Real-Time PCR Amplification of ERCC-0095 Insert in Saccharomyces cerevisiae for Proficiency Testing and Field Competency Assessment

1. General

1.1 Intended use

1.1.2 This procedure is intended for the extraction of genomic DNA from a modified yeast strain and subsequent testing by real-time PCR assay for the detection of a unique DNA insert in *Saccharomyces cerevisiae.* This procedure is a valuable mechanism for continued evaluation and improvement of an analytical process. Using a well-characterized material, the ability to detect and report an analytical result accurately and reproducibly is challenged. This procedure may be used to build confidence in a user's ability to achieve an appropriate instrument response and demonstrate the technology is working in the hands of the user.

1.2 Precautions

1.2.1 The yeast strain used in this procedure can be safely handled in a BSL1 laboratory facility.

1.2.2 Normal laboratory safety procedures and appropriate PPE should be used.

1.3 Procedure principal

1.3.1 This procedure uses a commercially available kit for extracting high molecular weight DNA that is free of PCR inhibitors. Cells are lysed using a solution containing guanidine hydrochloride and QIAGEN protease. The DNA is purified using a silica spin column and is eluted in AE buffer. Following extraction, the DNA extract is used in a TaqMan based real-time PCR assay to determine the presence or absence of the unique target sequence.

2. Sample

2.1 Sample components

2.1.1 Microcentrifuge tubes containing varying concentrations of yeast cells in 5% ethanol

2.1.2 Samples will be shipped in a dark plastic storage box

2.2 Sample requirements

2.2.1 Each microcentrifuge tube will be clearly labeled with its contents

2.2.2 Each sample will be labeled with its preparation date

2.2.3 Referral contact (name, address and phone number of submitter)

2.3 Rejection criteria

2.3.1 Tubes that have not been properly sealed and have leaked/been exposed to contamination

2.3.2 Tubes should contain 200uL of solution. Tubes with significantly less material should be considered contaminated.

3. Materials/Vendors

3.1 Reagents

3.1.1 Ethanol, 96-100%

3.1.2 QIAamp DNA Blood Mini Kit (QIAGEN; catalog #51104/51106)

3.1.3 Custom TaqMan primer/probe set (Invitrogen Assay ID AIKAKUQ)

3.1.4 TaqMan Environmental Master Mix 2.0 (Life Technologies, 4396838)

3.1.5 Nuclease-free water

3.2 Supplies
- 3.2.1 Laboratory marking pen
- 3.2.2 Disposable gloves
- 3.2.3 Sterile 1.5 or 1.7mL microcentrifuge tubes, nuclease-free, low bind
- 3.2.4 P2 aerosol barrier (plugged) pipette tips
- 3.2.5 P20 aerosol barrier (plugged) pipette tips
- 3.2.6 P200 aerosol barrier (plugged) pipette tips
- 3.2.7 P1000 aerosol barrier (plugged) pipette tips
- 3.2.8 Real-time PCR 96-well plate
- 3.2.9 Real-time PCR clear cover

3.3 Equipment
- 3.3.1 Microcentrifuge (capable of reaching speeds of 15,000xg)
- 3.3.2 Racks for microcentrifuge tubes
- 3.3.3 Vortex mixer
- 3.3.4 P2 pipette
- 3.3.5 P20 pipette
- 3.3.6 P200 pipette
- 3.3.7 P1000 pipette
- 3.3.8 Heating block or water bath set to 56°C
- 3.3.9 Real-time PCR system (Applied Biosystems 7900 or equivalent)

4. Quality Control

4.1 Follow applicable laboratory quality guidelines.

4.2 Carefully document procedures and processes performed, as well as reagents utilized. See Appendix C.

5. Procedure

5.1 Preparation
- 5.1.1 Personnel must be familiar with this procedure.
- 5.1.2 Equipment preparation
  - a. Equilibrate heating block or water bath to 56°C±2°C.
- 5.1.3 Reagent preparation
  - a. Prepare QIAGEN buffers AW1, AW2 and protease as instructed on the bottle. This only needs to be performed for new extraction kits or reagents.
  - b. Equilibrate QIAGEN buffer AE to room temperature, if necessary.
  - c. If precipitate has formed in QIAGEN buffer AL, dissolve by incubating at 56°C.
- 5.1.4 Important Note: To avoid cross-contamination, change gloves before opening each sample and change pipette tips between each sample.

5.2 Preparation of liquid samples for DNA extraction
- 5.2.1 Bring samples to room temperature
- 5.2.2 For each sample, label the following:
  - a. Two (2) 1.5 or 1.7mL microcentrifuge tubes
  - b. Three (3) 2mL QIAGEN collection tubes (provided in kit)
  - c. One (1) QIAamp Mini Column spin column
- 5.2.3 Pulse-vortex for 15 seconds to ensure homogenous suspension.
- 5.2.4 Briefly spin tubes to push solution to bottom of tube.

5.3 Extraction of genomic DNA from liquid samples

5.3.4 Add 20uL of QIAGEN protease to 1 microcentrifuge tube for each sample.
5.3.5 Gently mix then remove 200uL of each sample and add to the corresponding tube containing protease.
5.3.6 Add 200uL of QIAGEN buffer AL to each sample
5.3.7 Pulse-vortex for 15 seconds to thoroughly mix each sample.
5.3.8 Incubate at 56°C for 60 minutes.
5.3.9 Briefly spin tubes to push solution to the bottom of tubes.
5.3.10 Add 200uL 96-100% ethanol to each sample.
5.3.11 Pulse-vortex for 15 seconds to mix.
5.3.12 Briefly spin tubes to push solution to the bottom of tubes.
5.3.13 Transfer each sample from the microcentrifuge tubes into its respectively labeled QIAamp spin column with 2-ml collection tube; do not wet the rim.
5.3.14 Close the cap and centrifuge column/collection tube assembly at 6,000 x *g* for 1 minute.
5.3.15 Transfer the QIAamp column to a clean collection tube and discard the tube containing the filtrate.
5.3.16 Carefully open the QIAamp spin column and add 500uL Buffer AW1 without wetting the rim. Close the caps.
5.3.17 Centrifuge spin columns at 6,000 x *g* for 1 minute.
5.3.18 Place each spin column in a clean 2mL collection tube. Discard the collection tube containing the filtrate.
5.3.19 Carefully open the QIAamp spin column and add 500uL Buffer AW2 without wetting the rim. Close the cap.
5.3.20 Centrifuge spin columns at maximum speed (approximately 16,000 x *g*) for 3 minutes.
5.3.21 Place each spin column in a clean 2mL collection tube. Discard the collection tube containing the filtrate.
5.3.22 Centrifuge spin columns at maximum speed for 1 minute to dry the filter. This step helps to eliminate chance of possible carryover of Buffer AW2.
5.3.23 Place each spin column in a clean 1.5mL low bind collection tube (not provided in QIAGEN kit). Discard the collection tube containing the filtrate.
5.3.24 Carefully open the QIAamp spin column and add 200uL Buffer AE. Close the cap.
5.3.25 Incubate tubes at room temperature for 5 minutes.
5.3.26 Centrifuge spin columns at 6,000 x *g* for 1 minute.
5.3.27 The filtrate in the microcentrifuge tube contains the DNA extract. Discard the QIAamp spin column.
5.3.28 Proceed to real-time PCR detection procedure or store DNA for later analysis.

5.4 Storage of DNA extract

5.4.1 If DNA will not be used immediately in qPCR assay, store at 2-8°C for up to 7 days. If DNA will not be used in this time, store aliquots at ≤-20°C for longer periods.

6. Detection of target sequence using real-time PCR

6.1 Preparation

6.1.1 Turn real-time PCR system on to allow laser to warm up prior to use.
6.1.2 Label one microcentrifuge for preparing the mastermix.
6.1.3 Set up assay protocol on real-time PCR system and save (see Appendix A)
6.1.4 Wash hands to prevent carry-over contamination from real-time thermocycler

6.1.5 Remove mastermix reagents from the refrigerator
6.1.6 Prepare mastermix according to chart in Appendix B (in tube labeled mastermix).
6.1.7 Aliquot reactions into 96-well plate (16uL per well) (See Appendix B)
6.1.8 Add 4 uL sterile molecular grade water to the no template control well (See Appendix B)
6.1.9 Add 4 uL genomic DNA to the appropriate wells (see Appendix B)
6.1.10 Cover real-time PCR plate with clear film
6.1.11 Run real-time PCR assay

7. Interpretation/Results

7.1 Results will be reported as a C_t value, reported by the software
7.1.1 A C_t value of 37 or below will constitute a positive response
7.1.2 A C_t value above 37 will constitute a negative response
7.1.3 A reading of "undetermined" will constitute a negative response

8. Reporting/Action

8.1 Document any deviations from this procedure.
8.2 Report C_t value along with sample name to Lindsay Vang or Jayne Morrow
8.3 Export raw data to a compatible document type (CSV or Excel) and send to Lindsay Vang or Jayne Morrow.
8.4 See Appendix C for reporting format.

9. Performance characteristics

9.1 This method has been evaluated for the DNA extraction and detection of *Saccharomyces cerevisiae* with a novel insert and has been found to be acceptable.

10. Limitations

10.1 Cross contamination may occur if gloves are not changed between samples.
10.2 Extracted DNA may degrade if stored longer than 1 week at 2-8°C.

11. Procedure Notes

11.1 Send comments and questions regarding this procedure to lindsay.vang@nist.gov or jayne.morrow@nist.gov
11.2 Definitions and abbreviations
11.2.1 DNA – deoxyribonucleic acid
11.2.2 PCR – polymerase chain reaction
11.2.3 BSL1 – biosafety level 1
11.2.4 PPE – personal protective equipment
11.2.5 NTC – no template control
11.2.6 C_t – threshold cycle

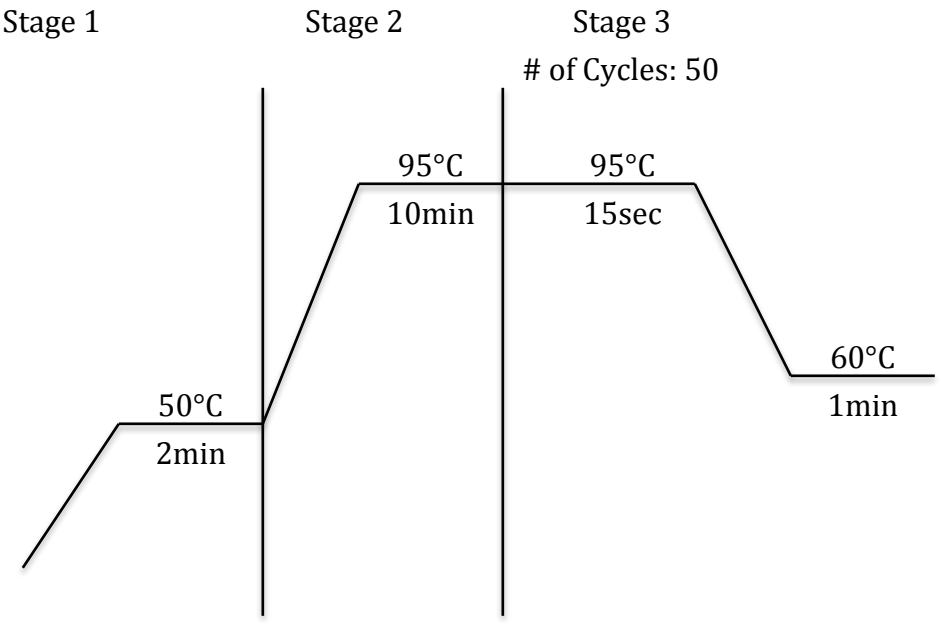
12. References/suggested reading

12.1 QIAamp DNA Mini Kit and QIAamp DNA Blood Mini Kit Handbook, QIAGEN, November, 2007

Appendix A – Real-time PCR assay setup

Assay parameters should be set up as follows:

- Standard curve (Absolute Quantification)
- 96-well clear plate
- Blank template
- Reaction volume – 20uL



Appendix B – qPCR assay template for assay preparation

Reagents	Recipe	Cocktail (9X)
Water	4uL	36uL
Master Mix	10uL	90uL
Primer	1uL	9uL
Template	4uL	N/A

qPCR assay description:
Date:
Assay/ Primers:
Samples/Plate Layout: This is an example layout of a qPCR plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C		A1	B1	C1	D1	E1	F1	G1	H1	I1	J1	NTC
D		A2	B2	C2	D2	E2	F2	G2	H2	I2	J2	NTC
E		A3	B3	C3	D3	E3	F3	G3	H3	I3	J3	NTC
F												
G												
H												

Procedure:

1. Add components of mastermix to a 1.5 or 1.7mL microcentrifuge tube
2. Pulse-vortex for 15 seconds to ensure components are properly mixed
3. Aliquot 16uL of mastermix into the appropriate wells
4. Add 4uL sterile water to the NTC wells
5. Add 4uL genomic DNA to the appropriate wells
6. Cover real-time PCR plate with clear film

Run real-time PCR assay

Appendix C – Data Reporting Sheet

Date sample received:
Sample processed by:
Date sample extracted:
Storage conditions prior to extraction:
Date sample run with qPCR:
Storage conditions prior to qPCR:
qPCR system used:
Lot number of primer/probe mix:
Lot number of Master Mix:
Lot number of QIAamp kit:

Initial evaluation:

Was the sample item received in a satisfactory condition? □ YES □ NO
If no, please explain why the sample was unsatisfactory.
Was the sample item tested according to the provided SOP? □ YES □ NO
If no, please explain where changes were made.
Was the qPCR assay set up the same as described in the SOP? □ YES □ NO
If no, please explain where changes were made.

Plate Layout: Please show how samples were loaded into your plate

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												

H												

Results: Please give the C_T values for each well that contained sample/NTC

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Following qPCR:

- If you are using an ABI platform/software please send your .sds file to nathanael.olson@nist.gov.
- If you are using another platform/software, please contact nathanael.olson@nist.gov to determine the best way to export the raw data to send to him.