The Fleischman Lab

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Title: Colony PCR – Jak2^{V617F} Screening

Introduction

This protocol is optimized for small amounts of DNA isolated from colonies grown in methylcellulose. For best results, pick colonies into 100µl of nuclease-free water and incubate in the thermal cycler at 97°C for 15 minutes with heated lid to lyse cells. Store plates at -20°C.

Materials

1. Nuclease-free water
2. Dream*Taq* green DNA polymerase and 10x PCR buffer
3. dNTPs (10mM)
4. First primer set (10mM):
   Forward: 5'-GGGTTTCCTCAGAACGTTGA-3'
   Reverse: 5'-TCATTCGTTTCCTTTTTCACAA-3'
5. Second primer set (10mM):
   Forward: 5'-ACACCTAGCTGTGATCCTGAAACT-3'
   Reverse: 5'-CATATAAAGGGACCAAAGCACATT-3'
6. 96-well plates and seals or PCR tube strips
7. Reagent reservoirs
8. BsaXI and CutSmart buffer (New England BioLabs)
9. Thermal cycler
10. 2% DNA gel and low-range DNA ladder (25-700bp)

Protocol

A. First PCR

		Notes
1.	Allow colony plate to thaw completely. Prepare master mix as follows:	*Be sure to make enough master mix for at least 1 additional well. For a full 96-well plate, make enough master mix for 110 wells and mix in a reagent reservoir.*

Reagent	1 well
10x PCR buffer	2.5 µl
dNTP mix (10 mM)	0.5 µl
Jak2 exon 12 primer mix (F+R) (10 mM)	1 µl
Nuclease-free H_2O	18.8 µl
DreamTaq DNA polymerase	0.2 µl
DNA	2 µl

2.	Transfer 23µl master mix to each well or tube. Add 2µl DNA per well. Seal plate well and briefly centrifuge in plate spinner.	*Always balance plate spinner. For tube strips, spin down using the tube strip adapter for the minicentrifuge .*
3.	Place plate into thermal cycler and run on the following program:	*For tube strips, be sure to use the green tube support insert.*

Lid temp: 105°C

1x	95°C	5 min
34x	95°C	30 sec
	58°C	30 sec
	72°C	40 sec
1x	72°C	5 min

B. Second (nested) PCR

1. Dilute product from first PCR with 75µl nuclease-free water per well.

2. Prepare master mix as follows:

Reagent	1 well
10x PCR buffer	2.5µl
dNTP mix (10mM)	0.5µl
Jak2 nested primer mix (F+R) (10mM)	1µl
Nuclease-free H_2O	18.8µl
DreamTaq DNA polymerase	0.2µl
DNA	2µl

Be sure to make enough master mix for at least 1 additional well. For a full 96-well plate, make enough master mix for 110 wells and mix in a reagent reservoir.

3. Transfer 23µl master mix to each well or tube. Add 2µl of the diluted product from the first PCR reaction. Seal plate well and briefly centrifuge in plate spinner.

Always balance plate spinner. For tube strips, spin down using the tube strip adapter for the minicentrifuge .

4. Place plate into thermal cycler and run on the following program:

Lid temp: 105°C

1x	95°C	5 min
21x	95°C	30 sec
	58°C	30 sec
	72°C	40 sec
1x	72°C	5 min

For tube strips, be sure to use the green tube support insert.

5. Run 10µl of the final PCR product on a 2% agarose gel to ensure there is product before proceeding to restriction digest step.

Each small gel is about 50 ml. Prepare 100ml 2% agarose by dissolving 2g agarose into 100ml TAE and microwaving. Add 7µl EtBR to gel.

C. Restriction Digest

1. Prepare master mix as follows:

Reagent	1 well
CutSmart Buffer	2.5µl
BsaXI	0.4µl
Nuclease-free H_2O	7.1µl
DNA	15µl

2. Transfer 10µl master mix to each well or tube. Add 15µl of the final PCR product. Seal plate well and briefly centrifuge in plate spinner.

Always balance plate spinner. For tube strips, spin down using the tube strip adapter for the minicentrifuge .

3. Place plate into thermal cycler and incubate at 37°C for 2 hours with heated lid on.

Prepare gels during this step. Each large gel uses 2 combs and is about 200ml. Use 12µl EtBR for each gel.

4. Run digested product on a 2% agarose gel alongside 8µl of low-range DNA ladder.
Homozygous mutants have a single band at 350bp.
Heterozygous mutants have a strong band at 350bp and weaker bands at 250bp and 100bp.
Wild types have strong bands at 250bp and 100bp, with a weak band at 350bp.