

pZERO-hTLR4A-HA

A plasmid expressing a HA-tagged TIR-deleted human TLR4A gene

Catalog # pzero-htlr4-ha

For research use only

Version # 05D01-SV

PRODUCT INFORMATION

Content:

- 1 disk of lyophilized GT110 bacteria transformed with pZERO-hTLR4A-HA.
- GT110 genotype is: *F*-, *mcrA*, Δ (*mrr-hsdRMS-mcrBC*), Δ *lacZ* Δ *M15*, Δ *lacX74*, *recA1*, *endA1*.
- 4 pouches of *E. coli* Fast-Media® Puro.

Storage and stability:

- Products are shipped at room temperature.
- Transformed bacteria should be stored at -20°C and are stable up to 1 year.
- Store *E. coli* Fast-Media® Puro at room temperature. Fast-Media® poche are stable 18 months when stored properly.

Quality control:

- Plasmid construct has been confirmed by restriction analysis and sequencing.
- Bacteria have been lyophilized, and their viability upon resuspension has been verified.

GENERAL PRODUCT USE

Toll-like receptors (TLRs) activate intracellular signaling pathways that share much in common with IL1-R signaling, owing to their conserved TIR (Toll/IL-1R) domains present in the cytoplasmic tails. The TIR family also includes the adapters MyD88, TIRAP (MAL) and a new member TRIF (TICAM). Upon ligand binding, TLRs recruit MyD88 via their TIR domains. The TLR/MyD88 complex then activates IRAK eliciting a signaling cascade leading to the activation of NF- κ B. Some TLRs also signal through a MyD88-independent pathway. Recently, TLR3 and TLR4 have been reported to interact with TRIF inducing the activation of the IRF-3 signaling pathway. Despite immense progress in the understanding of the TLR pathways, a lot remains unclear. To help you uncover the complex mechanisms governing TLR signaling, InvivoGen has engineered TIR domain-deleted TLR genes (TLR- Δ TIR). These mutant genes will serve as useful tools to study TLR signaling.

All ten human and nine murine TLR- Δ TIR genes are available in pZERO-TLR plasmids. Their expression is controlled by the strong EF1/HTLV composite promoter. pZERO-TLR plasmids are selectable with the potent antibiotic puromycin in both *E. coli* and mammalian cells.

PLASMID FEATURES

• Human TLR4A- Δ TIR-HA (2052 bp)

TLR4 is the receptor for Gram⁻ lipopolysaccharide (LPS). The TLR4 gene was shown to be mutated in C3H/HeJ and C57BL/10ScCr mice, both of which are low responders to lipopolysaccharide (LPS)¹. However, TLR4 alone is not sufficient to confer LPS responsiveness. TLR4 requires MD-2, a secreted molecule, to functionally interact with LPS². Furthermore, a third protein, called CD14, was shown to participate in LPS signaling, leading to NF- κ B translocation. This signaling is mediated through the adaptor protein MyD88 but also through a MyD88-independent pathways that involves the (TIR) domain-containing adapter protein (TIRAP)³.

TLR4A- Δ TIR-HA is a TIR-less form of the TLR4A gene generated by deleting the TIR domain (498 bp) and adding the influenza hemagglutinin epitope

tag (YPYDVPDYA) at the 3' end. The HA tag allows for simple and convenient detection of the expressed TLR- Δ TIR gene by Western blot using an HA primary antibody. InvivoGen offers an Anti-HA tag (catalog code: ab-hatag) that can be used to detect the expressed fusion protein.

- **EF1-HTLV prom** is a composite promoter comprising the Elongation Factor-1 α (EF-1 α) core promoter⁴ and the R segment and part of the U5 sequence (R-U5') of the Human T-Cell Leukemia Virus (HTLV) Type 1 Long Terminal Repeat⁴. EF-1 α is a 'housekeeping' gene ubiquitously expressed in eukaryotic cells. The EF-1 α promoter exhibits a strong activity, higher than viral promoters and, on the contrary to the CMV promoter, yields persistent expression of the transgene *in vivo*. The R-U5' has been coupled to the EF-1 α core promoter to enhance stability of DNA and RNA
- **SV40 pAn:** The SV 40 late polyadenylation signal enables efficient cleavage and polyadenylation reactions resulting in high levels of steady-state mRNA.
- **Ori** is a minimal *E. coli* origin of replication with the same activity as the longer Ori.
- **CMV prom:** The human cytomegalovirus immediate-early gene 1 promoter/enhancer was originally isolated from the Towne strain and was found to be stronger than any other viral promoters.
- **EM7** is a bacterial promoter that enables the constitutive expression of the antibiotic resistance gene in *E. coli*.
- **Pac:** The *Pac* gene encodes a puromycin N-acetyl-transferase that confers resistance to the antibiotic puromycin. Mammalian cells are generally sensitive to concentrations ranging from 1 to 10 μ g/ml depending on the cell line.
- **β Glo pAn:** The human beta-globin 3'UTR and polyadenylation sequence allows efficient arrest of the transgene transcription⁵.

References

1. Poltorak A. *et al.* (1998). Science, 282(5396):2085-8.
2. Shimazu R. *et al.* (1999). J Exp Med, 189(11):1777-82
3. Horng T. *et al.* (2001). Nat Immunol, 2(9):835-41
4. Kim *et al.* (1990). Gene 2: 217-223.
5. Yu J & Russell JE. (2001). Mol Cell Biol, 21(17):5879-88.

METHODS

Growth of pZERO-transformed bacteria:

Use sterile conditions to do the following:

- 1- Resuspend the lyophilized *E. coli* by adding 1 ml of LB medium in the tube containing the disk. Let sit for 5 minutes. Mix gently by inverting the tube several times.
- 2- Streak bacteria taken from this suspension on a puromycin LB agar plate prepared with the *E. coli* Fast-Media® Puro agar provided (see below).
- 3- Place the plate in an incubator at 37°C overnight.
- 4- Isolate a single colony and grow the bacteria in TB supplemented with puromycin using the Fast-Media® Puro liquid provided (see below).
- 5- Extract the pZERO plasmid DNA using the method of your choice.

Note: For long-term storage of the pZERO-transformed bacteria, prepare a 20% glycerol stock of the bacteria grown in the overnight liquid culture and freeze at -80°C.

TECHNICAL SUPPORT

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Selection of bacteria with *E. coli* Fast-Media Puro:

E. coli Fast-Media® Puro is a **new, fast and convenient** way to prepare liquid and solid media for bacterial culture by using only a microwave.

Method:

- 1- Pour the contents of a pouch into a clean borosilicate glass bottle or flask.
- 2- Add 200 ml of distilled water to the flask.
- 3- Heat in a microwave on MEDIUM power setting (about 400Watts), until bubbles start appearing (approximately 3 minutes). **Do not heat a closed container. Do not autoclave Fast-Media®.**
- 4- Swirl gently to mix the preparation. **Be careful, the bottle and media are hot, use heatproof pads or gloves and care when handling.**
- 5- Reheat the media for 30 seconds and gently swirl again. Repeat as necessary to completely dissolve the powder into solution. But be careful to avoid overboiling and volume loss.
- 6- Let agar medium cool to 45°C before pouring plates. Let liquid media cool to 37°C before seeding bacteria.

Note: Do not reheat solidified Fast-Media® as the antibiotic will be permanently destroyed by the procedure.

Preparation of cell extract for detection of HA-tagged® protein:

The buffer used to prepare the cell lysates is a modified RIPA buffer that is suitable for recovery of membrane receptors such as the TLRs.

- 1- Lift cells from 6-well plate and wash twice with PBS.
- 2- Add 100 µl of Cell Lysis Buffer and incubate 30 min. on ice.
- 3- Centrifuge 10 min @ 10,000 rpm (at 4°C if possible).
- 4- Transfer supernatant to a new tube and store @ -20°C.

Cell Lysis Buffer

- 5 ml of 100 mM Tris HCl, pH 7.4.
- 5 ml of 1M NaCl
- 100 µl of 0.5M EDTA
- 500 µl of 100mM NaF
- 500 µl of 10% SDS
- 2.5 ml of 10% Na-Deoxycholate
- 500 µl of 100% Triton-X 100
- 5 ml of 10% Glycerol
- H₂O to a final volume of 50 ml.

Store this stock buffer @ 4°C.

Determine amount of lysis buffer needed to make cell extracts and just before use add the following components at the final concentrations below:

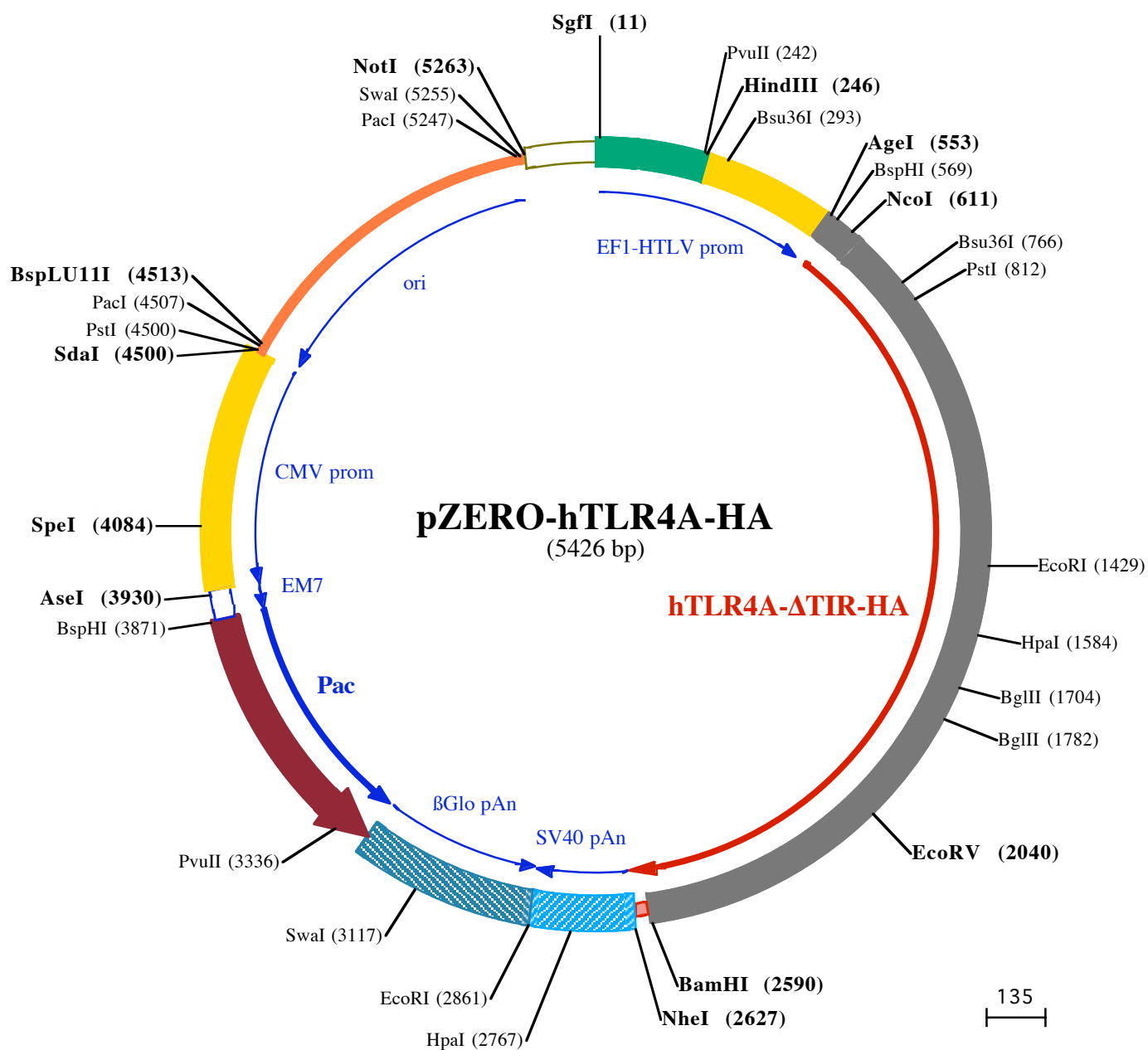
- 1 mM PMSF
- 1:100 Protease Inhibitor cocktail
- 2 mM of Na₃VO₄

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SgfI (11)
1 GGATCTGCGATCGCTCCGGTGCCCGTCAAGTGGGCGAGCCGACATCGCCACAGTCCCCGAGAAGTTGGGGGAGGGGTCGGCAATTGAACGGGTGCCTA
101 GAGAAGGTGGCGGGGTAACCTGGGAAAGTGATGTCGTGACTGGCTCCGCTTTTTCCCGAGGGTGGGGGAGAACCCTATATAAGTGCAGTAGTCGCC

HindIII (246) **PvuII (242)** **Bsu36I (293)**
201 GTGAACGTTCTTTTTCGCAACGGGTTTCCGCCAGAACACAGCTTGAAGCTTCGAGGGCTCGCATCTCTCCTTACGCGCCCGCCGCTACCTGAGGCC
301 GCCATCCACGCGGTTGAGTCCGCTTCTGCCGCTCCCGCTGTGGTGCCTCTGAACTGCGTCCGCCGTCTAGGTAAGTTTAAAGCTCAGGTCCGAGACC
401 GGGCCTTTGTCGGCGCTCCCTTGGAGCCTACTAGACTCAGCCGGCTCTCCACGCTTTGCTGACCCTGCTTGTCAACTCTACGCTTTGTTTCGTTT

AgeI (553) **BspHI (569)**
501 TCTGTTCTGCGCGTTACAGATCCAAGCTGTGACCGCGCTACCTGAGATCACCGGTAGGAGGCCATCATGATGTCTGCCTCGGCCCTGGCTGGGACT
1 MetMetSerAl aSerArgLeuAl aGl yThr

NcoI (611) **Bsu36I (766)**
601 CTGATCCAGCCATGGCCTTCTCTCTCTGCTGAGACCCGAAAGCTGGGAGCCCTGCTGGAGGTGGTTCCTAATATTACTTATCAATGCATGGAGCTGA
11 LeuI eP roAl aMe tAl aPheLeuSer CysVal A rgP roGl uSer TrpGl uP roCysVal I Gl uVal I Val I eThr TyrGl nCysMe tGl uLeuA
701 ATTTCTACAAAATCCCCGACAACCTCCCTTCTCAACCAAGAACCTGGACCTGAGCTTTAATCCCTGAGGCATTTAGGCAGCTATAGCTTCTTCAAGTTT
44 snPheTyrLysI l eP roAspAsnLeuP roPheSer Thr LysAsnLeuAspLeuSer PheAsnP roLeuArgHi sLeuGl ySer TyrSer PhePheSer Ph

PstI (812)
801 CCCAGAAGTGCAGGTGCTGGATTATCCAGGTGTGAAATCCAGACAATTGAAGTGGGCATATCAGAGCCTAAGCCACCTCTCTACCTTAATATTGACA
77 eP roGl uLeuGl nVal LeuAspLeuSer ArgCysGl uI l eGl nThr I l eGl uAspGl yAl aTyrGl nSer LeuSer Hi sLeuSer Thr LeuI l eLeuThr
901 GGAACCCATCCAGAGTTAGCCCTGGGAGCCTTTCTGGACTATCAAGTTACAGAAGCTGGTGGCTGTGGAGACAAATCTAGCATCTCTAGAGA
111 Gl yAsnP roI l eGl nSer LeuAl aLeuGl yAl aPheSer Gl yLeuSer Ser LeuGl nLysLeuVal I Al aVal I Gl uThrAsnLeuAl aSerLeuGl uAsnP
1001 TCCCCATTGGACATCTAAAACCTTGAAGAAGTAAATGTGGCTCACAATCTTCAAACTTTCAAATTTCAAACTTACCTGAGTATTTTCTAATCTGACCAATCT
144 heP roI l eGl yHi sLeuLysThr LeuLysGl uLeuAsnVal I Al aHi sAsnLeuI l eGl nSer PheLysLeuP roGl uTyrPheSer AsnLeuThrAsnLe
1101 AGAGTACTTGGACCTTCCAGCAACAAGATCAAAGTATTTATTCACAGACTTGGCGGTTCTACATCAAATGCCCTACTCAATCTCTTTAGACCTG
177 uGl uTyrLeuAspLeuSer SerAsnLysI l eGl nSer I l eTyrCysThrAspLeuArgVal I LeuHi sGl nMe tP roLeuLeuAsnLeuSer LeuAspLeu
1201 TCCTGAACCTATGAACCTTATCCAACAGGTGCATTTAAAGAAATTAGGCTTCATAAGCTGACTTTAAGAAATAATTTGATAGTTTAAATGTAATGA
211 Ser LeuAsnP roMe tAsnPheI l eGl nP roGl yAl aPheLysGl uI l eArgLeuHi sLysLeuThr LeuArgAsnAsnPheAspSer LeuAsnVal I Me tL
1301 AAACCTGTATCAAGGTCTGGCTGGTTAGAAAGTCCATCGTTTGGTCTGGGAGAATTTAGAAATGAAGGAAACTTGGAAAAGTTTGACAAATCTGCTCT
244 ysThr CysI l eGl nGl yLeuAl aGl yLeuGl uVal I Hi sArgLeuVal I LeuGl yGl uPheArgAsnGl uGl yAsnLeuGl uLysPheAspLysSer Al aLe

EcoRI (1429)
1401 AGAGGGCTGTGCAATTTGACCATTAAGAATTCGATTAGCATACTTAGACTACTACCTCGATGATATTATTGACTTATTTAATTGTTTACAAATGTT
277 uGl uGl yLeuCysAsnLeuThr I l eGl uGl uPheArgLeuAl aTyrLeuAspTyrTyrLeuAspAspI l eI l eAspLeuPheAsnCysLeuThrAsnVal I

HpaI (1584)
1501 TCTTCATTTTCCCTGGTGGTGTGACTATTGAAAGGTTAAAAGACTTTTCTTATAATTTCCGATGGCAACATTTAGAATTAGTTAACTGTAATTTGGAC
311 Ser Ser PheSer LeuVal Ser Val Thr I l eGl uArgVal I LysAspPheSer TyrAsnPheGl yTrpGl nHi sLeuGl uLeuVal I AsnCysLysPheGl yG
1601 AGTTTCCACATTAAGCTCAAATCTCTCAAAGGCTTACTTTCACTTCCAACAAAGTGGGAATGCTTTTTCAGAAGTTGATCTACCAAGCCTTGAGTT
344 I nPheP roThr LeuLysLeuLysSer LeuLysArgLeuThr PheThr Ser AsnLysGl yGl yAsnAl aPheSer Gl uVal I AspLeuP roSer LeuGl uPh

BglII (1704) **BglIII (1782)**
1701 TCTAGATCTCAGTAGAAATGGCTTGAGTTTCAAAGGTTGCTGTTCTCAAAGTGATTTTGGGACAACCAGCCTAAAGTATTTAGATCTGAGCTTCAATGGT
377 eLeuAspLeuSer ArgAsnGl yLeuSer PheLysGl yCysCysSer Gl nSerAspPheGl yThr Thr Ser LeuLysTyrLeuAspLeuSer PheAsnGl y
1801 GTTATTACCATGAGTTCAAACCTCTGGGCTTAGAACAACTAGAACATCTGGATTCCAGCATTCAAATTTGAAACAAATGAGTGAGTTTTCAGTATTC
411 Val I l eThr MetSer SerAsnPheLeuGl yLeuGl uGl nLeuGl uHi sLeuAspPheGl nHi sSerAsnLeuLysGl nMe tSer Gl uPheSer Val I PheL
1901 TATCACTCAGAAACCTCATTTACCTTGACATTTCTACACTCACACCAGAGTTGCTTCAATGGCATCTTCAATGGCTTGTCCAGCTCAGAACTTGA
444 euSer LeuArgAsnLeuI l eTyrLeuAspI l eSer Hi sThr Hi sThrArgVal I Al aPheAsnGl yI l ePheAsnGl yLeuSer Ser LeuGl uVal I LeuLy

EcoRV (2040)
2001 AATGGCTGGCAATCTTTCCAGGAAAACCTCTCCAGATATCTTCACAGAGCTGAGAACTTGACCTTCTGGACCTCTCTCAGTGTCAACTGGAGCAG
477 sMe tAl aGl yAsnSer PheGl nGl uAsnPheLeuP roAspI l ePheThr Gl uLeuArgAsnLeuThr PheLeuAspLeuSer Gl nCysGl nLeuGl uGl n
2101 TTGCTCCAACAGCATTAACTCACTCCAGTCTCAGTACTAAATATGAGCCACAACACTTCTTTTATTGGATACGTTTCTTATAAGTGTCTGA
511 LeuSer P roThr Al aPheAsnSer LeuSer Ser LeuGl nVal I LeuAsnMetSer Hi sAsnAsnPhePheSer LeuAspThr PheP roTyrLysCysLeuA
2201 ACTCCCTCCAGGTTCTTGATTACAGTCTCAATCACATAATGACTTCAAAAAACAGGAACTACAGCATTTCCAAGTAGTCTAGCTTTCTTAAATCTTAC
544 snSer LeuGl nVal I LeuAspTyrSer LeuAsnHi sI l eMe tThr Ser LysLysGl nGl uLeuGl nHi sPheP roSer Ser LeuAl aPheLeuAsnLeuTh
2301 TCAGAATGACTTTGCTTGTACTTGTGAACACCAGAGTTTCTGCAATGGATTAAGGACAGAGGCAGCTCTTGGTGAAGTTGAACGAATGGAATGTGGC
577 r Gl nAsnAspPheAl aCysThr CysGl uHi sGl nSer PheLeuGl nTrpI l eLysAspGl nArgGl nLeuLeuVal I Gl uVal I Gl uArgMetGl uCysAl a
2401 ACACCTTCAGATAAGCAGGCATGCCTGTGCTGAGTTTGAATATCACCTGTGAGTGAATAAGACCATCATTTGGTGTGCTGGTCTCAGTGTGCTGTAG
611 Thr P roSer AspLysGl nGl yMe tP roVal I LeuSer LeuAsnI l eThr CysGl nMe tAsnLysThr I l eI l eGl yVal I Ser Val I LeuSer Val I LeuVal V

BamHI (2590)
2501 TATCTGTTGTAGCAGTTCTGGTCTATAAGTTCTATTTTACCTGATGCTTCTGCTGGCTGCATAAAGATGGTAGAGGTGAAAACATCGGATCTACCC
644 al Ser Val I Val I Al aVal I LeuVal I TyrLysPheTyrPheHi sLeuMetLeuLeuAl aGl yCysI l eLysTyrGl yArgGl yGl uAsnI l eGl ySer TyrP r

NheI (2627)
2601 CTATGATGTGCCAGACTACGCCTAAAGCTAGCTGGCCAGACATGATAAGATACATTGATGAGTTTGGACAACCAACTAGAATGCAGTGAAAAAATG
677 oTyrAspVal P roAspTyrAl a●●●

HpaI (2767)
2701 CTTTATTTGTGAAATTTGTGATGCTATTGCTTTATTTGTAACCAATTAAAGTGAATAAACAAGTTAAACAACAATTGCATTCATTTATGTTTCAG

EcoRI (2861)
2801 GTTCAGGGGGAGGTGTGGGAGGTTTTTAAAGCAAGTAAACCTCTACAAATGTGGTATGGAATTTAAAATACAGCATAGCAAACTTTAACCTCCAAA
2901 TCAAGCTCTACTTGAATCTTTTCTGAGGGATGAATAAGGCATAGGCATCAGGGGCTGTTGCCAATGTGCATTAGCTTTTGCAGCCTCACCTCTTTTC
3001 ATGGAGTTTAAAGATATAGTGATTTTTCCAAAGTTTGAACCTGCTTTCATTTCTTTATGTTTTAAATGCACTGACCTCCACATTCCTTTTTAGTAAA

SwaI (3117)
3101 ATATTCAGAATAATTTAAATACATCATTGCAATGAAAATAAATGTTTTTTATTAGGCAGAATCCAGATGCTCAAGGCCCTTCAATAATATCCCCAGTTT

3201 AGTAGTTGGACTTAGGGAACAAAGGAACCTTTAATAGAAATTGGACAGCAAGAAAGCGAGCTTCTAGCTCAGGTTAAAGCTCCAGGCTTCCTTGTCATGC
200AAlaGlyProLysArgThrMetCys

PvuII (3336)

3301 ACCAAGTCTCTGGGCTTCTGGAACCTCAACATCAGCTGTACAGTGAATCCAGTCTTTTATAAAAAGGCAGGTTTCTGGGAGCAGAAGTTCCAGAAA
191sTrpThrArgProGlyGluProValGluValAspAlaThrValThrPheGlyLeuArgGluTyrPheProLeuAsnArgProAlaSerThrGluLeuPhe
3401 GGCAGGAAGCTCCAGCCCTTTCAGCAGCTTCAACTCCAGGCAGAACACAGCAGATCCCAGACCCTTCCCTGGTGGTCAGGGCTCACTCCAACAGTTGCC
158AlaProValGlyAlaArgGluAlaGluValGlyProLeuValValAlaSerGlyLeuGlyLysGlyGluHisAspProSerValGlyValThrAlaL
3501 AGAAACCAAGCTGGCTCTTTGGCCTGTGTGGTCCAGCAGACCTCCATTTGTTGTTGTGCTGCCAGCCTGCTTCCAGAGAGCTCAGCCATTCTGGTC
124euPheTrpAlaProGluLysProArgHisProAlaLeuLeuGlyGluMetGluGluGluAlaLeuArgSerGlySerLeuGluAlaMetArgProGly
3601 CAATTCAGCAAAAACAGCACCAGCTTCAACAGACTCAGGTGTGTCCAACTGCAACAGCAGCTCCATCATCTGCAACCCAACTTTTCCAATGTCCAG
91yIleGluAlaPheValAlaGlyAlaGluValSerGluProThrThrTrpValAlaValAlaAlaGlyAspAspAlaValTrpValLysGlyIleAspLeu
3701 TCCCACTCTGGTGAGGAAGATTCTTGCAGTCTGTCCACCTCTCAATGTGCCTGTCAGGGTCAACTGTGTGCTTGTTCAGGGTAGTCTGCAAAAGCA
58GlyValArgThrLeuPheLeuGluGluLeuGluThrValArgGluIleHisArgAspProAspValThrHisArgThrAlaProTyrAspAlaPheAlaA

BspHI (3871)

3801 GCAGCCAGTGTCTCACAGCTCTTGAACATCATCTCTGGTGGCCAGCCTCACTGTGGGTTTGTACTCAGTCATGATGGCCCTCCTATAGTGAGTGTAT
24IleAlaLeuThrArgValAlaArgProValAspAspArgThrAlaLeuArgValThrProLysTyrGluThrMet

AseI (3930)

3901 TATACTATGCCGATATACTATGCCGATGATTAATTGTCAAACACGCGTGGATGGCGTCTCCAGCTTATCTGACGGTTCCTAAACGAGCTCTGCTTATAT

SpeI (4084)

4001 AGACCTCCCACCGTACACGCCTACCGCCATTTGCGTCAATGGGGCGGAGTTGTTACGACATTTTGAAAGTCCCCTGTTGATTAAGTCAAAACAAACT
4101 CCCATTGACGTCAATGGGGTGGAGACTTGGAAATCCCCGTGAGTCAAACCGCTATCCACGCCATTGATGTACTGCCAAAACCGCATCATCATGGTAATA
4201 GCGATGACTAATACGTAGATGTACTGCCAAGTAGGAAAGTCCATAAGTGCATGTACTGGGCATAATGCCAGGCGGGCCATTACCCTGATTGACGTCAA
4301 TAGGGGGCGTACTTGGCATATGATACACTTGTACTGCAAGTGGGCAGTTTACCCTAAATACTCCACCATTGACGTCAATGGAAAGTCCCTATTGG

PstI (4500)

SdaI (4500)

4401 CGTTACTATGGGAACATACGTCATTATTGACGTCAATGGGCGGGGTCGTTGGGCGGTGAGCCAGGCGGGCCATTACCCTAAGTTATGTAACGCCTGCA

PaeI (4507) BspLU11I (4513)

4501 GGTTAATTAAGAACATGTGAGCAAAAAGGCCAGCAAAAAGGCCAGGAACCGTAAAAAGGCCGCTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGA
4601 GCATCACA AAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTTCCCTGGAAGCTCCCTCGTGCCTCTCCT
4701 GTTCCGACCCTGCCGTTACCGGATACCTGTCCGCTTTCTCCCTTCGGGAAGCGTGGCGCTTCTCATAGCTCAGCTGTAGGTATCTCAGTTCGGTGT
4801 AGGTCGTTGCTCCAAGCTGGGCTGTGTGCACGAACCCCGTTCAGCCGACCGCTGCGCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGTAAG
4901 ACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGCGGTGCTACAGAGTCTTGAAGTGGTGGCCTAACTAC
5001 GGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCG
5101 CTGGTAGCGGTGTTTTTTTTGTTTGAAGCAGCAGATTACGCGCAGAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCA

PaeI (5247) SmaI (5255) NotI (5263)

5201 GTGGAACGAAAACCTCACGTTAAGGGATTTTGGTCATGGCTAGTTAATTAACATTTAAATCAGCGGCCAATAAAAATCTTTATTTTCATTACATCTGT
5301 GTGTTGGTTTTTTGTGTGAATCGTAACATACTCCTCCATCAAAACAAAACGAAACAAAACAACTAGCAAAATAGGCTGTCCCAGTGCAAGTGC
5401 AGTGCCAGAACATTTCTCTATCGAA