	FOOD SAFETY LAB / MILK QUALITY IMPROVEMENT PROGRAM *Standard Operating Procedure*	

Title: ITS Yeast and Mold PCR			
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ITS Yeast and Mold PCR SOP

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SECTION 1 INTRODUCTION

1.1 Purpose

This protocol describes the standardized method to amplify a ~700 bp portion of the ITS gene for yeast and mold by PCR for sequencing and subsequent organism identification.

This SOP is derived from an earlier protocol titled "Yeast and Mold PCR SOP". The original protocol can be found in the archived section of the Food Safety Wiki.

1.2 Scope

This SOP applies to the Food Safety Lab and the Milk Quality Improvement Program. The protocols may also be used by laboratory members from other locations.

1.3 Definitions

BP: base pair
dNTPs: deoxyribonucleotide monomers, which are the building blocks of DNA. There are four dNTPs, cytosine, adenine, guanine and thymine
GoTaq Green: A PCR kit containing thermostable Taq polymerase, $MgCl_2$, and a PCR buffer which contains a green loading dye. This loading dye does not interfere with the PCR and saves the step of loading dye incorporation prior to running gel electrophoresis.
PCR: polymerase chain reaction, used to amplify a specific region within a DNA sequence

1.4 Safety

Wear gloves, safety glasses, and other appropriate personal protective equipment for the entire procedure.

SECTION 2 MATERIALS

Single Tube Reactions

- Sterile water - Room 358B and Room 350A
- Sterile 0.2 mL tubes - Room 358B and Room 350A
- ITS 4 (forward) Primer, 10 uM (5' - TCC TCC GCT TAT TGA TAT GC - 3') -Room 352C, chest freezer
- ITS 5 (reverse) Primer 10 uM (5' - GGA AGT AAA AGT CGT AAC AAG G - 3') - Room 352C, chest freezer
- GoTaq Flexi Polymerase - Room 352, "Dumb" freezer
- GoTaq Green 5x PCR Buffer - Room 352, "Dumb" freezer
- dNTP solution consisting of 1 mM each dATP, dGTP, dCTP, dTTP dNTP stocks in Room 352, "Dumber" freezer, in covered cryoblock
- $MgCl_2$, 25 mM - Room 352, "Dumb" freezer
- Vortex Mixer - Room 358B
- Miniature Centrifuge - Room 358B
- Thermocycler - Room 356
- Micropipette and sterile filter tips
- Crushed Ice

High Throughput Reactions (96-well plate)

- Sterile reservoir basin - Room 358B
- Sterile 96-well plates - Room 358B
- Multi-channel pipette
- Sterile water - Room 358B and Room 350A
- ITS 4 (forward) Primer, 10 uM (5' - TCC TCC GCT TAT TGA TAT GC - 3') -Room 352C, chest freezer
- ITS 5 (reverse) Primer 10 uM (5' - GGA AGT AAA AGT CGT AAC AAG G - 3') - Room 352C, chest freezer
- GoTaq Flexi Polymerase - Room 352, "Dumb" freezer
- GoTaq Green 5x PCR Buffer - Room 352, "Dumb" freezer
- dNTP solution consisting of 1 mM each dATP, dGTP, dCTP, dTTP. Stocks in Room 352, "Dumber" freezer, in covered cryoblock
- $MgCl_2$, 25 mM - Room 352, "Dumb" freezer
- Vortex Mixer - Room 358B
- Miniature Centrifuge - Room 358B
- Thermocycler - Room 356
- Adhesive aluminum foil lids - Room 358B
- Micropipette and sterile filter tips
- Crushed Ice

SECTION 3 PROCEDURES

3.1 Lysate Preparation

Refer to the "Preparing Cell Lysates for PCR" SOP on the Food Safety Lab wiki.

3.2 PCR Amplification

3.2.1 To prepare 200µL of the 1mM dNTP working solution:

3.2.1.1 Always make at least 200 µL to avoid pipetting 1 µL volumes, which can be inaccurate.

3.2.1.2 Thaw the individual 100mM dNTP tubes on ice.

3.2.1.3 Fill a 1.5ml tube with 192 µL of dH2O.

3.2.1.4 Transfer 2 µL of each dNTP into the 1.5mL tube and gently vortex to mix.

3.2.1.5 If only small portions of the 200µL will be used in your PCRs, aliquot this dNTP solution into 1.5ml tubes at appropriate volumes for your needs to reduce freeze thaw cycles.

3.2.1.6 This solution should be frozen at -20°C after use.

3.2.2 To prepare 100 µL of the 10uM primer working solutions:

3.2.2.1 If the primer stock has not been reconstituted, refer to the "8.1.1.1.7- Primer Ordering and Reconstitution" SOP on the Food Safety Lab wiki.

3.2.2.2 Thaw the 100µM primer stock solutions on ice.

3.2.2.3 Fill two 1.5ml tubes with 90ul of 10mM Tris-HCL(pH8.0).

3.2.2.4 Add 10 µL of your forward primer to one of the 1.5ml tubes, and 10µL of your reverse primer to the other 1.5ml tube. Label the tubes appropriately.

3.2.2.5 Gently vortex each tube to mix.

3.2.2.6 These solutions should be frozen at -20°C after use.

3.2.3 Working in Room 358B, prepare a master mix of the components listed in Table 1 by pipetting each component into a sterile 1.5 mL microcentrifuge tube.

3.2.3.1 **IMPORTANT**: Do not vortex the stock Taq polymerase solution or the mastermix after Taq is added, it will be damaged by vortexing. The Taq can be the last component added to the master mix.

3.2.3.2 Calculate master mix volumes required to include positive and negative controls and 1-2 additional reactions to compensate for pipetting errors

3.2.3.3 Store all master mix components and the prepared

master mix on crushed ice.

3.2.3.4 Vortex and centrifuge down the contents of all reagent containers before opening (except Taq)

3.2.3.5 Pipette up and down to mix after each addition and to ensure that no reagent clings to the pipette tip

Reagent	**Volume per reaction (µL)**	**Final reaction concentration**
Sterile ddH_2O	28.5	
GoTaq Green 5x PCR Buffer	10	
dNTPs, 1 mM each	5	200uM
$MgCl_2$, 25 mM	2	2mM
ITS 4 Primer 10µM	1	0.4uM
ITS 5 Primer 10µM	1	0.4uM
GoTaq Flexi Polymerase	0.5	
Total	**48.0µL**	

3.2.4 Briefly shake the tube containing the prepared master mix, but do not vortex.

3.2.5 Spin briefly in the mini centrifuge to concentrate all contents at the bottom of the tube.

3.2.6 Dispense 48 µL of prepared master mix into one well per lysate in a 96-well PCR plate or into individual 0.2 mL PCR tubes. Cover the plate loosely or cover the tubes and hold on ice.

3.2.7 Remove the plate or tubes containing the master mix from room 358B to a lab bench.

3.2.8 Briefly spin down the previously prepared lysates in a centrifuge, then add 2µL of lysate to its corresponding PCR well or tube. Pipette up and down to mix.

3.2.9 Briefly centrifuge the PCR plate or tubes to concentrate reagents at the bottom of the tube.

3.2.10 Set up the thermocycler using the parameters described in 3.2.10.1.

3.2.10.1 **Thermocycling Conditions for ITS PCR**

Time (minutes:seconds)	Temperature (°C)	Number of Cycles
5:00	95	1
1:00	95	35
1:00	56	
1:00	72	
10:00	72	1
∞	4	1

3.2.11 Promptly remove the reactions from the thermocycler when complete. PCR product can be stored at 4°C for up to 1 week or at -20°C for up to 6 months.

3.3 Gel Electrophoresis Confirmation of Amplification

Refer to the "Gel Electrophoresis of PCR Product" SOP on the Food Safety Lab wiki.

3.4 ExoSAP Purification and Sequencing Submission

Refer to the "PCR Product Purification and Sanger Sequencing Submission" SOP on the Food Safety Lab wiki.

3.5 Sequence Analysis

Refer to the "Automated Sequence Editing" SOP on the Food Safety Lab wiki.

SECTION 4 TROUBLESHOOTING

4.1 Issues with $MgCl_2$

4.1.1 $MgCl_2$ forms a concentration gradient when frozen and needs to be vortexed prior to use.

4.1.2 Every PCR reaction has an optimal $MgCl_2$ concentration range, usually between 1-4 mM. Since Mg^{+2} forms complexes with dNTPs and acts as a cofactor for polymerase you may need to try several conditions to optimize your concentration.

4.2 Too Much Enzyme

4.2.1 Excess enzyme in your PCR can lead to smearing of PCR products.

4.3 Wrong Primer Concentration

4.3.1 If you have too little primer you won't see any product.

4.3.2 Too much primer may result in primer dimerization, which may reduce amplification.

4.4 Wrong PCR Program

4.4.1 Check your program while it's cycling to make sure it is the right program.

4.5 Excess or Insufficient Template

4.5.1 Too much template can inhibit PCR by binding all of the primers. If the lysate appears very turbid, dilute the template 1:10 or 1:100 and then use 2 µL of the diluted template for the PCR.

4.5.2 Too little template and amplification may be undetectable. Make sure you correctly prepared your dirty lysate and that you took a sufficiently large sample from your culture.

4.6 Too Much dNTP or Degraded dNTP

4.6.1 Excess dNTP inhibits PCR due to MgCl2 depletion. Between 40-200 µM final concentration is optimal. dNTPs are sensitive to repeated freeze-thaw cycles. Make small aliquots (but no less than 200ul) when you get a fresh batch and turn over your stock frequently.

4.6.2 Note that the dNTP solution concentration suggested by the GoTaq Flexi manufacturer Promega suggests having each dNTP at 10mM , while this protocol uses 1mM due to less MgCl2 being used.

4.6.3 The 1mM dNTP concentration has been validated for this PCR protocol, but should not be transferred for use in other PCRs, especially if the fragment length is much longer

4.7 Suggested Positive control: FSL E2-0320 (*Penicillium decumbens*), stored in freezer 3, tower 25. Amplifies with both yeast and mold primer sets.

4.8 The **NL** gene can be amplified for PCR troubleshooting if the ITS gene fails to amplify. Use the same reagents and volumes as listed in Materials but use the NL primer set and thermocycle settings:

- NL 1 (forward) Primer, 10 uM (5' - ATA TCA ATA AGC GGA GGA AAA G -3') - Room 352C, chest freezer
- NL 4 (reverse) Primer 10 uM (5' - GGT CCG TGT TTC AAG ACG G - 3') - Room 352C, chest freezer

Thermocycling Conditions for NL PCR

Time (minutes:seconds)	Temperature (ºC)	Number of Cycles
5:00	94	1
1:00	94	36
1:00	52	
1:00	72	
10:00	72	1
∞	4	1

SECTION 5 REFERENCES

Yeast and Mold DNA

Extraction SOP

Primer Dilution SOP

dNTPs PCR Nucleotide Mix SOP

SECTION 6 METHOD VERSION & CHANGES

VERSION	DATE	EDITOR	COMMENTS
Version 1	11/12/2019	Jordan	Original SOP
Version 2	4/15/2020	Rachel E.	Formatting changes to fit new SOP format. No content was changed.