PRCs to test Quadruple sigma factor Mutant

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Authored by: Barbara Bowen
Last Modified on: Date
Approved by: Martin Wiedmann

EFFECTIVE DATE: Date of Approval

APPROVED BY:

_____ _____
Dr. Martin Wiedmann (date)

AUTHORED BY:

_____ _____
(Name) (date)

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SECTION 1 INTRODUCTION

1.1 Purpose

The purpose of this document is to set forth **standard** guidelines for checking quadruple mutant sigma factor C3-0135 to be sure it possesses deletions in all four sigma factors.

1.2 Scope

This SOP applies to the Food Safety Lab, including the Laboratory for Food Microbiology and Pathogenesis of Foodborne Diseases.

1.3 Definitions

1.4 Safety

C3-0135 is a *Listeria monocytogenes* 10403S strain. *L. monocytogenes* is a BSL-2 pathogen. Appropriate protective measures need to be taken when working with *L. monocytogenes*, including wearing gloves and labcoat. Benchtops need to be cleaned with appropriate cleaning solutions (10% bleach; 70% ethanol; Lysol) before and after performing procedures. All waste from these experiments needs to be treated as BSL-2 waste.

SECTION 2 MATERIALS

- **Primers:**

Primer Name	Sequence
LM sigB-18	GGGTATAGTACTTCGAATCG A should be T
LM sigB-19	CGAATATTACCCATACCAG

Primer Name	Sequence
SC17 sigL CHK_F	ACCAATGATGGCGTGGCTG
SC18 sigL CHK_R	GACCCACTTAGTCATCTGAC

Primer Name	Sequence
SC15 sigH CHK_F	GATAAGCTTGTTGAATGGATG
SC16 sigH CHK_R	TCCTAGCACTACACGATAAG

SigC = lmo0423

Primer Name	Sequence
SC13 lmo0423 CHK_F2	TCGGGATGCTGACATGCGC
SC14 lmo0423 CHK_R	AACTCATAAAGAAGCCTCCTC

- **Polymerase and buffers, stock dNTPs.**
- **DNA preps of each strain to be tested, plus control DNAs.**
- **Positive controls**
  Single mutants, if used for controls in addition to 10403S, are as follows:
  ΔsigB = A1-0254
  ΔsigL = B2-0124
  ΔsigC = C3-0113
  ΔsigH = C3-0123
  The quadruple mutant is C3-0135.

  They are located in the mutant tower of Freezer 1, Tower 112, Box 1.

SECTION 3 PROCEDURES

3.1. PCR for sigB mutant or wild type

1) Begin with a fresh DNA prep for template. A lysate can be used, however best results are derived with DNA.

2) Please note that there is a single base mismatch in LmSigB18 according to the Broad Institute 10403S sequence (incorrect base highlighted in sequence), nonetheless, the primer pair works well. These primer sets were designed years ago.

3) The following PCR was set up for Q5 polymerase (NEB). If you use another Taq, you may have to recalculate the Tm of your primers (for help, see IDTDNA's OligoAnalyzer tool section https://www.idtdna.com/analyzer/Applications/OligoAnalyzer/).

4) The recommended positive controls are: 10403S wild type and the corresponding single mutant for each (see Section 2 for list).

Rxn	Sample (for example)	Primers
1	Your DNA	LmSigB18 and 19
2	Negative control	
3	Positive control	

SET UP REAGENTS	µl per reaction	Cocktail for x # rxns
diH2O	32.75	
5 X Q5 Buffer	10	
(MgCl2 is included in buffer)	---	
dNTP (10 mM)	1	
Primer F: 10 µM LmsigB18	2.5	
Primer R: 10 µM LmsigB19	2.5	
Polymerase: NEB Q5	0.25	

Template: 1µl template DNA 10 – 100 ng (use the lesser amount if sample has been RNAased, the higher if it has not); see also NEB's instructions for suggested template amount with Q5 DNA Polymerase**.**

Total volume per reaction: 50 µl

PCR in (name of thermal cycler)	
Initial hold	98°C 30 sec.
35 cycles	98°C 10 sec, 57°C 20 sec., 72°C 30 sec.
Final extension	72°C, 2 min.
Final hold	4°C ∞

5) Run the PCR amplicons on a 1.5% agarose gel with pGEM markers. The wild type band will run at 1760 bp. The mutant will be some 300 bp smaller.

3.2 PCR for sigH to determine mutant from wild type.

1) Perform the following PCR

Rxn	Sample (for example)	Primers
1	Your DNA	**SC15 sigH CHK_F & SC16 sigH CHK_R**
2	Negative control	
3	Positive control	

SET UP REAGENTS	µl per reaction	Cocktail for x # rxns
diH2O	32.75	
5 X Q5 Buffer	10	
(MgCl2 is included in buffer)	---	
dNTP (10 mM)	1	
Primer F: 10 µM sc15sigHF	2.5	
Primer R: 10 µM sc16sigHR	2.5	
Polymerase: NEB Q5	0.25	

Template: 1µl template DNA 10 – 100 ng (use the lesser amount if sample has been RNAased, the higher if it has not); see also NEB's instructions for suggested template amount with Q5 DNA Polymerase**.**

Total volume per reaction: 50 µl

PCR in (name of thermal cycler)	
Initial hold	98°C 30 sec.
30 cycles	98°C 10 sec, 60°C 20 sec., 72°C 30 sec.
Final extension	72°C, 2 min.
Final hold	4°C ∞

2) Run the PCR amplicons on a 1.5% agarose gel with pGEM markers. The wild type band will run at 1566 bp and the mutant at 1220 base pair.

3.3 PCR for SigL

Repeat the above, using SC17 sigL CHK_F and SC18 sigL CHK_R primers, and the following PCR conditions.

PCR in (name of thermal cycler)	
Initial hold	98°C 30 sec.
30 cycles	98°C 10 sec, 62°C 20 sec., 72°C 40 sec.
Final extension	72°C, 2 min.
Final hold	4°C ∞

When you run the PCR amplicon on a gel, expect a band of 2150 bp for the wild type, and 1450 for the mutant.

3.4 PCR for SigC (also called SigECF or lmo0423)

Repeat the above, using sc13 lmo0423 CHK_F2 and sc14 lmo0423_R primers, and the following PCR conditions.

PCR in (name of thermal cycler)	
Initial hold	98°C 30 sec.
30 cycles	98°C 10 sec, 62°C 20 sec., 72°C 30 sec.
Final extension	72°C, 2 min.
Final hold	4°C ∞

When you run the PCR amplicons on a gel, expect a band of 1380 bp for wild type, and 872 for mutant.

Quick Reference Table

mutant	Primers	PCR conditions (for Q5 polymerase)	Size of amplicons In base pairs
sigB	LmsigB 18 and LmsigB 19	98°C 30”initial denat. 98°C 10”,57° 20”,72° 30” (35 cycles) 72°C 2’ final hold	Wt: 1760 Mutant: 1463
sigC (ECF or lmo 0423)	Sc13 lmo0423 CHK_F2 sc14 lmo0423 CHK_R	98°C 30”initial denat. 98°C 10”,62° 20”,72° 30” (35 cycles) 72°C 2’ final hold	Wt: 1380 Mutant: 872
sigH	sc15 sigH CHK_F and sc16 sigH CHK_R	98°C 30”initial denat. 98°C 10”,60° 20”,72° 30” (35 cycles) 72°C 2’ final hold	Wt: 1566 Mutant: 1220
sigL	Sc17 sigL CHK_F and Sc18 sigL CHK_R	98°C 30”initial denat. 98°C 10”,62° 20”,72° 40” (35 cycles) 72°C 2’ final hold	Wt: 2150 Mutant: 1423

SECTION 4 TROUBLESHOOTING

Problems previously encountered with this assay include:

(1) Difficulty getting robust bands from dirty lysates. If this happens, take the time to make DNA preps. Streak a fresh plate from -80°C stocks and use a single colony.
(2) The usual PCR problems. Please look over the suggested reading of Current Protocols: Polymerase Chain Reaction 15.0.1-15.1.13. This can be found in room 260.

SECTION 5 REFERENCES

NEB Q5 Polymerase Protocol: https://www.neb.com/protocols/2013/12/13/pcr-using-q5-high-fidelity-dna-polymerase-m0491