

Supplementary Figure S1. Amplified nucleotide sequences of potential off-targets loci. Positions of primers used for PCR amplification (underlined) and sequences complementary to gRNA (bold) are shown.

OFF-TARGET 1

mm4_intron_ESYT2_chr2_9588710

GAGCTGAAGTTCACCCTGCAGGGGAAGCCCGGCCTGCCGCCGCTCCGCTGCTGTCGGGCCG
GCGGGCAGCGCCGGGCTCAGGCTAACGGCGACGGGGACGCGATGCCGGGGCGGAGGGATGCT
CCCCGGGCTCGCCCAACATCGCACCCAGGCAGGACGTGCCCCCGGGTACGGCATCCCCGCAC
CTCCTCCCTCCCGGCCCGCAG**CCAGCCGCTACCCGCAGGAGGGG**CCGAGGCCCCGGCCCCGCC
CCACAGCCGCGCTGCCCGCCGGGCTCGGAGCCGCCTCGGAAGCGCCCCGAAGGGTGTGCGCCG
CGCGCTCCCTCACTACCCACGCAGGCAGATCGGCGGAGGAGACGCTGAGCCGCACCGCCTC
CTCCTCGTCCTCCAGGAAGGCGAGCGCCCGGCCAGGCGGGAGGTCTTGCCGCCGCGGTGCC
GCCGGATCCAGAAGAGCCCGCACAGGGCGATGAGCACCCAGCTGAAGCTCAGCCCCAGGTA

1F GAGCTGAAGTTCACCCTGCA

1R TACCTGGGGCTGAGCTTCA

OFF-TARGET 2

mm4_intergenic_ENSGALT00000044901.2|VWA5B2_chr9_15903196

AAGCAGCGAACAGAACAGGAGCGGCAGAAAGTTCAGGTGCGGCTTCTAGGCTAGGGCTTTGC
CAGCAGGGTTTGGCTGGCCCAGCCAAAGCCGAGCGAGCGTCCGGGAGAGCGACGAGCGGCTC
TACTCCTCCTTGGCCCAGGCCGAGCCGGCGGCCCGCTGCTGCTGCAGGTGCGGAGCGACCGC
CTCGGTGTAGAACTTCTCCAGCTTGGGACCCGTCTTCCCCAGAACTCGGCGTCAACTCCA
CGGGCGCCACGGCCGTCTCCTTTTTTGGTGTGCACCACGAAGTCGGCGTGCTGCAGGCCGGTG
GCCGCCAGCTGGCACTGCACCTGGGTGAAGTAGCCGTGAT**CCGCTTCAGCGCGTAGGAGTC**
GCCGTTTCAGCTCCAGGCAGAAAGTCCCTGTCCTTGACAGGCCTCGCGCACCGTCCTGTTGCGGT
GCTTGTAGGGACACTT

2F AAGCAGCGAACAGAACAGGA

2R AAGTGTCCCTACAAGCACCG

OFF-TARGET 3

mm4_intron_ENSGALT00000082879.1/ENSGALT00000074100.1_chr10_12351360

AAGCCCCGAGGAACTAAGGAGAAAGTGAACGAAAGGCCGATTGCACGCAGCCACCTCCAAAG
CCACGAGCAAACCACGGCACCCAGCAGAGCCCAGGCGGGCGGTACGCCTGCAGCT**CCCTCCC**
AGCGGGCAGCGGTCCGTGCCGGCAGCCGCACCCACCCCCGGCAGGGGCAGCCCCAGCCCGG
CGGCACGGAGCTCCCATCGCCGCCTTTAGGGCAGCCCCAGCCAGCTGGGCTACAGACGAG
GAGCCCCGGGCGGAGCGGCACCGCCTTCTCTCCTCTCTCCTCCTCCCCCGCCAGCACT
GCCACCCCCCTCTCTCCCCGGAGGCCGTACCGTATCGCCGTTGTCTGGGGACGCTCCTCGC
CGCCTCGGGCCGGGCTCCCAGCACCGCCAGCAGCACCAGCACCAGGGCGCAGCTCCTCGGGG
GGCCCGCGGCCGTCCCCAGCCGGGGGGGGGCACCTGCAACACACACAG

3F AAGCCCCGAGGAACTAAGGA

3R CGTGTGTGTGTTGCAGGTG

OFF-TARGET 4

mm4_intergenic_HRH2|SPINK5_chr13_10537122

ACATTCCCAGCAGAAGAGGCAATTGGCTGATGCAGCGTTAGGCATTACTGCTCCCATCCCTG
AGGAGTGTGCTTTTGTGGTTCATCCCTTGACCCAAATCACTCCTCAGTTACTCCACTCTGG
CCAATTGCTTTCACATCCTGCTGGCACTTCTAGGACTGTATTTTATGCTCTTGAATATAACA
TAAAGTCCT**CCCCTGCAGCGGGTAGCAATTTG**CAGCTGTATGCAAATTAAGTGC GTATTCT
TTTCTCATGAATAATGGCCCTTGCAATGAGTTTTGAACTTTCTGCTCACTGCTCCCAAGGCA
AGAAGAGCTCAGAGTGCAGCAAGGGGTGGGGGAGACGAGTGTGGCAGCCCCCAGACAACCAA
ATGTGGATGAACTGTTTGCAGGACCTGGATGGCCTCCGGGAGAGCCCTCCGCTGACCGTCCT
CCTCCGTGCATTTCA

4F ACATTCCCAGCAGAAGAGGC
4R TGAAATGCACGGAGGAGGAC

OFF-TARGET 5

mm4_intron_DGKK_chr4_1853057
TGTGCAGCTCCTTTACCTGGGAGTGAAGTGTGCTCTTTTGCTTCTGTGCCTTGTCTTCCT
GGGCTTGGGGATGTTGAGGCGAAGCTTGAAGCTGCCCTTATTGGCACTGCTGGCACTCTCCT
GCAAGAAACAGGGATGAGCCACCCCTACAGCCCAACAGCCCCCCCCACCACCCCTATGCG
CCCACCTCCTCGCTGGGGTGGGCGATGGGTCCAGCCAGTGGATGTCCAGCAGCCGCTGCAG
CTCCTGGCCCAACGCACTCAGGGGGGCCCTGCAGCGCTTCCTCCTCCTGGGGTGAGTCCAACT
GCGGGGGACAGGGACCGTCCCCAGC**CCCCTGCCACCTGCTGGTGGGGGGGACACCTGGGCC**
GGAGGGCTCGGGGGGGTCCCCAGCCCCACACTACCGCCTTCCCCTCCAGGAACGCTCTGGT
CTCGGTATAGAGCGCT

5F TGTGCAGCTCCTTTACCTGG
5R AGCGCTCTATACCGAGACCA

OFF-TARGET 6

mm4_exon_MIXL1_chr3_17105323
GTTCTCCTCCCACTCCGTTCCCAGCTCCGAGAA
GCCGCCGCCGGCCGGGAGGGGAACGCGGGGCGGGGCGGGTAGGGCCCCGCCGGGGCGCCTC
CGCTCCCCCGTGCGGGCGCAGCGCGGAGCGGGCGGGCCGGCGGCGCGGGGCGGCCACTCGC
GGTGCTCCTCCGGGCGCGGAGCAGCGGGGCGCGCCGCACGGCGAGCGCTGGGGCGGGGGC
GGTGGCGGTGCGGGGGCACCCGGCCGGGGCGGTCCCCGCTGCCGGCGAGACTTGGCGCGGGC
GTTCTGGAACCAGACCTGGGGAGAGAGGGCGGCCGTGAGGGCGCGGTGAGGGCGGGCGGCGA
GGAGGGCGGGCACCCCTGCCCTCACCTGGATGCGGGACTCGGGGATCTGCGTGGCGTCGG
CCAG**CCGCTCCCGCAGGTAGATGT**CGGGGTACATGGTGTCTTGGAACACCA

6F GTTCTCCTCCCACTCCGTTT
6R TGGTGTTCAGGACACCATG

OFF-TARGET 7

mm4_intergenic_gga-mir-6560|ENSGALT00000022251.5_chr6_32490020
CTAAGCTGGGCGATAGTGCATGGCAAACAAAGCCTACACCATTTTATTTATGGCTGGCTATT
TACTTTATGCAACTCA**CCACTGCAGTGGGTATCAGTAGGG**ATTTATAACAGATTGGATTTT
GAAATGTTTCATGTCCTCAGTGGCACACCATTTGCTTTGTCAACTTTTACATCAGTTTTTCCCG
TGTCAGCTGGAACAGTCAGCAAACACCTAAACTTGTAGTGTGTTCCAGTTACAGGCATCT
GTGACTTGGAACCAGTCCAAAACATGCTGATCTACAGAACCACATCCACTGCCTTGCTGATA

CTTCCAGTTCTGCTTAGTGGTCCCAGTCATAGCAAGAGGCAACAAAAGTCAAAGCACAACGC
AAGGCCCTGCCGTGAGGAGTGAAGCATTTCCAGCAGTGTAGCACAGTG

7F CTAAGCTGGGCGATAGTGCA

7R CACTGTGCTACACTGCTGGA

Supplementary Figure S2. Nucleotide sequences of potential off-target sites amplified from PGC *tva* ^{-/-} clone 2 and ejaculate of recipient rooster No. 16 aligned to the NCBI chicken genome. Sequencing primers (either forward or reverse) are highlighted with green color, the gRNA complementary sequence with red color.

OFF-TARGET 1

NCBI chicken	GAGCTGAAGTTCACCTTCAGGGGAAGCCCGGCCTGCCGCCCTCCGCTGCTGTCTCGGGC
PGC	-----
ejaculate 16	-----GGGGAAGCCCGGCCTGCCGCCCTCCGCTGCTGTCTCGGGC
NCBI chicken	CGGCGGGCAGCGCCGGGCTCAGGCTAACGGCGACGGGGACGCGATGCCGGGGCGGAGGGA
PGC	-----
ejaculate 16	CGGCGGGCAGCGCCGGGCTCRGGCTAACGGCGACGGGGACGCGATGCCGGGGCGGAGGGA
NCBI chicken	TGCTCCCCGGGCTCGCCCAACATCGCACCAGGCAGGACGTGCCCCCGGGTACGGCATCC
PGC	-----TCGCCCAACATCGCACCAGGCAGGACGTGCCCCCGGGTACGGCATCC
ejaculate 16	TGCTCCCCGGGCTCGCCCAACATCGCACCAGGCAGGACGTGCCCCCGGGTACGGCATCC

NCBI chicken	CCGCACCTCCTCCCTCCCGGCCCGCAG TCAGCCGCTACCCGCAGGAGGGG CCGAGGCCCC
PGC	CCGCACCTCCTCCCTCCCGGCCCGCAGCCAGCCGCTACCCGCAGGAGGGGCCGAGGCCCC
ejaculate 16	CCGCACCTCCTCCCTCCCGGCCCGCAGCCAGCCGCTACCCGCAGGAGGGGCCGAGGCCCC

NCBI chicken	GGCCCGCCCCACAGCCGCGCTGCCCGCCGGGCTCGGAGCCGCCTCGGAAGCGCCCGAAGG
PGC	GGCCCGCCCCACAGCCGCGCTGCCCGCCGGGCTCGGAGCCGCCTCGGAAGCGCCCGAAGG
ejaculate 16	GGCCCGCCCCACAGCCGCGCTGCCCGCCGGGCTCGGAGCCGCCTCGGAAGCGCCCGAAGG

NCBI chicken	GTGTTGGCCGCGCGCTCCCTCACTCACCACGCAGGCAGATCGGCGGAGGAGACGCTGAG
PGC	GTGTTGGCCGCGCGCTCCCTCACTCACCACGCAGGCAGATCGGCGGAGGAGACGCTGAG
ejaculate 16	GTGTTGGCCGCGCGCTCCCTCACTCACCACGCAGGCAGATCGGCGGAGGAGACGCTGAG

NCBI chicken	CCGCACCGCCTCCTCCTCGTCTCCAGGAAGGCGAGCGCCCGGCCAGGCGGGAGGTCTT
PGC	CCGCACCGCCTCCTCCTCGTCTCCAGGAAGGCGAGCGCCCGGCCAGGCGGTGAGCGTA
ejaculate 16	CCGCACCGCCT-----

NCBI chicken	GCCGCCGCGGTGCCGCCGATCCAGAAGAGCCCGCACAGGGCGATGAGCACCAGC TGAA
PGC	CGAACATCGTTAC-----
ejaculate 16	-----
NCBI chicken	GCTCAGCCCCAGGTA
PGC	-----
ejaculate 16	-----

OFF-TARGET 2

NCBI chicken	AAGCAGCGAACAGAACAGGA GCGGCAGAAAGTTCAGGTGCGGCTTCTAGGCTAGGGCTTT
PGC clone 2	-----GTGCGGCTTCTAGGCTAGGGCTTT

NCBI chicken	GCCAGCAGGGTTTGCGTGGCCCAGCCAAAGCCGAGCGAGCGTCCGGGAGAGCGACGAGCG
PGC clone 2	GCCAGCAGGGTTTGCGTGGCCCAGCCAAAGCCGAGCGAGCGTCCGGGAGAGCGACGAGCG

NCBI chicken	GCTCTACTCCTCCTTGCGCCAGGCCGAGCCGGCGGCCCGCTGCTGCTGCAGGTGCGGAGC
PGC clone 2	GCTCTACTCCTCCTTGCGCCAGGCCGAGCCGGCGGCCCGCTGCTGCTGCAGGTGCGGAGC

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NCBI chicken      GACCGCCTCGGTGTAGAACTTCTCCAGCTTGGGCACCGTCTTCCCCAGAACTCGGCGTC
PGC clone 2       GACCGCCTCGGTGTAGAACTTCTCCAGCTTGGGCACCGTCTTCCCCAGAACTCGGCGTC
                  *****

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NCBI chicken CAGGCCGGTGGCCGCCAGCTGGCACTGCACCTGGGTGAAGTAGCCGTGATCCGCTTCAG
PGC clone 2 CAGGCCGGTGGCCGCCAGCTGGCACTGCACCTGGGTGAAGTAGCCGTGATCCCGCTTCAG

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NCBI chicken      CGTCCTGTTGCGGTGCTTGTAGGGACACTT
PGC clone 2       CGTCCTGTTG-----
                  *****

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NCBI chicken      AAGCCCGAGGAACTAAGGA GAAAGTGAACGAAAGGCCGATTGCACGCAGCCACCTCCAA
PGC clone 2      -----CGATTGCACGCAGCCACCTCCAA
                  *****

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NCBI chicken TCCCAGCGGGCAGCGGTTCGGTGCCGGCAGCCGCACCCACCCCCCGGCAGGGGCAGCCCCA
PGC clone 2 TCCCAGCGGGCAGCGGTTCGGTGC CGGCAGCCGCACCCACCCCCCGGCAGGGGCAGCCCCA

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NCBI chicken      CAGACGAGGAGCCCCGGGCCGGAGCGGCACCGCCTTCCTCTCCCTCTCTCCTCCTCCCC
PGC clone 2      CAGACGAGGAGCCCCGGGCCGGAGCGGCACCGCCTTCCTCTCCCTCTCTCCTCCTCCCC
                  *****
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NCBI chicken      GACGCTCCTCGCCGCCCTCGGGCCGGGCTCCCAGCACC GCCAGCAGCACCAGCACCAGGGC
PGC clone 2      GACGCTCCTCGCCGCCCTCGGGCCGGGCTCCCAGCACC GCCAGCAGCACCAGCACCAGGGC
*****

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NCBI chicken	ACG
PGC clone 2	---

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NCBI chicken      ACATTCCCAGCAGAAGAGGCAATTGGCTGATGCAGCGTTAGGCATTACTGCTCCCATCCC
PGC               -----CGTTAGGCATTACTGCTCCCATCCC
PGC clone 2      -----CGTTAGGCATTACTGCTCCCATCCC
                  *****

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NCBI chicken      CTGGCCAATTGCTTTTCACATCCTGCTGGCACTTCTAGGACTGTATTTTATGCTCTTGAAT
PGC               CTGGCCAATTGCTTTTCACATCCTGCTGGCACTTCTAGGACTGTATTTTATGCTCTTGAAT
PGC clone 2       CTGGCCAATTGCTTTTCACATCCTGCTGGCACTTCTAGGACTGTATTTTATGCTCTTGAAT
*****

NCBI chicken      ATAACATAAAGTCCTCCCTGCAGCGGGTAGCAATTGCAGCTGTATGCAAATTAAGTGC
PGC               ATAACATAAAGTCCTCCCCTGCAGCGGGTAGCAATTGCAGCTGTATGCAAATTAAGTGC
PGC clone 2       ATAACATAAAGTCCTCCCCTGCAGCGGGTAGCAATTGCAGCTGTATGCAAATTAAGTGC
*****

NCBI chicken      GTATTCCCTTTTCTCATGAATAATGGCCCTTGCAATGAGTTTGAACCTTCTGCTCACTGC
PGC               GTATTCCCTTTTCTCATGAATAATGGCCCTTGCAATGAGTTTGAACCTTCTGCTCACTGC
PGC clone 2       GTATTCCCTTTTCTCATGAATAATGGCCCTTGCAATGAGTTTGAACCTTCTGCTCACTGC
*****

NCBI chicken      TCCAAGGCAAGAAGAGCTCAGAGTGCAGCAAGGGGTGGGGGAGACGAGTGTGGCAGCCC
PGC               TCCAAGGCAAGAAGAGCTCAGAGTGCAGCAAGGGGTGGGGGAGACGAGTGTGGCAGCCC
PGC clone 2       TCCAAGGCAAGAAGAGCTCAGAGTGCAGCAAGGGGTGGGGGAGACGAGTGTGGCAGCCC
*****

NCBI chicken      CCAGACAACCAAATGTGGATGAAGTGTTCAGGACCTGGATGGCCTCCGGGAGAGCCCT
PGC               CCAGACAACCAAATGTGGATGAAGTGTTCAGGACCTGGATGGCCTCCGGGAGAGCCCT
PGC clone 2       CCAGACAACCAAATGTGGATGAAGTGTTCAGGACCTGGATGGCCTCCGGGAGAGCCCT
*****

NCBI chicken      CCGCTGACCGTCCTCCTCCGTGCATTTC
PGC               CCGCTGACC-----
PGC clone 2       CCGCTGACC-----
*****

OFF-TARGET 5
PGC clone 2       -----GCTTCTGTGCCTTGTCTCTTC
NCBI chicken      TGTGCAGCTCCTTTACCTGGGAGTGAAGTGTTCCTTTGCTTCTGTGCCTTGTCTCTTC
*****

PGC clone 2       CTGGGCTTGCGGATGTTGAGGCGAAGCTTGAAGCTGCCCTTATTGGCACTGCTGGCACTC
NCBI chicken      CTGGGCTTGCGGATGTTGAGGCGAAGCTTGAAGCTGCCCTTATTGGCACTGCTGGCACTC
*****

PGC clone 2       TCCTGCAAGAAACAGGGATGAGCCACCCCTACAGCCCAACAGCCCCCCCCACCACCC
NCBI chicken      TCCTGCAAGAAACAGGGATGAGCCACCCCTACAGCCCAACAGCCCCCCCCACCACCC
*****

PGC clone 2       TATGCGCCACCTCCTCGCTGGGGTGGGCGATGGGTCCAGCCAGTGGATGTCCAGCAGC
NCBI chicken      TATGCGCCACCTCCTCGCTGGGGTGGGCGATGGGTCCAGCCAGTGGATGTCCAGCAGC
*****

PGC clone 2       CGCTGCAGCTCCTGGCCCAATGCATTACAGGGGGCCCTGCAGCGCTTCTCCTCCTGGGGT
NCBI chicken      CGCTGCAGCTCCTGGCCCAACGCATCAGGGGGCCCTGCAGCGCTTCTCCTCCTGGGGT
*****

PGC clone 2       GAGTCCAAGTGCAGGGGACAGGGACCGTCCCCAGCCCCACTGCCACCTGCTGGTGGGGG
NCBI chicken      GAGTCCAAGTGCAGGGGACAGGGACCGTCCCCAGCCCCACTGCCACCTGCTGGTGGGGGG
*****

PGC clone 2       GACACCTGGGCGGAGGGCTCGGGGGGTCCCCAGCCCCACACTACCGCCTTCCCTCC
NCBI chicken      GACACCTGGGCGGAGGGCTCGGGGGGTCCCCAGCCCCACACTACCGCCTTCCCTCC
*****

PGC clone 2       AGGAACGCTC-----
NCBI chicken      AGGAACGCTCTGGTCTCGGTATAGAGCGCT
*****

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OFF-TARGET 7

PGC clone 2	-----TGGCAAACAAAGCCTACACCATTTTATTTATGGCTGGCTA
NCBI chicken	CTAAGCTGGGCGATAGTGCATGGCAAACAAAGCCTACACCATTTTATTTATGGCTGGCTA
PGC	-----TGGCAAACAAAGCCTACACCATTTTATTTATGGCTGGCTA *****
PGC clone 2	TTTACTTTATGCAACTCACCAGTGCAGTGGGTATCAGTAGGGATTTATAACAGATTGGAT
NCBI chicken	TTTACTTTATGCAACTCA CACCTGCAGTGGGTATCAGTAGG GATTTATAACAGATTGGAT
PGC	TTTACTTTATGCAACTCACCAGTGCAGTGGGTATCAGTAGGGATTTATAACAGATTGGAT *****
PGC clone 2	TTTCGAAATGTTTCATGTCCTCAGTGGCACACCATTGCTTTGTCAACTTTTACATCAGTTT
NCBI chicken	TTTCGAAATGTTTCATGTCCTCAGTGGCACACCATTGCTTTGTCAACTTTTACATCAGTTT
PGC	TTTCGAAATGTTTCATGTCCTCAGTGGCACACCATTGCTTTGTCAACTTTTACATCAGTTT *****
PGC clone 2	TTCCCGTGTCAGCTAGAAATAGTCAGCAAACACCTAAAACTTGTAGTGTTGTTCCAGTTAC
NCBI chicken	TTCCCGTGTCAGCTGGAACAGTCAGCAAACACCTAAAACTTGTAGTGTTGTTCCAGTTAC
PGC	TTCCCGTGTCAGCTGGAACAGTCAGCAAACACCTAAAACTTGTAGTGTTGTTCCAGTTAC ***** ** *
PGC clone 2	AGTCATCTGTGACTTGGAACCAAGTCCAAAACATGCTGATCTACAGAACCACATCCACTGC
NCBI chicken	AGGCATCTGTGACTTGGAACCAAGTCCAAAACATGCTGATCTACAGAACCACATCCACTGC
PGC	AGTCATCTGTGACTTGGAACCAAGTCCAAAACATGCTGATCTACAGAACCACATCCACTGC ** *****
PGC clone 2	CTTGCTGGTACTGCCAGTTCTGCTTAGTGCTCCAGTCATAGCAAGAGGCAACAAAAGTC
NCBI chicken	CTTGCTGATACTTCCAGTTCTGCTTAGTGCTCCAGTCATAGCAAGAGGCAACAAAAGTC
PGC	CTTGCTGGTACTTCCAGTTCTGCTTAGTGCTCCAGTCATAGCAAGAGGCAACAAAAGTC ***** ** *
PGC clone 2	AAAGCACAAACGCAAGG-----
NCBI chicken	AAAGCACAAACGCAAGGCCCTGCCGTGAGGAGTGAAGCAT TCCAGCAGTGTAGCACAGTG
PGC	AAAGCACAAACGCAAGG----- *****

Supplementary Table S1. List of potential off-target sites with the highest CFD score and the core sequence of gRNA. The mismatches between gRNA and the off-target site are in bold and the protospacer adjacent motifs are in italics.

OFF-TARGET SITE NUMBER	SEQUENCE	CFD SCORE
<i>tva</i>	CCGACTGCTACCCGCTGGAG <i>TGG</i>	1.00
1	CC AGCC GCTACCCGC AG GAG <i>GGG</i>	0.38
2	GCGACTCCTACGCGCTGAAG <i>CGG</i>	0.19
3	CCGAC CGCTG CCCGCTGG GA <i>GGG</i>	0.17
4	CC CACTGCCACCTG CTGG TG <i>GGG</i>	0.16
5	CCGAC ATCTACCTGC GGGAG <i>CGG</i>	0.13
6	CC TACTGATA CCC ACTGC AG <i>TGG</i>	0.12
7	CC TACTGATA CCC ACTGC AG <i>TGG</i>	0.11