### Supplementary protocol 2 - Inverse PCR individual inserts

There is a very nice and comprehensive protocol that covers how to map Mos1 insertions by Boulin & Bessereau (2007) in *Nature Protocols*. This protocol is meant as a complement to their protocol because we changed and optimized several parameters which in our hands improve the reliability of inverse PCR reactions. It is the protocol that we currently (December 2012) use in the lab. Please see www.wormbuilder.org for the latest updates to protocols or vectors.

**Use aerosol resistant tips for all steps!! Contamination is a real problem when doing two sequential PCR reactions on small amounts of template. And it only gets worse with every reaction you do.**

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#### Reagents

Molecular Biology Reagents

Genomic DNA isolation kit from Zymo Research. Catalog # D6016

Ligase from Enzymatics: Catalog # L6030-LC-L

DpnII from NEB: Catalog # R0543L

Phusion DNA Polymerase: Catalog #M0530S

Oligos sequences (5’ → 3’ )

**5’ end**

oCF1587 ATAGTTTGGCGCGAATTGAG

oCF1588 GGTGGTTCGACAGTCAAGGT

oCF1589 AGAGCAAACGCGGACAGTAT

oCF1590 CGATAAATATTTACGTTTGCGAGAC

**3’ end**

oCF1591 AAAAATGGCTCGATGAATGG

oCF1592 TAAGAATCGAAGCGCTGCTC

oCF1593 AGCTAGCGACGGCAAATACT

oCF1594 CATCGAAGCGAATAGGTGGT

### Protocol

#### 1. Isolate genomic DNA

We use the kit from Zymo Research but any method that generates genomic DNA should give similar results. Follow manufacturer’s protocol.

*The protocol can work but not as efficiently on crude genomic DNA lysates generated with freezing and proteinase K digest. It’s much easier to get a good inverse PCR product with decent quality DNA.*

#### 2. Digest 150 ng of genomic DNA in 25 ul volume for 3 hours.

Digest genomic DNA with the DpnII enzyme.

*DpnII cuts the same sequence as MboI but is slightly cheaper and works better over extended digests. It’s important to use the DpnII buffer because there is a lot of star activity in the regular NEB buffers. In our hands, DpnII and MboI work well possibly because the enzymes leave a 4 bp overhang after cutting compared to the often 1 bp or blunt ends that most four-cutter enzymes leave. The protocol also works with HpaII - adjust digest conditions.*

Component 1x

DNA sample (150 ng - add water to 10ul) 10 ul

Restriction buffer DpnII (10x) 2.5 ul

Restriction enzyme (DpnII 10U/ul) 1.0 ul

H20 11.5 ul

Reaction conditions: Digest at 37ºC for three hours to overnight.

Heat inactivate the enzyme after restriction diges at 80ºC for 20 min.

#### 3. Ligate the digested DNA for 2 hours at room temperature

Set up ligation in large volume to favor intra-molecular reactions. Use the 10x ligation buffer from Enzymatics.

Set up 25 ul reactions with:

Component 1x

Digested DNA from step 2 2.5 ul

10x ligation buffer 2.5 ul (Enzymatics ligase buffer)

T4 ligase 1.0 ul (Enzymatics ligase)

H20 19.0 ul

The ligation reactions can be frozen indefinitely before proceeding to the next step.

#### 4. Do first round of inverse PCR

Set up a **10** ul PCR reaction with the following components:

Component 1x

Ligation mix from step 3 2.0 ul

Primer oCF1587 (10 uM) 1.0 ul

Primer oCF1588 (10 uM) 1.0 ul

dNTPs (10 mM) 0.2 ul

Phusion 5x GC buffer 2.0 ul

**NEB Phusion Polymerase** 0.1 ul

H20 3.7 ul

Make master mix of PCR ingredients and add “ligation mix” individually to each tube. It is very difficult (read = impossible) to accurately pipette only 0.2 ul and 0.1 ul.

PCR settings:

Initial denaturation: 2 minutes @ 98C

PCR cycles: 30x

Annealing temperature: **64ºC**

Elongation time: 1 min

*If you use another polymerase than the Phusion polymerase, you will probably want use the appropriate PCR buffer and decrease the annealing temperature to 60C. The higher temperature works well for getting specific bands.*

#### 5. Second round of inverse PCR.

Dilute the first round of PCR product 100 fold. Transfer 1 ul of PCR product to new PCR tube, add 99 ul of destilled water. Mix with vortexer. Spin down to avoid contamination.

Set up a 25 ul PCR reaction with the following components:

Component 1x (20ul)

PCR from step 4 1.0 ul

Primer oCF1589 (10uM) 2.5 ul

Primer oCF1590 (10uM) 2.5 ul

dNTPs (10 mM) 0.5 ul

Phusion 5x GC buffer 5.0 ul

**NEB phusion polymerase** 0.2 ul

H20 13.0 ul

PCR settings:

Initial denaturation: 2 minutes @ 98C

PCR cycles: 30x

Annealing temperature: **64ºC**

Elongation time: 1 min

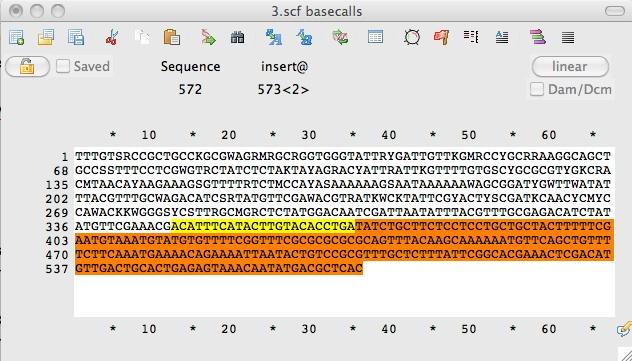
*If you use another polymerase than the Phusion polymerase, you will probably want use the appropriate PCR buffer and decrease the annealing temperature to 60C.*

#### 6. Run the PCR products on a 1% agarose gel, excise clear bands from gel and gel purify.

Only excise one band from each reaction. Do not excise bands that are not clearly distinct or when there is a smear. The sequence read will come back garbled. Only excise bands that are larger than 100bp. Send the gel purified product for sequencing with oCF1590.

*Alternatively, you can run only 10 ul of the PCR reaction to determine if the band is specific. If there is only a single band, we use the ExoSAP protocol (ExonucleaseI digest to remove oligos and Shrimp Alkaline Phosphatase removal of dNTPs) to purify the PCR reaction and submit for sequencing.*

#### 7. Determine insertion site

Once you get the sequence read back, you can determine the insertion site. Search the sequence read for the following sequence: ACATTTCATACTTGTACACCTGA. Allow for two mismatches to accommodate poor sequence calls. This is the end of the Mos1 transposon (in yellow below). The next two nucleotides should be a “TA”, where the Mos1 transpson inserted. The rest of the read is the genomic DNA insertion site (in orange below).

A) Go to wormbase and blast search.

Change “Query Type” to Nucleotide.

Change “E-value Threshold” to 1E-4

Unclick “Filter”

B) Identify the correct match to your insertion site. Typically it will be the best match but make sure the query match starts at position “1”. Otherwise the read is probably finding part of the *unc-119* rescue gene or the transgene you put in. Some insertions cannot be mapped to unique locations because of repetitive regions in the genome or too short reads.

#### 8. No bands?

Redo the PCR reactions with oligos that anneal at the other end of the transposon. Start with the ligated DNA from step3.

**Do first round of inverse PCR**

Set up a **10** ul PCR reaction with the following components:

Component 1x

Ligation mix from step 3 2.0 ul

Primer oCF1591 (10 uM) 1.0 ul

Primer oCF1592 (10 uM) 1.0 ul

dNTPs (10 mM) 0.2 ul

Phusion 5x GC buffer 2.0 ul

**NEB phusion polymerase** 0.1 ul

H20 3.7 ul

Make master mix of PCR ingredients and add “ligation mix” individually to each tube. It is very difficult (read = impossible) to accurately pipette only 0.2 ul and 0.1 ul.

PCR settings:

Initial denaturation: 2 minutes @ 98C

PCR cycles: 30x

Annealing temperature: **62ºC**

Elongation time: 1 min

**Second round of inverse PCR.**

Dilute the first round of PCR product 100 fold. Transfer 1 ul of PCR product to new PCR tube, add 99 ul of destilled water. Mix with vortexer. Spin down, so you don’t get contamination.

Set up a 25 ul PCR reaction with the following components:

Component 1x (20ul)

PCR from step 4 1.0 ul

Primer oCF1593 (10uM) 2.5 ul

Primer oCF1594(10uM) 2.5 ul

dNTPs (10 mM) 0.5 ul

Phusion 5x GC buffer 5.0 ul

**NEB phusion polymerase** 0.2 ul

H20 13.0 ul

PCR settings:

Initial denaturation: 2 minutes @ 98C

PCR cycles: 30x

Annealing temperature: **62ºC**

Elongation time: 1 min

Sequence the PCR product with oCF1593.

#### 9. Still no bands?

Repeat protocol with another restriction enzyme, for example HpaII.

References:

Boulin T & Bessereau JL. (2007). Mos1-mediated insertional mutagenesis in Caenorhabditis elegans. Nature Protocols.