}$ (WY3711) and *STA*$^{-}$ strain (A60) with a commercial NEB WarmStart® LAMP Kit. An incubation temperature of 65°C (with no DMSO) was used in the first trials. Set 1 produced an amplified product only in the positive control, while set 2 did not result any amplification in either sample (Figure 3). The amplified product, which is formed from repeats of the desired target sequence, can be seen as bright bands of different lengths. Different incubation temperatures (65, 67, and 69°C) and DMSO contents (0, 5, and 7.5%) were further tested. However, no further improvement in specificity and amplicon intensity was obtained compared to the initial trial (Supplementary Figure 3). Primer set 1, with an incubation temperature of 65°C, and without DMSO was selected for further experiments. As single mismatches had been detected for two of the primers against the *STA1* sequences from all ten of the *STA1*$^{+}$ strains with a deletion in the *STA1* promoter (Krogerus et al. 2019), the primer set was further tested against two of these strains to ensure amplification. The primer set successfully amplified the product in these strains despite the single mismatches (Supplementary Figure 4).

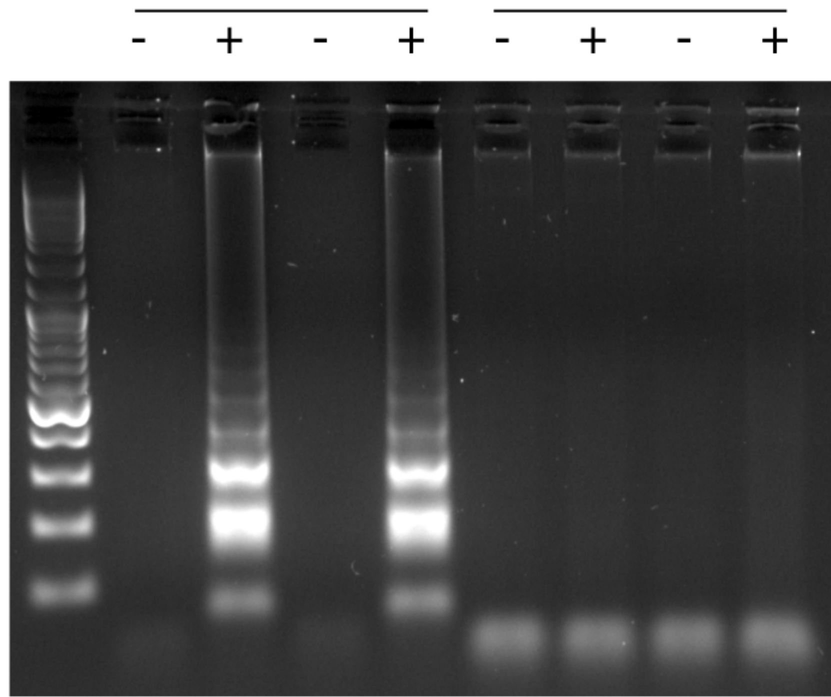

Figure 3.

LAMP reactions using DNA from the *STA1*$^{-}$ control *S. cerevisiae* A60 and *STA1*$^{+}$ control *S. cerevisiae* WY3711 with primer set 1 and 2.

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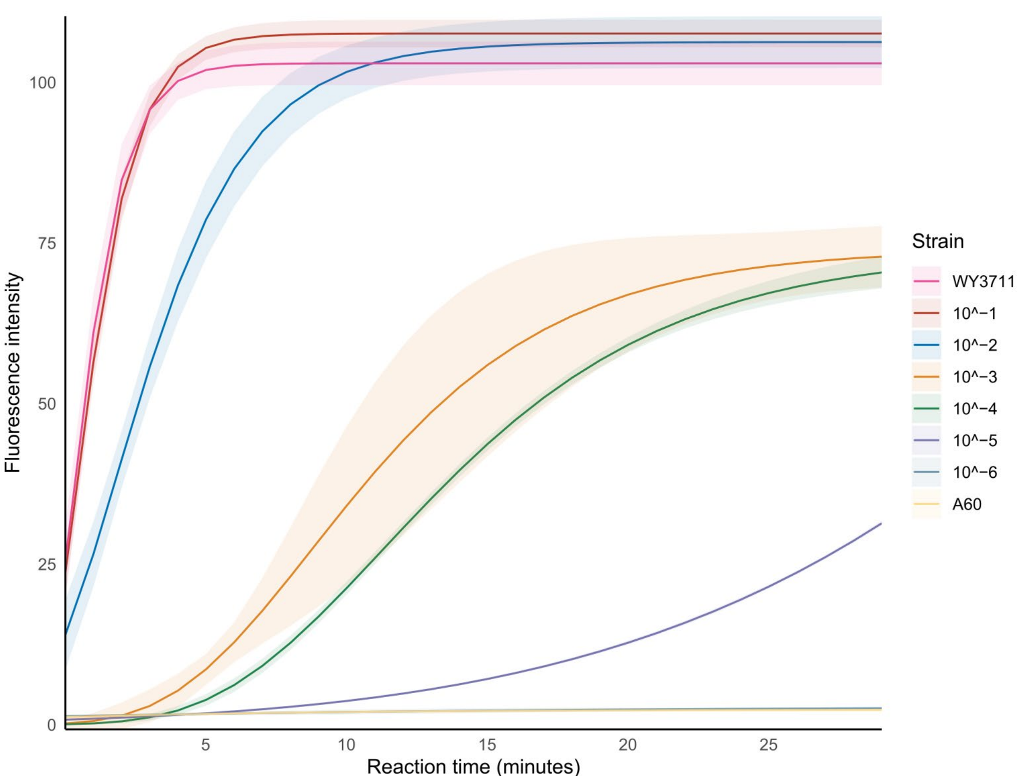

Figure 4.

Change in fluorescence during Cas12a reactions using DNA pre-amplified with LAMP from the dilution series of *STA1⁺ S. cerevisiae* WY3711 in the *STA1⁻ S. cerevisiae* A60. Values are replicates of four independent reactions, and the standard deviation is represented as the lighter shaded area.

Discussion of pre-amplification experiments

Of the three pre-amplification methods, LAMP was the most promising in that it could clearly differentiate a *STA1⁺* from a *STA1⁻* strain, and the reaction could be performed isothermally. LAMP-based protocols for the detection of beer spoilage microorganisms have been reported previously (Tsuchiya et al. 2007; Hayashi et al. 2009). PCR and LAMP primers for *STA1* gene detection have been developed (Yamauchi et al. 1998; Hayashi et al. 2009), but the primers could not be used here as they do not produce amplicons containing the Cas12a protospacer sequence. Primer design to produce amplicons containing the Cas12a protospacer sequence proved challenging, evident from the poor specificity during the RPA amplifications and some of the PCR primers, and the failed amplification for the second set of LAMP primers. The main challenge in primer design was that the *STA1* gene is chimeric, consisting of fragments from both *FLO11* and *SGA1* (Yamashita et al. 1987; Krogerus and Gibson 2020). The protospacer sequence is located just upstream of *STA1*, and the region is homologous to that upstream of *FLO11*. Hence, to develop primers specific for *STA1*, off-target activity against *FLO11* had to be assessed.

CRISPR-Cas12a assay fluorescent read out

The first CRISPR-Cas12a reactions were performed on DNA samples with or without pre-amplification using PCR or RPA. The DNA samples were obtained from serial dilutions of *STA1⁺* WY3711 in *STA⁻* A60. A control, where the template DNA was replaced with water, was also included. A reporter molecule containing a fluorophore and quencher was used such that Cas12a activity could be monitored by an increase in fluorescence. Reactions were incubated for 30 minutes, and fluorescence intensity was measured after incubating for 3 and 30 minutes (Table 5). The results indicated that pre-amplification of DNA samples was necessary, as no Cas12a activity was observed in any of the non-pre-amplified samples. Differences between the positive and negative controls were observed by the increasing fluorescence with pre-amplification with either PCR or RPA. However, PCR-amplified samples showed a clearer increase in fluorescence compared to RPA-amplified samples which produced only a modest fluorescence in the diluted samples after 30 minutes of incubation. Accordingly, RPA pre-amplification was considered unsuitable for reliable detection using the CRISPR-Cas12a assay.

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Table 5.

Fluorescence intensity (RFU) after Cas12a reactions using DNA pre-amplified with PCR or RPA from serial dilution of yeasts *STA1*$^{+}$ WY3711 in *STA1*$^{-}$ A60.

Amplification	Time	WY3711	10^{-1}	10^{-2}	10^{-3}	A60	Water
PCR	3 min	54.2	40.8	57.9	20.3	1.1	2.7 (3 min)
	30 min	46.4	49.8	70.7	45.9	1.0	
RPA	3 min	25.5	6.1	5.7	3.6	1.9	
	30 min	47.4	15.8	12.7	7.1	2.5	2.5 (30 min)
No pre-amplification	3 min	2.6	2.1	2.2	2.4	1.9	
	30 min	2.1	1.7	2	2	2	

Table 6.

Fluorescence intensity (RFU) after Cas12a reactions using DNA pre-amplified with LAMP or PCR from serial dilution of yeasts *STA1*$^{+}$ WY3711 in *STA1*$^{-}$ A60.

Amplification	Time	WY3711	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	A60	Water
LAMP	1 min	38.4	32.9	30.2	26.3	29.9	1	1	1.3	1.3 (1 min)
	10 min	69.7	81.1	67.5	71.4	82.5	1.9	2.1	1.9	
PCR	1 min	15.5	12.1	8.7	3.2	2	1.1	1.2	1	
	10 min	49.5	53	54.7	25	7.8	2.6	4	1.4	2.3 (10 min)
No pre-amplification	1 min	1.6	2	-	-	-	-	-	1.8	
	10 min	3.9	3	-	-	-	-	-	3.9	

As RPA was not suitable for isothermal pre-amplification, CRISPR-Cas12a reactions were carried out on samples pre-amplified with LAMP. DNA was extracted from an extended dilution series (WY3711, 10^{-1} - 10^{-6}, A60), and the genomic DNA was pre-amplified with both LAMP and PCR. DNA samples without pre-amplification (positive control, 10^{-1} dilution and negative control) and a non-template control were included. Fluorescence intensity was measured after one and 10 minutes of incubation with the Cas12a enzyme (Table 6). In samples pre-amplified with LAMP, an increase in fluorescence intensity was observed for WY3711, with samples diluted to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. After 10 minutes, these samples pre-amplified with PCR yielded lower fluorescence intensity. LAMP therefore appeared to be the most sensitive of the tested pre-amplification methods, and also beneficially was an isothermal method.

As pre-amplification with LAMP was promising, the dynamics of fluorescence increase during the Cas12a reactions was studied using DNA extracted from the dilution series (WY3711, 10^{-1} to 10^{-6} and A60) and replicate reactions. Four replicate reactions for each DNA sample were incubated with Cas12a for 30 minutes at 37°C, and fluorescence intensity was continuously monitored (Figure 4).

A non-template control was included, but results are excluded as the fluorescence was same as the negative control (A60). The increase in fluorescence (Cas12a activity) was rapid, with fluorescence plateauing after around five minutes with the WY3711, 10^{-1} and 10^{-2} samples. An increase in fluorescence was also observed with the 10^{-3}, 10^{-4}, and 10^{-5} samples, but was slower, with a longer lag time. Fluorescence did not increase at 10^{-6} or the negative control A60 during a 30-minute incubation.

Lateral flow read out

Following the successful detection of *STA1* using Cas12a, it was assessed whether assay results could be visualised with lateral flow strips instead of fluorescence for a more user-friendly assay. A HybriDetect Universal Lateral Flow Assay Kit was used, and prior to testing visualisation of Cas12a activity with lateral flow strips, the concentration of the biotin-labelled ssDNA reporter was optimised with aqueous solutions of the reporter. If the reporter concentration is not optimal, this can result in the 'hook effect', where the reporter is not retained at the control and a test line becomes unintentionally visible. Reporter dilutions (5 µL) were directly added to dipsticks and strips were incubated for one minute in the manufacturer's

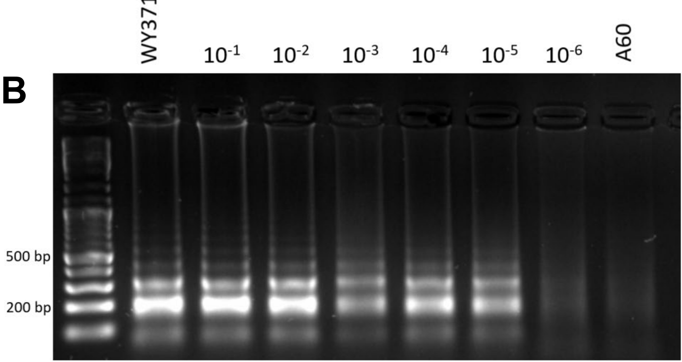
assay buffer. The objective was to minimise the intensity of the test line, while retaining a visible control line (C). The T line remained visible at all tested reporter concentrations (Supplementary Figure 5). While it was not possible to minimise the T line completely, the best results were obtained with a reporter concentration of 200-500 nM. Therefore, in subsequent experiments, a reporter concentration of 400 nM was used. Further, the addition of 5% polyethyleneglycol (PEG) to the buffer was tested, but this failed decrease the strength of the T line.

As the T line could not be completely minimised in an aqueous solution of just the reporter, it was evaluated whether Cas12a-treated positive and negative samples could be distinguished with lateral flow strips. CRISPR-Cas12a reactions with the biotin-labelled reporter were performed on LAMP pre-amplified samples of DNA extracted from the above dilution series. The CRISPR-Cas12a reactions were incubated at 37°C for 30 minutes and the results immediately read with lateral flow strips (Figure 5A). Clear differences between positive and negative controls could be observed despite the T line not being eliminated. With the strips loaded with Cas12a-treated samples from the positive control (dilutions 10^{-1} to 10^{-5}), the T line was more intense than the C line, which had disappeared completely. LAMP amplicons were also separated with gel electrophoresis to compare the result, and these samples showed clear amplification during LAMP (Figure 5B). Therefore, based on these results, a positive result with the lateral flow strips (i.e. *STA1* detection) was obtained where the T line is more intense than the C line.

One-pot assay

To minimise pipetting and reduce cross-contamination, the LAMP and CRISPR-Cas12a reactions were combined in a single reaction vessel. The LAMP reaction was assembled in the bottom of an Eppendorf tube and sealed with mineral oil, while the CRISPR-Cas12a reaction mix was prepared in the cap. Reactions were tested with positive (WY3711) and negative (A60) controls using both the FQ and biotin-labelled reporters. LAMP reactions were first incubated for 30 minutes at 65°C, after which the Cas12a reaction mix was combined with the LAMP reaction by inverting the tube and brief centrifugation. The tubes were then incubated a further 30 minutes at 37°C. The diastatic *S. cerevisiae* and non-diastatic *S. cerevisiae* were successfully identified using the 'one-pot'-method with both reporters (Figure 5C-D). With the

A
WY3711 10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6} A60 Water control
T→
C→



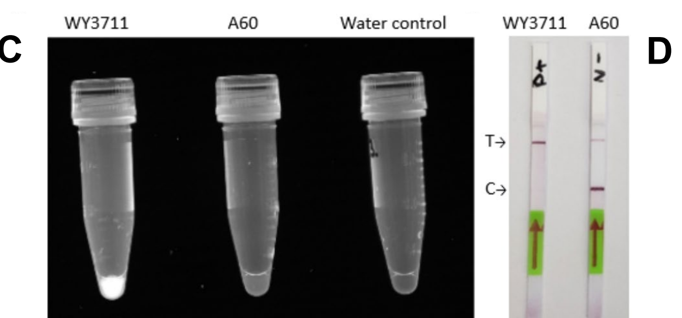


Figure 5.

(A) Lateral flow read-out from the Cas12a reactions using DNA pre-amplified with LAMP from the dilution series (10^{-1} to 10^{-6}) of *STA1⁺ S. cerevisiae* WY3711 in the *STA1⁻ S. cerevisiae* A60. A NTC control was included where the DNA template was replaced with water. The T and C lines are marked next to lateral flow strips.
(B) The corresponding LAMP products separated with gel electrophoresis.
(C) Cas12a and LAMP reactions performed in the same tube using the FQ reporter and photographed under UV light.
(D) Cas12a and LAMP reactions performed in the same tube using the biotin-labelled reporter and visualised with lateral flow read out.

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fluorescent reporter, the positive sample biotin-labelled reporter, the positive control produced a strong T line on the lateral flow strips (Figure 5D). A full protocol of the assay is available in Supplementary Note 1.

The CRISPR-Cas12a assay

The pre-amplification of template DNA was required for *STA1* detection using the Cas12a system. Of the three tested methods, isothermal LAMP amplification was the most promising based on sensitivity. Ultimately, a successfully amplified *STA1* gene could be detected with a CRISPR-Cas12a reaction combined with two different visual detection methods (fluorescence and lateral flow). The *STA1* gene could also be detected directly using LAMP amplification and gel electrophoresis (Figure 3), as previously demonstrated (Hayashi et al. 2009), or through a change in fluorescence or colour with DNA-binding dyes (Dao Thi et al. 2020). However, because of the high amplification efficiency of LAMP, and products with multiple repeats, the technique is prone to non-specific amplification and false positives (Ho et al. 2018; Bao et al. 2020; Zhang et al. 2021). However, as the CRISPR-Cas system, has high specificity and sensitivity, LAMP was combined with CRISPR-Cas12a to improve reliability and accuracy (Li et al. 2019; Mahas et al. 2021)

Detection of diastatic *S. cerevisiae* in wort samples

To test whether the assay was applicable to wort or beer samples, wort fermentations contaminated with diastatic yeast were performed. Three different wort (12°P) fermentations in duplicate were inoculated with either 1.2 x 10^7 cells/mL of diastatic *S. cerevisiae* (TPB109), non-diastatic *S. cerevisiae* (WLP023), or the control yeast (WLP023) contaminated with 120 cells/mL of diastatic TPB109 (10^{-5} contamination). Based on sugar consumption and ethanol formation, the TPB109 fermentation could be differentiated from WLP023 (Figure 6). This was particularly evident from the wort glucose concentration, which was rapidly consumed by WLP023, but present in low amounts during fermentation by the diastatic TPB109 due to glucoamylolytic activity. The contaminated fermentation could not be distinguished from the control (WLP023) fermentation from sugar and profiles alone. DNA was extracted with 'GC Prep' during early (2nd day), middle (4th and 7th day), and the end of fermentation (11th day). The samples included one of the two duplicate positive controls (TPB109), both duplicates of the contaminated fermentations (C1 and C2) and a negative control WLP023 from the last fermentation day. Sampling on 7th day failed from one of the duplicate contaminated fermentations (C1), and this was replaced with a sample on the 8th day (C1). Extracted DNA samples were pre-amplified with both PCR and LAMP. The PCR results acted as a control for the LAMP-Cas12a assay, and both the commercially used SD5A/SD6B (Yamauchi et al. 1998) and the newly designed F2/R2 PCR primers were used.

With the SD5A/SD6B primers, contamination could only be detected from day 7 onwards (Figure 7A), whereas with the F2/R2 primers, contamination could be detected in the 4th day samples (Figure 7B). With LAMP, a product could be detected with gel electrophoresis in the day 4 samples onwards (Figure 7C). However, the bands were weak in some samples and difficult to distinguish from the negative control. After incubating the LAMP products with Cas12a, and visualising results on lateral flow strips, clear positive results were obtained for all contaminated samples from day 4 onwards (Figure 7D). Contaminated samples from day 2 had slightly more intense T lines than the negative controls, indicating the potential for increased sensitivity of the assay compared to PCR. However, as the T-line could not be completely diminished from the negative controls, the C1 and C2 samples from the 2nd day could not be reliably interpreted as positive. The assay sensitivity could be further improved by optimising and purifying the reporter oligo, to completely diminish the 'hook effect', or using different lateral flow strips.

The DNA samples from the second and fourth day of fermentation were retested with the one-pot method with a lateral flow read-out. To test whether the results could be obtained more quickly or with greater sensitivity, LAMP incubation time was either decreased from 30 minutes to 20 minutes or increased to 40 minutes. The results showed a shorter 20 minute incubation time was sufficient for a positive result in samples with high amounts of diastatic yeast, such as the positive controls (Fig.7E).

However, only one of the 4th day contaminated samples (C2) was detected as positive with the shorter incubation time. Results with the 40 minute incubation time during LAMP were identical to those obtained with 30 minute incubation (data not shown), and therefore sensitivity could not be improved by increasing the incubation time.

Figure 6.

The concentrations (g/L) of (A) glucose, (B) maltose, (C) maltotriose, and (D) ethanol during fermentations in 12 °P wort. Fermentations were inoculated with *STA1*⁻ *S. cerevisiae* WLP023 (red), *STA1*⁺ *S. cerevisiae* TUM PI BA 109 (blue), or WLP023 intentionally contaminated with TUM PI BA 109 (orange; contamination level of 10^{-5}). Values are an average from two replicate fermentations and error bars represent the standard deviation.

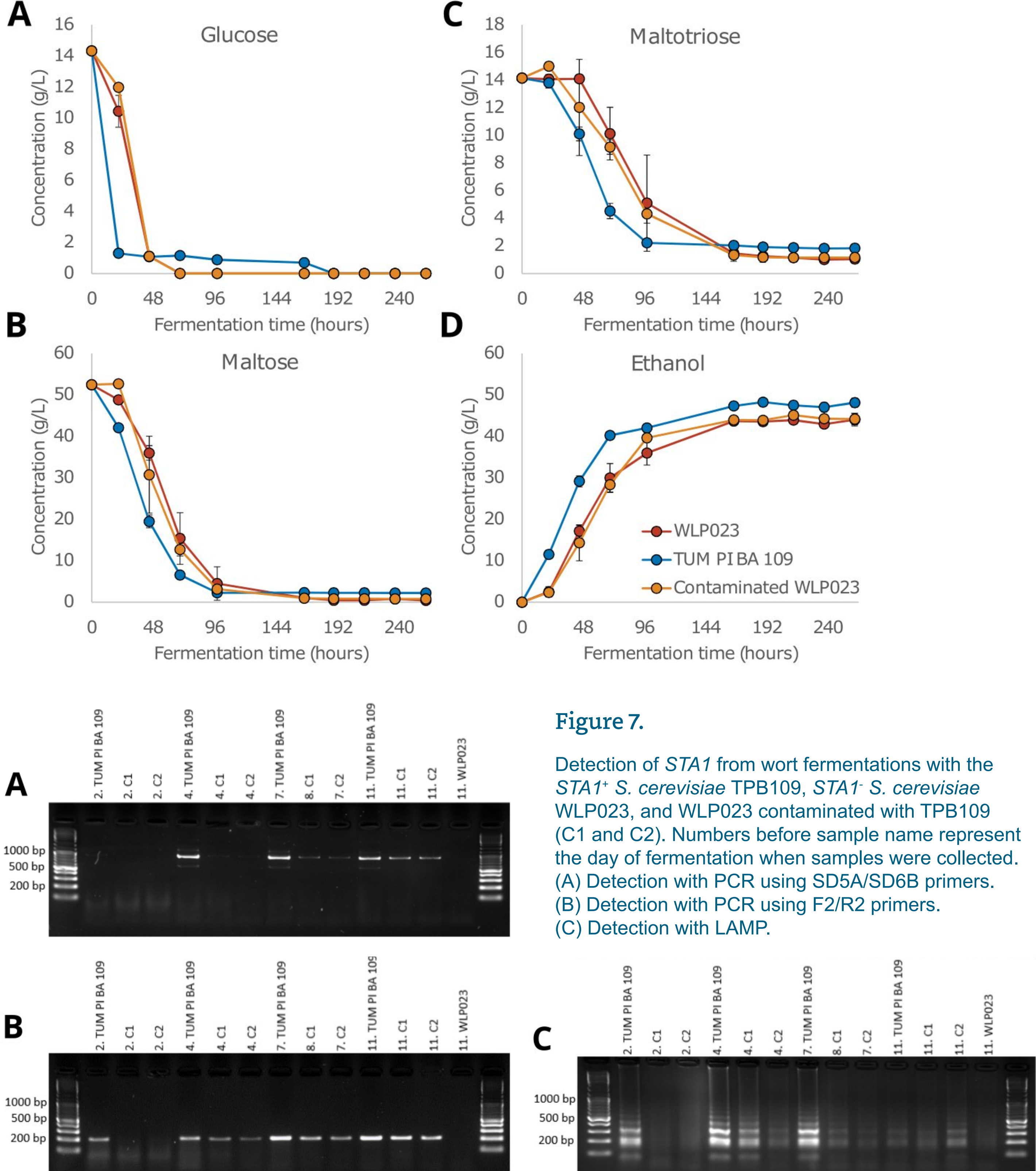
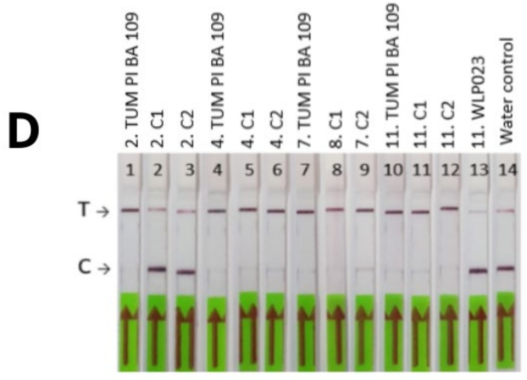


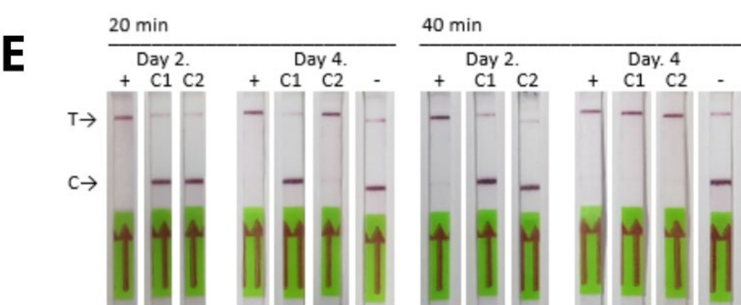
Figure 7.

Detection of *STA1* from wort fermentations with the *STA1*⁺ *S. cerevisiae* TPB109, *STA1*⁻ *S. cerevisiae* WLP023, and WLP023 contaminated with TPB109 (C1 and C2). Numbers before sample name represent the day of fermentation when samples were collected. (A) Detection with PCR using SD5A/SD6B primers. (B) Detection with PCR using F2/R2 primers. (C) Detection with LAMP.

Figure 7.

(D) Detection with Cas12a reactions with lateral flow read-out on LAMP-pre-amplified DNA.
(E) Detection with Cas12a reactions with lateral flow read-out on LAMP-pre-amplified DNA using different incubation times (20 and 40 minutes) for the LAMP reaction.





Conclusions

Diastatic *Saccharomyces cerevisiae* (var. *diastaticus*) is a common contaminating microorganism in brewery fermentations and beer, especially in smaller breweries with less stringent quality control and lack of pasteurisation. To ensure high beer quality, it is vital to enable rapid and accurate detection of these yeasts in samples, preferably without the need of expensive equipment. The objective of this study was to develop an easy and rapid detection assay for diastatic *S. cerevisiae* using basic laboratory equipment. Such an assay can be easily implemented for quality control in both microbreweries and larger-scale breweries.

Here, a rapid detection assay for diastatic *S. cerevisiae* using nucleic acid detection by CRISPR-Cas12a was successfully developed. The assay consisted of three steps: DNA extraction with 'GC Prep', isothermal pre-amplification with LAMP, and CRISPR-Cas12a based nucleic acid detection with lateral flow readout. With this assay, visually detectable results were achieved within 75 minutes from yeast samples using pipettes, pipette tips, tubes, a vortex mixer, a heat block, a lateral flow kit and reagents involved in DNA extraction and the LAMP-Cas12a assay. Compared to other molecular detection techniques, such as PCR or quantitative PCR, this assay does not need a thermal cycler, gel electrophoresis or a qPCR machine. While the new assay yields rapid results, we were unsuccessful in our aim to achieve results within one hour. However, the reactions and timing may be subject to further optimisation to shorten the assay time.

The assay could detect contamination at levels of 10^{-5} with both fluorescence and lateral flow strips with yeast suspensions in water. The assay was also evaluated on wort fermentations contaminated with diastatic *S. cerevisiae*, and with an initial loading of 10^{-5}, contamination could be detected from day 4. These detection limits were equivalent to those obtained using PCR and are similar to the report of Michel et al. (2016) where brewing samples were spiked with diastatic *S. cerevisiae* and a contamination level of 0.001% was detected with real-time PCR. Curiously, when samples from the wort fermentations were analysed, detection was more reliable with the assay reported here compared to the detection by PCR using the widely used SD-5A/SD-6B primers. Overall, this new assay can be applied for rapid detection of diastatic yeast, and will be useful for small breweries looking to implement better quality control without having well-equipped laboratories.

Author contributions

Ida Uotila: Investigation, methodology, formal analysis, writing (original draft, review and editing).

Kristoffer Krogerus: Conceptualisation, resources, methodology, formal analysis, supervision, writing (original draft, review and editing).

Acknowledgements

IU was funded by Tor-Magnus Enari Foundation, and the work was performed as a part of her MSc thesis.

Conflict of Interest

KK is employed by VTT Technical Research Centre of Finland Ltd. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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