JMJ-Group – Microbiology – BMB – SDU

Title: Primer design for gene Knock Out (KO) in pathogenic *E.coli* **SOP number:** SOP00XX_v01 **Version number:** 01	**Date issued:** 2013.12.03 **Review date:** 2014.12.03 **Written by:** Tina Kronborg

1. Purpose

To knock out specific genes in pathogenic *E. coli*

2. Area of application

This procedure is valid for some pathogenic *E. coli* strains (AIEC, UPEC, ETEC)

3. Apparatus and equipment

Program	Web add	Used for
ECOCYC	http://ecocyc.org/	Search for the gene of interest
Primer3	http://bioinfo.ut.ee/primer3-0.4.0/	Primer design
Bioinformatics organization Sequence Manipulation Suite (SMS) Reverse Complement	http://www.bioinformatics.org/sms/rev_comp.html or http://www.bioinformatics.org/sms2/rev_comp.html new version	Reverse and complementary the sequence

4. Materials and reagents – their shelf life and risk labelling

Name	Components (Concentrations)	Manufacturer/Cat. #	Room	Safety considerations
pKD3 fw primer (cml)	TTATACGCAAGGCGACAAGG	JMJ99 Wanner C1	V18-405a-2	20 pMol
pKD3 rev primer (cml)	GATCTTCCGTCACAGGTAGG	JMJ100 Wanner C2	V18-405a-2	20 pMol
pKD4 fw primer (kan)	CGGTGCCCTGAATGAACTGC	JMJ71 Wanner K1	V18-405a-2	20 pMol
pKD4 rev primer (kan)	CGGTGCCCTGAATGAACTGC	JMJ72 Wanner K2	V18-405a-2	20 pMol
KO Reverse primer	Made specific to the template, to KO the gene	Order at Sigma		Around 90 bp incl pKD3/4 fw primer
KO Forward primer	Made specific to the template, to KO the gene	Order at Sigma		Around 90 bp incl pKD3/4 rev primer
Primer **Up**stream of the gene in question	To check if the gene are KO	Order at Sigma		Around 23 bp
Primer **Down**stream of the gene in question	To check if the gene are KO	Order at Sigma		Around 23 bp

5. QC – Quality Control

pKD3 has Cml cassette is 1100 bp

pKD4 has Kan cassette is 1400 bp

Be aware of the size of the cassettes contra the gene in question, if they are the same size use one of the following primers together with the Up or Down primer for the gene in question

For pKD3:

JMJ99 C1 oligo, Wanner (fw)

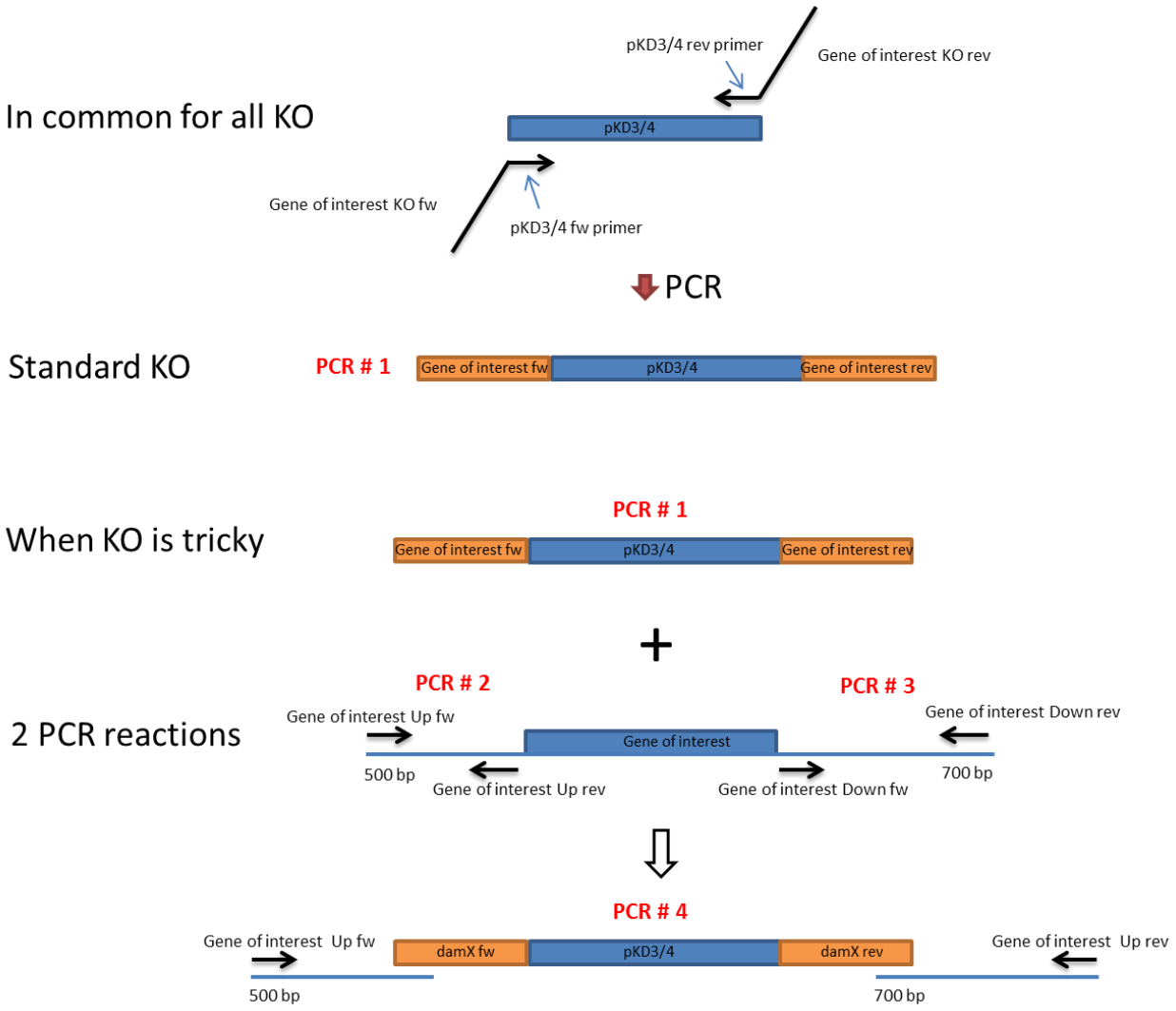
JMJ100 C2 oligo, Wanner (rev)

For pKD4:

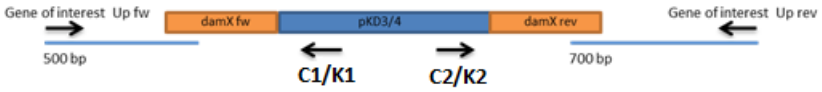
JMJ71 K1 oligo, Wanner (fw)

JMJ72 K2 oligo, Wanner (rev)

If the gene I KO there will be a product with the listing primers



Be aware when checking inserts!!!
C1 and K1 are reverse primers whereas C2 and K2 are forward primers!!



6. List of other SOPs relevant to this SOP

JMJ_SOP0006_v01_MM_Agarose_gel_DNA

JMJ_SOP0001_v01_MM_ON_culture_of_E.coli

JMJ_SOP0011_v01_TK_Colony_PCR_with_MyTaq

JMJ_SOP0010_v01_TK_Electroporation_of_pathogenic_E.coli_pKD46

JMJ_SOP0013_v01_TK_LA_plates_with_antibiotic

JMJ_SOP0004_v01_MM_Bacterial_freezing_stock

7. Environmental conditions required

8. Procedure

8.1 **Up and down primers, used to check for KO of the gene**:
8.2 Find the gene of interest in: http://ecocyc.org/
8.3 Change the organism to fx. UTI89
8.4 Use Chromosome 1
8.5 Type the gene name and click on the gene and chose Nucleotide Sequence, Advanced
8.6 Type 400 bp upstream and 400 bp downstream and show sequence
8.7 Copy the sequence to http://bioinfo.ut.ee/primer3-0.4.0/
8.8 Chose primer size at 23, Tm at 62 °C and the Product Size Ranges according to gene size, be sure that the primers start outside the gene.
8.9 The boxes with Pick left primer, or use left primer below: and Pick right primer, or use right primer below (5' to 3' on opposite strand) shall have a mark.
8.10 Push Pick Primers.
8.11 Primers for pKD3 and pKD4 used to KO the gene with:
8.12 pKD3/4 fw primer : GTGTAGGCTGGAGCTGCTTC
8.13 pKD3/4 rev primer: CATATGAATATCCTCCTTA
8.14 Find the gene of interest again.
8.15 Type 100 bp upstream and 100 bp downstream and show sequence
8.16 From the start (fw primer) and stop (rev primer) codon you pick 6 codons and then go backwards until 70 bp in all
8.17 Use: http://www.bioinformatics.org/sms/rev_comp.html to make the rev primer sequence reverse and complementary
8.18 Pair the sequences with pKD3/4 fw and rev primer respectively
8.19 **For difficult genes**:
8.20 1st set of primers – Up primers:
8.21 Follow 8.1 – 8.5
8.22 Type 500 bp upstream and show sequence
8.23 Copy the sequence from the 500 bp extra and into the start codon and paste it into Primer 3. It is important that the region include the sequence from the gene of interest fw primer
8.24 Chose a product size range from 470-480, primer size on 22 and pick primers
8.25 2nd set of primers – Down primers:
8.26 Follow 8.1 – 8.5
8.27 Type 700 bp downstream and show sequence
8.28 Copy the sequence from the 700 bp extra and into the stop codon and paste it into Primer 3. It is important that the region include the sequence from the gene of interest rev primer
8.29 Chose a product size range from 670-680, primer size on 22 and pick primers

9. Waste handling

Not required

Chemical name	Concentration	Type of waste (C, Z…)	Remarks

10. Time consumption

- Total-time 2 hours
- Hands-on-time 1 hour

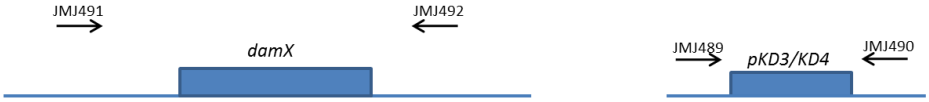
11. Scheme of development

Date / Initials	Version No.	Description of changes
14.01.15 / TK	01	The SOP has been written

12. Appendixes

Procedure with an example:

Primer design for KO



Knock out of damX
damX 1287 bp
JMJ489 KOfw
JMJ490 KOrev
JMJ491 up
JMJ492 down

PCR product sizes: 2174
JMJ489 + JMJ490 = 1100/1400bp depending on pKD3/pKD4 template

Find the gene of interest in:

http://ecocyc.org/

Change the organism to fx. UTI89

Use Chromosome 1

Type the gene name and click on the gene and chose Nucleotide Sequence, Advanced

Type 400 bp upstream and 400 bp downstream and show sequence

Copy the sequence to http://bioinfo.ut.ee/primer3-0.4.0/

Chose primer size at 23, Tm at 62 °C and the Product Size Ranges according to gene size, be sure that the primers start outside the gene.

The boxes with Pick left primer, or use left primer below: and Pick right primer, or use right primer below (5’ to 3’ on opposite strand) shall have a mark.

Push Pick Primers.

Ex. damX:

Nucleotide sequence:

```
>gnl|ECOL364106|GHPQ-3849 damX "GHPQ-3849-MONOMER"
(complement(3769974..3768688)) Escherichia coli UTI89 Chromosome 1

atgGATGAAT TCAAACCAGA AGACGAGCTG AAACCCGATC CCAGCGATCG TCGTACTGGT
CGTTCTCGTC AATCTTCTGA ACGTTCTGAG CGTACTGAAC GTGGCGAACC GCAGATCAAT
TTTGATGATA TTGAACTTGA TGACACTGAC GATCGCCGTC CGACTCGTGC GCAAAAAGCG
CGTAATGAGG AACCGGAAAT CGAAGAAGAA ATTGACGAAT CCGAAGATGA AACCGTGGAT
GAAGAGCGCG TAGAGCGTCG TCCGCGTAAG CGCAAAAAAG CAGCCAGTAA ACCCGCTTCT
CGTCAGTATA TGATGATGGG TGTCGGCATT CTGGTTCTAC TGCTGTTGAT CATCGGTATC
GGTTCTGCGC TAAAAGCCCC CTCGACCTCT TCCAGCGATC AAACCGCGTC TGGCGAGAAG
AGTATTGATC TTGCAGGCAA TGCGACCGAT CAGGCCAATG GCGTGCAGCC AGCGCCGGGA
ACCACGTCTG CGGAAAATAC TCAGCAGGAT GTTTCTCTGC CGCCGATCTC TTCTACGCCG
ACTCAAGGGC AAACCCCGGT GGCAACGGAT GGTCAACAAC GTGTTGAAGT GCAGGGTGAC
CTGAACAATG CGCTGACCCA GCCACAAAAT CAGCAACAGT TGAACAATGT GGCGGTCAAT
TCTACATTGC CGACTGAACC CGCAACGGTT GCGCCTGTTC GCAATGGCAA TGCATCGCGT
GACACGGCGA AAACGCAAAC CGCTGAACGT CCGGCCACTA CGCGCCCGGC TCGTCAGCAG
GCGGTGATTG AACCGAAAAA ACCGCAAGCA ACCGTGAAAA CGGAACCGAA GCCAGTCGCA
CAGACGCCGA AGCGAACTGA ACCAGCTGCC CCTGTGGCGA GCACAAAGGC ACCGGCTGCG
ACTTCTACGC CAGCACCAAA AGAGACGGCG ACTACGGCTC CAGTACAGAC GGCATCCCCG
GCGCAAACCA CGGCAACACC AGCCGCTGGA GGGAAGACCG CAGGTAATGT TGGTTCGTTG
AAATCGGCAC CGTCCAGCCA TTACACTCTG CAGCTGAGCA GTTCCTCTAA CTACGACAAC
CTGAACGGTT GGGCGAAGAA AGAGAATCTG AAAAACTACG TTGTCTATGA AACGACGCGT
AATGGTCAGC CGTGGTATGT CCTGGTTTCT GGCGTGTATG CTTCGAAAGA AGAGGCGAAA
AAAGCGGTAT CTACATTGCC AGCAGATGTC CAGGCCAAAA ACCCGTGGGC GAAACCGCTG
CGTCAGGTAC AGGCCGATCT GAAGtaa
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With 400 extra bp on each side of the gene:

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AATTGGTTAC ATGGTGAAGC GGTCGCTGCG GGTATGGTGA TGGCGGCGCG GACGTCGGAA
CGTCTCGGGC AGTTTAGTTC TGCCGAAACG CAGCGTATTA TAACCCTGCT CACGCGGGCT
GGGTTACCGG TCAATGGGCC GCGCGAAATG TCCGCGCAGG CGTATTTACC GCATATGCTG
CGTGACAAGA AAGTCCTTGC GGGAGAGATG CGCTTAATTC TTCCGTTGGC AATTGGTAAG
AGTGAAGTTC GCAGCGGCGT TTCGCACGAG CTTGTTCTTA ACGCCATTGC CGATTGTCAA
TCAGCGTAAC AACAAGAAAG GTCAGGCCGC TTATCAAGCG GTCTATTAGC TTCAGGTTAA
TTGCAACGTG GTAAGCATTA ACCTTTTAGT GGGGTGTTAA atgGATGAAT TCAAACCAGA
AGACGAGCTG AAACCCGATC CCAGCGATCG TCGTACTGGT CGTTCTCGTC AATCTTCTGA
ACGTTCTGAG CGTACTGAAC GTGGCGAACC GCAGATCAAT TTTGATGATA TTGAACTTGA
TGACACTGAC GATCGCCGTC CGACTCGTGC GCAAAAAGCG CGTAATGAGG AACCGGAAAT
CGAAGAAGAA ATTGACGAAT CCGAAGATGA AACCGTGGAT GAAGAGCGCG TAGAGCGTCG
TCCGCGTAAG CGCAAAAAAG CAGCCAGTAA ACCCGCTTCT CGTCAGTATA TGATGATGGG
TGTCGGCATT CTGGTTCTAC TGCTGTTGAT CATCGGTATC GGTTCTGCGC TAAAAGCCCC
CTCGACCTCT TCCAGCGATC AAACCGCGTC TGGCGAGAAG AGTATTGATC TTGCAGGCAA
TGCGACCGAT CAGGCCAATG GCGTGCAGCC AGCGCCGGGA ACCACGTCTG CGGAAAATAC
TCAGCAGGAT GTTTCTCTGC CGCCGATCTC TTCTACGCCG ACTCAAGGGC AAACCCCGGT
GGCAACGGAT GGTCAACAAC GTGTTGAAGT GCAGGGTGAC CTGAACAATG CGCTGACCCA
GCCACAAAAT CAGCAACAGT TGAACAATGT GGCGGTCAAT TCTACATTGC CGACTGAACC
CGCAACGGTT GCGCCTGTTC GCAATGGCAA TGCATCGCGT GACACGGCGA AAACGCAAAC
CGCTGAACGT CCGGCCACTA CGCGCCCGGC TCGTCAGCAG GCGGTGATTG AACCGAAAAA
ACCGCAAGCA ACCGTGAAAA CGGAACCGAA GCCAGTCGCA CAGACGCCGA AGCGAACTGA
ACCAGCTGCC CCTGTGGCGA GCACAAAGGC ACCGGCTGCG ACTTCTACGC CAGCACCAAA
AGAGACGGCG ACTACGGCTC CAGTACAGAC GGCATCCCCG GCGCAAACCA CGGCAACACC
AGCCGCTGGA GGGAAGACCG CAGGTAATGT TGGTTCGTTG AAATCGGCAC CGTCCAGCCA
TTACACTCTG CAGCTGAGCA GTTCCTCTAA CTACGACAAC CTGAACGGTT GGGCGAAGAA
AGAGAATCTG AAAAACTACG TTGTCTATGA AACGACGCGT AATGGTCAGC CGTGGTATGT
CCTGGTTTCT GGCGTGTATG CTTCGAAAGA AGAGGCGAAA AAAGCGGTAT CTACATTGCC
AGCAGATGTC CAGGCCAAAA ACCCGTGGGC GAAACCGCTG CGTCAGGTAC AGGCCGATCT
```

```
GAAGtaaTCA AGGTTATCTC CCGCAATGGT TTATCATTGC GGGCGTTGCC TGAAGCGCTG
GATGCTGTCG GAGCTTTCTC CACAGCCGGA GAAGGTGTAA TTAGTTAGTC AGCATGAAGA
AAAATCGCGC TTTTTTGAAG TGGGCAGGGG GCAAGTATCC CCTGCTTGAT GATATTAAAC
GGCATTTGCC CAAGGGCGAA TGTCTGGTTG AGCCTTTTGT AGGTGCCGGG TCGGTGTTTC
TCAACACCGA CTTTTCTCGT TATATCCTTG CCGATATCAA TAGCGACCTG ATCAGTCTCT
ATAACATTGT GAAGATGCGT ACTGATGAGT ACGTACAGGC CGCACGCGAG CTGTTTGTTC
CCGAAACAAA TTGCGCCGAG GTTTACTATC AGTTCCGCGA AGAGTTC
```

Copied to Primer3 and chose a product size ranges from 2000-2050, primer size of 23 bp and Tm opt at 62, Tm max at 63 gives the followed result:

Primer3 Output

```
No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm     gc%   any   3' seq
LEFT PRIMER         59   23   62.62   47.83  4.00  2.00 AACGTCTCGGGCAGTTTAGTTCT
RIGHT PRIMER      2080   23   61.79   47.83  4.00  0.00 TCGCGGAACTGATAGTAAACCTC
SEQUENCE SIZE: 2174
INCLUDED REGION SIZE: 2174

PRODUCT SIZE: 2022, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 3.00

    1 AATTGGTTACATGGTGAAGCGGTCGCTGCGGGTATGGTGATGGCGGCGCGGACGTCGGAA
                                                                >>

   61 CGTCTCGGGCAGTTTAGTTCTGCCGAAACGCAGCGTATTATAACCCTGCTCACGCGGGCT
      >>>>>>>>>>>>>>>>>>>>>

  121 GGGTTACCGGTCAATGGGCCGCGCGAAATGTCCGCGCAGGCGTATTTACCGCATATGCTG


  181 CGTGACAAGAAAGTCCTTGCGGGAGAGATGCGCTTAATTCTTCCGTTGGCAATTGGTAAG


  241 AGTGAAGTTCGCAGCGGCGTTTCGCACGAGCTTGTTCTTAACGCCATTGCCGATTGTCAA


  301 TCAGCGTAACAACAAGAAAGGTCAGGCCGCTTATCAAGCGGTCTATTAGCTTCAGGTTAA


  361 TTGCAACGTGGTAAGCATTAACCTTTTAGTGGGGTGTTAAatgGATGAATTCAAACCAGA


  421 AGACGAGCTGAAACCCGATCCCAGCGATCGTCGTACTGGTCGTTCTCGTCAATCTTCTGA


  481 ACGTTCTGAGCGTACTGAACGTGGCGAACCGCAGATCAATTTTGATGATATTGAACTTGA


  541 TGACACTGACGATCGCCGTCCGACTCGTGCGCAAAAAGCGCGTAATGAGGAACCGGAAAT
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601 CGAAGAAGAAATTGACGAATCCGAAGATGAAACCGTGGATGAAGAGCGCGTAGAGCGTCG

661 TCCGCGTAAGCGCAAAAAAGCAGCCAGTAAACCCGCTTCTCGTCAGTATATGATGATGGG

721 TGTCGGCATTCTGGTTCTACTGCTGTTGATCATCGGTATCGGTTCTGCGCTAAAAGCCCC

781 CTCGACCTCTTCCAGCGATCAAACCGCGTCTGGCGAGAAGAGTATTGATCTTGCAGGCAA

841 TGCGACCGATCAGGCCAATGGCGTGCAGCCAGCGCCGGGAACCACGTCTGCGGAAAATAC

901 TCAGCAGGATGTTTCTCTGCCGCCGATCTCTTCTACGCCGACTCAAGGGCAAACCCCGGT

961 GGCAACGGATGGTCAACAACGTGTTGAAGTGCAGGGTGACCTGAACAATGCGCTGACCCA

1021 GCCACAAAATCAGCAACAGTTGAACAATGTGGCGGTCAATTCTACATTGCCGACTGAACC

1081 CGCAACGGTTGCGCCTGTTCGCAATGGCAATGCATCGCGTGACACGGCGAAAACGCAAAC

1141 CGCTGAACGTCCGGCCACTACGCGCCCGGCTCGTCAGCAGGCGGTGATTGAACCGAAAAA

1201 ACCGCAAGCAACCGTGAAAACGGAACCGAAGCCAGTCGCACAGACGCCGAAGCGAACTGA

1261 ACCAGCTGCCCCTGTGGCGAGCACAAAGGCACCGGCTGCGACTTCTACGCCAGCACCAAA

1321 AGAGACGGCGACTACGGCTCCAGTACAGACGGCATCCCCGGCGCAAACCACGGCAACACC

1381 AGCCGCTGGAGGGAAGACCGCAGGTAATGTTGGTTCGTTGAAATCGGCACCGTCCAGCCA

1441 TTACACTCTGCAGCTGAGCAGTTCCTCTAACTACGACAACCTGAACGGTTGGGCGAAGAA

1501 AGAGAATCTGAAAAACTACGTTGTCTATGAAACGACGCGTAATGGTCAGCCGTGGTATGT

1561 CCTGGTTTCTGGCGTGTATGCTTCGAAAGAAGAGGCGAAAAAAGCGGTATCTACATTGCC

1621 AGCAGATGTCCAGGCCAAAAACCCGTGGGCGAAACCGCTGCGTCAGGTACAGGCCGATCT

1681 GAAGtaaTCAAGGTTATCTCCCGCAATGGTTTATCATTGCGGGCGTTGCCTGAAGCGCTG

1741 GATGCTGTCGGAGCTTTCTCCACAGCCGGAGAAGGTGTAATTAGTTAGTCAGCATGAAGA

```
 1801 AAAATCGCGCTTTTTTGAAGTGGGCAGGGGGCAAGTATCCCCTGCTTGATGATATTAAAC

 1861 GGCATTTGCCCAAGGGCGAATGTCTGGTTGAGCCTTTTGTAGGTGCCGGGTCGGTGTTTC

 1921 TCAACACCGACTTTTCTCGTTATATCCTTGCCGATATCAATAGCGACCTGATCAGTCTCT

 1981 ATAACATTGTGAAGATGCGTACTGATGAGTACGTACAGGCCGCACGCGAGCTGTTTGTTC

 2041 CCGAAACAAATTGCGCCGAGGTTTACTATCAGTTCCGCGAAGAGTTCAAAGCGGTATCTA
                      <<<<<<<<<<<<<<<<<<<<<<<

 2101 CATTGCCAGCAGATGTCCAGGCCAAAAACCCGTGGGCGAAACCGCTGCGTCAGGTACAGG

 2161 CCGATCTGAAGtaa


KEYS (in order of precedence):
>>>>>> left primer
<<<<<< right primer

ADDITIONAL OLIGOS
                    start  len      tm     gc%   any   3' seq

 1 LEFT PRIMER         59   23   62.62   47.83  4.00  2.00
AACGTCTCGGGCAGTTTAGTTCT
   RIGHT PRIMER      2070   23   62.65   43.48  4.00  3.00
GATAGTAAACCTCGGCGCAATTT
   PRODUCT SIZE: 2012, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 1.00

 2 LEFT PRIMER         59   23   62.62   47.83  4.00  2.00
AACGTCTCGGGCAGTTTAGTTCT
   RIGHT PRIMER      2081   23   61.31   43.48  4.00  0.00
TTCGCGGAACTGATAGTAAACCT
   PRODUCT SIZE: 2023, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 3.00

 3 LEFT PRIMER         59   23   62.62   47.83  4.00  2.00
AACGTCTCGGGCAGTTTAGTTCT
   RIGHT PRIMER      2082   23   61.31   47.83  4.00  0.00
CTTCGCGGAACTGATAGTAAACC
   PRODUCT SIZE: 2024, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 3.00

 4 LEFT PRIMER         58   22   61.86   50.00  4.00  0.00
GAACGTCTCGGGCAGTTTAGTT
   RIGHT PRIMER      2080   23   61.79   47.83  4.00  0.00
TCGCGGAACTGATAGTAAACCTC
   PRODUCT SIZE: 2023, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 3.00

Statistics
         con   too    in    in          no    tm    tm  high  high        high
         sid  many   tar  excl   bad    GC   too   too   any    3'  poly   end
        ered    Ns   get   reg   GC% clamp   low  high compl compl     X  stab
ok
```

```
Left     929     0     0     0     3     0   102   693     0     1     0    30
100
Right    916     0     0     0     0     0   257   398     0     2     0    33
222
Pair Stats:
considered 50, unacceptable product size 41, high end compl 1, ok 8
primer3 release 1.1.4
```

So **damX up** is:

AACGTCTCGGGCAGTTTAGTTCT

And **damX down** is:

TCGCGGAACTGATAGTAAACCTC

It will give a product size on 2174 bp

Primers for pKD3 and pKD4 and damX:

pKD3/4 fw primer : GTGTAGGCTGGAGCTGCTTC

pKD3/4 rev primer: CATATGAATATCCTCCTTA

Find the gene of interest again.

Type 100 bp upstream and 100 bp downstream and show sequence

Organism: **Escherichia coli UTI89**
Chromosome: **Chromosome 1**
Region: 3768588 - 3770074 (reverse complement)

```
TCAGCGTAAC AACAAGAAAG GTCAGGCCGC TTATCAAGCG GTCTATTAGC TTCAGGTTAA
TTGCAACGTG GTAAGCATTA ACCTTTTAGT GGGGTGTTAA atgGATGAAT TCAAACCAGA
AGACGAGCTG AAACCCGATC CCAGCGATCG TCGTACTGGT CGTTCTCGTC AATCTTCTGA
ACGTTCTGAG CGTACTGAAC GTGGCGAACC GCAGATCAAT TTTGATGATA TTGAACTTGA
TGACACTGAC GATCGCCGTC CGACTCGTGC GCAAAAAGCG CGTAATGAGG AACCGGAAAT
CGAAGAAGAA ATTGACGAAT CCGAAGATGA AACCGTGGAT GAAGAGCGCG TAGAGCGTCG
TCCGCGTAAG CGCAAAAAAG CAGCCAGTAA ACCCGCTTCT CGTCAGTATA TGATGATGGG
TGTCGGCATT CTGGTTCTAC TGCTGTTGAT CATCGGTATC GGTTCTGCGC TAAAAGCCCC
CTCGACCTCT TCCAGCGATC AAACCGCGTC TGGCGAGAAG AGTATTGATC TTGCAGGCAA
TGCGACCGAT CAGGCCAATG GCGTGCAGCC AGCGCCGGGA ACCACGTCTG CGGAAAATAC
TCAGCAGGAT GTTTCTCTGC CGCCGATCTC TTCTACGCCG ACTCAAGGGC AAACCCCGGT
GGCAACGGAT GGTCAACAAC GTGTTGAAGT GCAGGGTGAC CTGAACAATG CGCTGACCCA
GCCACAAAAT CAGCAACAGT TGAACAATGT GGCGGTCAAT TCTACATTGC CGACTGAACC
CGCAACGGTT GCGCCTGTTC GCAATGGCAA TGCATCGCGT GACACGGCGA AAACGCAAAC
CGCTGAACGT CCGGCCACTA CGCGCCCGGC TCGTCAGCAG GCGGTGATTG AACCGAAAAA
ACCGCAAGCA ACCGTGAAAA CGGAACCGAA GCCAGTCGCA CAGACGCCGA AGCGAACTGA
ACCAGCTGCC CCTGTGGCGA GCACAAAGGC ACCGGCTGCG ACTTCTACGC CAGCACCAAA
AGAGACGGCG ACTACGGCTC CAGTACAGAC GGCATCCCCG GCGCAAACCA CGGCAACACC
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AGCCGCTGGA GGGAAGACCG CAGGTAATGT TGGTTCGTTG AAATCGGCAC CGTCCAGCCA
TTACACTCTG CAGCTGAGCA GTTCCTCTAA CTACGACAAC CTGAACGGTT GGGCGAAGAA
AGAGAATCTG AAAAACTACG TTGTCTATGA AACGACGCGT AATGGTCAGC CGTGGTATGT
CCTGGTTTCT GGCGTGTATG CTTCGAAAGA AGAGGCGAAA AAAGCGGTAT CTACATTGCC
AGCAGATGTC CAGGCCAAAA ACCCGTGGGC GAAACCGCTG CGTCAGGTAC AGGCCGATCT
GAAGtaaTCA AGGTTATCTC CCGCAATGGT TTATCATTGC GGGCGTTGCC TGAAGCGCTG
GATGCTGTCG GAGCTTTCTC CACAGCCGGA GAAGGTGTAA TTAGTTA

From the start and stop codon you pick 6 codons and then go backwards until 70 bp in all

For damX fw it will give:

GCTTCAGGTTAATTGCAACGTGGTAAGCATTAACCTTTTAGTGGGGTGTTAAatgGATGAAT TCAAACCA

And for damX rev:

CAGGCCGATCTGAAGtaaTCAAGGTTATCTCCCGCAATGGTTTATCATTGCGGGCGTTGCCT GAAGCGCT

Use:

http://www.bioinformatics.org/sms/rev_comp.html

to make the sequence reverse and complementary:

AGCGCTTCAGGCAACGCCCGCAATGATAAACCATTGCGGGAGATAACCTTGAttaCTTCAGA TCGGCCTG

So damX KO fw is:

GCTTCAGGTTAATTGCAACGTGGTAAGCATTAACCTTTTAGTGGGGTGTTAAatgGATGAAT TCAAACCA **GTGTAGGCTGGAGCTGCTTC**

So damX KO rev is:

AGCGCTTCAGGCAACGCCCGCAATGATAAACCATTGCGGGAGATAACCTTGAttaCTTCAGA TCGGCCTG **CATATGAATATCCTCCTTA**

If it is difficult to knock out the gene it can be helpful to make the PCR product around 1000 bp longer in all. You can do so by making 2 more set of primers and run additional 2 PCR

1th set of primers:

Find the gene in question, in Nucleotide Sequence, Advanced type 500 bp upstream and show sequence. Copy the sequence from the 500 bp extra and into the start codon (marked with yellow) and paste it into Primer 3. It is important that the region include the sequence from the damX fw primer.

AGAAGTTGTC GCCGCCGACG AGCGCGAAAC CGGGTTACGT GCTTTACTGA ATCTGGGACA
CACCTTTGGT CATGCCATTG AAGCTGAAAT GGGGTATGGC AATTGGTTAC ATGGTGAAGC
GGTCGCTGCG GGTATGGTGA TGGCGGCGCG GACGTCGGAA CGTCTCGGGC AGTTTAGTTC
TGCCGAAACG CAGCGTATTA TAACCCTGCT CACGCGGGCT GGGTTACCGG TCAATGGGCC
GCGCGAAATG TCCGCGCAGG CGTATTTACC GCATATGCTG CGTGACAAGA AAGTCCTTGC
GGGAGAGATG CGCTTAATTC TTCCGTTGGC AATTGGTAAG AGTGAAGTTC GCAGCGGCGT
TTCGCACGAG CTTGTTCTTA ACGCCATTGC CGATTGTCAA TCAGCGTAAC AACAAGAAAG
GTCAGGCCGC TTATCAAGCG GTCTATTAGC TTCAGGTTAA TTGCAACGTG GTAAGCATTA
ACCTTTTAGT GGGGTGTTAA atgGATGAAT TCAAACCAGA AGACGAGCTG AAACCCGATC
CCAGCGATCG TCGTACTGGT CGTTCTCGTC AATCTTCTGA ACGTTCTGAG CGTACTGAAC
GTGGCGAACC GCAGATCAAT TTTGATGATA TTGAACTTGA TGACACTGAC GATCGCCGTC
CGACTCGTGC GCAAAAAGCG CGTAATGAGG AACCGGAAAT CGAAGAAGAA ATTGACGAAT
CCGAAGATGA AACCGTGGAT GAAGAGCGCG TAGAGCGTCG TCCGCGTAAG CGCAAAAAAG
CAGCCAGTAA ACCCGCTTCT CGTCAGTATA TGATGATGGG TGTCGGCATT CTGGTTCTAC
TGCTGTTGAT CATCGGTATC GGTTCTGCGC TAAAAGCCCC CTCGACCTCT TCCAGCGATC
AAACCGCGTC TGGCGAGAAG AGTATTGATC TTGCAGGCAA TGCGACCGAT CAGGCCAATG
GCGTGCAGCC AGCGCCGGGA ACCACGTCTG CGGAAAATAC TCAGCAGGAT GTTTCTCTGC
CGCCGATCTC TTCTACGCCG ACTCAAGGGC AAACCCCGGT GGCAACGGAT GGTCAACAAC
GTGTTGAAGT GCAGGGTGAC CTGAACAATG CGCTGACCCA GCCACAAAAT CAGCAACAGT
TGAACAATGT GGCGGTCAAT TCTACATTGC CGACTGAACC CGCAACGGTT GCGCCTGTTC
GCAATGGCAA TGCATCGCGT GACACGGCGA AAACGCAAAC CGCTGAACGT CCGGCCACTA
CGCGCCCGGC TCGTCAGCAG GCGGTGATTG AACCGAAAAA ACCGCAAGCA ACCGTGAAAA
CGGAACCGAA GCCAGTCGCA CAGACGCCGA AGCGAACTGA ACCAGCTGCC CCTGTGGCGA
GCACAAAGGC ACCGGCTGCG ACTTCTACGC CAGCACCAAA AGAGACGGCG ACTACGGCTC
CAGTACAGAC GGCATCCCCG GCGCAAACCA CGGCAACACC AGCCGCTGGA GGGAAGACCG
CAGGTAATGT TGGTTCGTTG AAATCGGCAC CGTCCAGCCA TTACACTCTG CAGCTGAGCA
GTTCCTCTAA CTACGACAAC CTGAACGGTT GGGCGAAGAA AGAGAATCTG AAAAACTACG
TTGTCTATGA AACGACGCGT AATGGTCAGC CGTGGTATGT CCTGGTTTCT GGCGTGTATG
CTTCGAAAGA AGAGGCGAAA AAAGCGGTAT CTACATTGCC AGCAGATGTC CAGGCCAAAA
ACCCGTGGGC GAAACCGCTG CGTCAGGTAC AGGCCGATCT GAAGtaa

Chose a product size range from 470-480, primer size on 22 and pick primers

```
No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm      gc%   any   3'  seq
LEFT PRIMER         31   22   60.41   45.45  4.00  2.00 CGGGTTACGTGCTTTACTGAAT
RIGHT PRIMER       509   22   60.46   40.91  4.00  1.00 TTCATCcatTTAACACCCCACT
SEQUENCE SIZE: 510
```

```
INCLUDED REGION SIZE: 510

PRODUCT SIZE: 479, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 3.00

    1 AGAAGTTGTCGCCGCCGACGAGCGCGAAACCGGGTTACGTGCTTTACTGAATCTGGGACA
                                    >>>>>>>>>>>>>>>>>>>>>>

   61 CACCTTTGGTCATGCCATTGAAGCTGAAATGGGGTATGGCAATTGGTTACATGGTGAAGC

  121 GGTCGCTGCGGGTATGGTGATGGCGGCGCGGACGTCGGAACGTCTCGGGCAGTTTAGTTC

  181 TGCCGAAACGCAGCGTATTATAACCCTGCTCACGCGGGCTGGGTTACCGGTCAATGGGCC

  241 GCGCGAAATGTCCGCGCAGGCGTATTTACCGCATATGCTGCGTGACAAGAAAGTCCTTGC

  301 GGGAGAGATGCGCTTAATTCTTCCGTTGGCAATTGGTAAGAGTGAAGTTCGCAGCGGCGT

  361 TTCGCACGAGCTTGTTCTTAACGCCATTGCCGATTGTCAATCAGCGTAACAACAAGAAAG

  421 GTCAGGCCGCTTATCAAGCGGTCTATTAGCTTCAGGTTAATTGCAACGTGGTAAGCATTA

  481 ACCTTTTAGTGGGGTGTTAAatgGATGAAT
            <<<<<<<<<<<<<<<<<<<<<<

KEYS (in order of precedence):
>>>>>> left primer
<<<<<< right primer

ADDITIONAL OLIGOS
                    start  len      tm     gc%   any    3'  seq

 1 LEFT PRIMER         31   22   60.41   45.45  4.00  2.00
CGGGTTACGTGCTTTACTGAAT
   RIGHT PRIMER       508   22   59.20   40.91  4.00  2.00
TCATCcatTTAACACCCCACTA
   PRODUCT SIZE: 478, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 2.00

 2 LEFT PRIMER         40   22   59.24   45.45  3.00  1.00
TGCTTTACTGAATCTGGGACAC
   RIGHT PRIMER       509   22   60.46   40.91  4.00  1.00
TTCATCcatTTAACACCCCACT
   PRODUCT SIZE: 470, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 0.00

 3 LEFT PRIMER         39   22   59.24   45.45  3.00  1.00
GTGCTTTACTGAATCTGGGACA
   RIGHT PRIMER       509   22   60.46   40.91  4.00  1.00
TTCATCcatTTAACACCCCACT
   PRODUCT SIZE: 471, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 1.00

 4 LEFT PRIMER         27   22   61.11   45.45  7.00  1.00
AAACCGGGTTACGTGCTTTACT
```

```
   RIGHT PRIMER       496   22   59.77   45.45  4.00  2.00
CACCCCACTAAAAGGTTAATGC
   PRODUCT SIZE: 470, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 2.00

Statistics
         con   too    in    in          no    tm    tm  high  high        high
         sid  many   tar  excl   bad    GC   too   too   any    3'  poly   end
        ered    Ns   get   reg   GC% clamp   low  high compl compl     X  stab
ok
Left     383     0     0     0     4     0    42   288     0     2     0     4
43
Right    402     0     0     0     0     0   147    83     0     0     0     3
169
Pair Stats:
considered 366, unacceptable product size 355, high end compl 2, ok 9
primer3 release 1.1.4
```

damX up fw: CGGGTTACGTGCTTTACTGAAT
damX up rev: TTCATCcatTTAACACCCCACT

2nd set of primers

Find the gene in question, in Nucleotide Sequence, Advanced type 700 bp downstream and show sequence. Copy the sequence from the 700 bp extra and into the stop codon (marked with yellow) and paste it into Primer 3. It is important that the region include the sequence from the damX rev primer.

```
atgGATGAAT TCAAACCAGA AGACGAGCTG AAACCCGATC CCAGCGATCG TCGTACTGGT
CGTTCTCGTC AATCTTCTGA ACGTTCTGAG CGTACTGAAC GTGGCGAACC GCAGATCAAT
TTTGATGATA TTGAACTTGA TGACACTGAC GATCGCCGTC CGACTCGTGC GCAAAAAGCG
CGTAATGAGG AACCGGAAAT CGAAGAAGAA ATTGACGAAT CCGAAGATGA AACCGTGGAT
GAAGAGCGCG TAGAGCGTCG TCCGCGTAAG CGCAAAAAAG CAGCCAGTAA ACCCGCTTCT
CGTCAGTATA TGATGATGGG TGTCGGCATT CTGGTTCTAC TGCTGTTGAT CATCGGTATC
GGTTCTGCGC TAAAAGCCCC CTCGACCTCT TCCAGCGATC AAACCGCGTC TGGCGAGAAG
AGTATTGATC TTGCAGGCAA TGCGACCGAT CAGGCCAATG GCGTGCAGCC AGCGCCGGGA
ACCACGTCTG CGGAAAATAC TCAGCAGGAT GTTTCTCTGC CGCCGATCTC TTCTACGCCG
ACTCAAGGGC AAACCCCGGT GGCAACGGAT GGTCAACAAC GTGTTGAAGT GCAGGGTGAC
CTGAACAATG CGCTGACCCA GCCACAAAAT CAGCAACAGT TGAACAATGT GGCGGTCAAT
TCTACATTGC CGACTGAACC CGCAACGGTT GCGCCTGTTC GCAATGGCAA TGCATCGCGT
GACACGGCGA AAACGCAAAC CGCTGAACGT CCGGCCACTA CGCGCCCGGC TCGTCAGCAG
GCGGTGATTG AACCGAAAAA ACCGCAAGCA ACCGTGAAAA CGGAACCGAA GCCAGTCGCA
CAGACGCCGA AGCGAACTGA ACCAGCTGCC CCTGTGGCGA GCACAAAGGC ACCGGCTGCG
ACTTCTACGC CAGCACCAAA AGAGACGGCG ACTACGGCTC CAGTACAGAC GGCATCCCCG
GCGCAAACCA CGGCAACACC AGCCGCTGGA GGGAAGACCG CAGGTAATGT TGGTTCGTTG
AAATCGGCAC CGTCCAGCCA TTACACTCTG CAGCTGAGCA GTTCCTCTAA CTACGACAAC
CTGAACGGTT GGGCGAAGAA AGAGAATCTG AAAAACTACG TTGTCTATGA AACGACGCGT
AATGGTCAGC CGTGGTATGT CCTGGTTTCT GGCGTGTATG CTTCGAAAGA AGAGGCGAAA
AAAGCGGTAT CTACATTGCC AGCAGATGTC CAGGCCAAAA ACCCGTGGGC GAAACCGCTG
CGTCAGGTAC AGGCCGATCT GAAGtaaTCA AGGTTATCTC CCGCAATGGT TTATCATTGC
GGGCGTTGCC TGAAGCGCTG GATGCTGTCG GAGCTTTCTC CACAGCCGGA GAAGGTGTAA
TTAGTTAGTC AGCATGAAGA AAAATCGCGC TTTTTTGAAG TGGGCAGGGG GCAAGTATCC
CCTGCTTGAT GATATTAAAC GGCATTTGCC CAAGGGCGAA TGTCTGGTTG AGCCTTTTGT
AGGTGCCGGG TCGGTGTTTC TCAACACCGA CTTTTCTCGT TATATCCTTG CCGATATCAA
```

```
TAGCGACCTG ATCAGTCTCT ATAACATTGT GAAGATGCGT ACTGATGAGT ACGTACAGGC
CGCACGCGAG CTGTTTGTTC CCGAAACAAA TTGCGCCGAG GTTTACTATC AGTTCCGCGA
AGAGTTCAAC AAAAGCCAGG ATCCGTTCCG TCGGGCGGTA CTGTTTTTAT ATTTGAACCG
CTACGGTTAC AACGGCCTGT GTCGTTACAA TCTGCGCGGT GAGTTTAACG TGCCGTTTGG
CCGCTACAAA AAACCCTATT TCCCGGAAGC AGAGTTGTAT CACTTCGCTG AAAAAGCGCA
GAATGCCTTT TTCTATTGTG AGTCTTACGC CGATAGCATG GCGCGCGCAG ATGATGCATC
TGTCGTCTAT TGCGATCCGC CTTATGCGCC GCTGTCTGCG ACTGCCAACT TTACGGCGTA
TCACACA
```

Chose a product size range from 670-680, primer size on 22 and pick primers

```
No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm     gc%   any   3' seq
LEFT PRIMER         14   22   60.17   40.91  3.00  1.00 TTATCTCCCGCAATGGTTTATC
RIGHT PRIMER       693   21   61.91   47.62  4.00  2.00 TAAAGTTGGCAGTCGCAGACA
SEQUENCE SIZE: 707
INCLUDED REGION SIZE: 707

PRODUCT SIZE: 680, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 2.00

    1 GAAGtaaTCAAGGTTATCTCCCGCAATGGTTTATCATTGCGGGCGTTGCCTGAAGCGCTG
                >>>>>>>>>>>>>>>>>>>>>

   61 GATGCTGTCGGAGCTTTCTCCACAGCCGGAGAAGGTGTAATTAGTTAGTCAGCATGAAGA

  121 AAAATCGCGCTTTTTTGAAGTGGGCAGGGGGCAAGTATCCCCTGCTTGATGATATTAAAC

  181 GGCATTTGCCCAAGGGCGAATGTCTGGTTGAGCCTTTTGTAGGTGCCGGGTCGGTGTTTC

  241 TCAACACCGACTTTTCTCGTTATATCCTTGCCGATATCAATAGCGACCTGATCAGTCTCT

  301 ATAACATTGTGAAGATGCGTACTGATGAGTACGTACAGGCCGCACGCGAGCTGTTTGTTC

  361 CCGAAACAAATTGCGCCGAGGTTTACTATCAGTTCCGCGAAGAGTTCAACAAAAGCCAGG

  421 ATCCGTTCCGTCGGGCGGTACTGTTTTTATATTTGAACCGCTACGGTTACAACGGCCTGT

  481 GTCGTTACAATCTGCGCGGTGAGTTTAACGTGCCGTTTGGCCGCTACAAAAAACCCTATT

  541 TCCCGGAAGCAGAGTTGTATCACTTCGCTGAAAAAGCGCAGAATGCCTTTTTCTATTGTG

  601 AGTCTTACGCCGATAGCATGGCGCGCGCAGATGATGCATCTGTCGTCTATTGCGATCCGC

  661 CTTATGCGCCGCTGTCTGCGACTGCCAACTTTACGGCGTATCACACA
                <<<<<<<<<<<<<<<<<<<<<
```

```
KEYS (in order of precedence):
>>>>>> left primer
<<<<<< right primer

ADDITIONAL OLIGOS
                    start  len      tm      gc%   any   3'  seq

 1 LEFT PRIMER         14   22   60.17   40.91  3.00  1.00
TTATCTCCCGCAATGGTTTATC
   RIGHT PRIMER       691   19   60.61   52.63  4.00  2.00 AAGTTGGCAGTCGCAGACA
   PRODUCT SIZE: 678, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 2.00

 2 LEFT PRIMER         14   22   60.17   40.91  3.00  1.00
TTATCTCCCGCAATGGTTTATC
   RIGHT PRIMER       692   20   61.97   50.00  4.00  2.00 AAAGTTGGCAGTCGCAGACA
   PRODUCT SIZE: 679, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 2.00

 3 LEFT PRIMER         15   22   61.51   40.91  3.00  2.00
TATCTCCCGCAATGGTTTATCA
   RIGHT PRIMER       694   22   62.63   50.00  4.00  2.00
GTAAAGTTGGCAGTCGCAGACA
   PRODUCT SIZE: 680, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 1.00

 4 LEFT PRIMER         13   22   59.25   40.91  3.00  2.00
GTTATCTCCCGCAATGGTTTAT
   RIGHT PRIMER       691   19   60.61   52.63  4.00  2.00 AAGTTGGCAGTCGCAGACA
   PRODUCT SIZE: 679, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 1.00

Statistics
         con   too    in    in          no    tm    tm  high  high        high
         sid  many   tar  excl   bad    GC   too   too   any    3'  poly   end
        ered    Ns   get   reg   GC% clamp   low  high compl compl     X  stab
ok
Left     302     0     0     0     0     0    40   188     2     8     0    11
53
Right    335     0     0     0     0     0    16   274     0     7     0     6
32
```

damX down fw: TTATCTCCCGCAATGGTTTATC
damX down rev:TAAAGTTGGCAGTCGCAGACA