

According to

[https://www.ncbi.nlm.nih.gov/nucore/328349772?from=1335369&to=1336727&sat=2&sat\\_key=46463764](https://www.ncbi.nlm.nih.gov/nucore/328349772?from=1335369&to=1336727&sat=2&sat_key=46463764)

EHT1 mRNA: Available without EcorI and NotI sites, available

**ATGCCATCTTGGGGGTTC**ATAATCAGATTCATGCTACCTACAGCGATAAATCGGTG  
GCACTCAAGCTGAAGTCTGGCAAGAACGACTTTGCACCTTTGAATGTCATTCCTTTT  
TCTACATTTATTAAGACTTTAGTACCTGAACTCAGCGATGGCTACAAATCAACCTT  
CACCTTTTCTGTTCAATGGCGCTCTCCAGACTTTATACGCGTCCAAAGCTGACTAT  
AGTAAAGAATTTCAAATATACTATGGAAGATGCATGGTTCGATATCCTGAGGCAAAT  
CATGAGTATCCACATTTACATCTTGGTCAAGCGACTGCGGATTTTGTAGTTGATCCA  
CCAACAGAAATCGATGAATGGAATTATTTATACAATCAGACTTTACCTGAAGGATGG  
CCTCGATTGCATCCGCGGACTAGATTCCTAACAGTGCAAGAAGAACAAGACCTGGAA  
GCTGACTGGGCCTCAAACCTCTGGATCAATTGTTGTAATTATTCACGGATTGGCCGGT  
GGCTCGCACGAACCGGGAATACGAGATGTGAGCCAGCATCTTCATCAAGCTGGTTTTT  
AATGTTGTGACACTCAACTCTCGTGGATGTTGTGCGCTCTAAATTGTCAACAGGAAGA  
CTTTACTCGGCCGTGAAACAGATGATCTCCGTTACTTTATTGATGAGCTACATAAG  
AAAATACCAACAAGCCTATCTATCTTTTGGGATTTTCTTTAGGGTCTGCACTGGTT  
CTCAATTATCTTGGCGAGGAAGGAAAGAAGAGCTTCATCAAAGTGCGGTCACTATC  
GGAGCACCTGTAGACCTCCTGGACTCCCATTATCATTTGAACCACAGTTACTCTGGA  
AAGTATCTATTCGATCCTGCAGTGGCTTCATTCTTGTCAAATTTGGTAAAAGTTAAC  
TTCCAATTCTAAACCGTGATAAACCGGAAGTATTCTCATACGAAAAGAAGTCCAAA  
GATTACTTGGTACGGAGAATCGTCGACTTTGATCGAAAATATACTGCCCCAGCGTAT  
GGATTTGCAAGTCCAAAATATTACTATCAAGCTGGATCTCCCTTAACCAAGCTAGTA  
AATATTTACTCTCAACAGTACTGCTGAACTCCTTAGATGATCCGGTAGTATCGTGT  
AATTTACCAGTGGATGAAGTTAAAGCAAACCCATATCTATATCTAGCGGCCTCAGAT  
TTAGGTGGTCATTTGGCTTATATTCAGTGGGATGGTGGATTCTGGTTTAGCGAAGCC  
GTGTCTGCATACTTCATGGCATTGGAATCGGAACTGTCAAACGAACCTGCACAAAGT  
GATTACGAGCCTCAGCTAAGTGAATA**CAGCTATAAACTGGAATT**TGA

EHT1 EcorI F: TTTGAATTCATGCCATCTTGGGGGTTC

EHT1 NotI R: ATAAGAATGCGGCCGAATTCCAGTTTTATAGCTG

### Reaction system: 50 µL

5 X PCR buffer	10 µL
cDNA	2 µL
EHT1 Primer F	1 µL
EHT1 Primer R	1 µL
Primastar	0.5 µL
ddH <sub>2</sub> O	35.5 µL

**PCR reaction conditions:**

98 ° C	2 min	
98 ° C	10 S	
56 ° C	15 S	35cycles
72 ° C	1.5 min	

**EHT1 gene knockout**

**1. Amplification the left and right recombinant arms**

CCCAGGGCGCTCCAGCCCAGCCGACATTCACCAATTCAACGTTTCGTCACGATGATCAGAATATCTATA  
TCAACGATACTGCCATTCCAAAAGAAGAGTTCATGTACGCTTTTGGGGGTACCTTAACCACTGGTCGTC  
AGTCTGTGCATCGCAGCTTTGGCAACCCTGTACCCGCTGGACTTGCAGGATTCCTTAGGAACTTTTG  
CCCTGGGTCTAATTCTTCTCAGGCCAGAGGCGTAAACGGACGTTAGCATTCTCATTGGAATGTTCCTTT  
TTGTTTCAGGAGTAGTTGAAAATAATTGCTGGTATTTGGTGTATCGTGGTGGAAAATACCTGGGCCAGTG  
TAGTATTTCTGGGCTATGGTCTTTCTGGCTTGGATATGCTGCAATTGTAACCTCCGGCATTGGTGTTGC  
TGCTGCCTATGAAACAGCTGAAGATTTCAACAATGCCGTTGGAATGTACCTATGCCCTTGGGCAGTCTT  
CAGTTTTATTCTCTGGAGTTGTACTTGGAAATCGACCTGGCCGCTCACCGCCTTAGTCTTCTGCATTTTT  
ATGATATTTCTAGTATTGACAATTGGGTATTTTGTAGCTCGGTGCCAACAATTAAGCTGGAGGAGTCT  
TTTGTTTCGTGGGTAGTCTCTTGGGATTTTACAACATGTATGCAGGTTTGGCTAATCCAACCAACAGTTA  
TGTTGTTGTCCATCTCTATTCATGCCTAATGCAGCCAAGCCAGCTAGTTAAGTAGTTTATTTACTTGAG  
GAAATCCAACAACCTAGTTAACAGTTAACGTGTTTTTTTTTCTCGGACTCCTTATGGAGCCGCACCC  
CCGGTGTGAAACCCCATCCACATATGATCTCCCCCGCACATGAAGACTTAACAAGTCAGCCACCC  
CCTCCTCCGCACCCCTCAGTTATACGAAAATGGTAAGGAGGAATTACGTTTTCTCGGACTCCTAACCC  
CTGACACCCATTTTTAGATGTCCCTCTAAACCCCTCCTCAGCTTGCAGCACCCCATGATAGTTTTCAGTT  
CATTTTCGTAGGTTAGTGACCATTTTCTTGCAATATATATTGAAACGATTTCTCCCTTAGCCATATTT  
ATTTCTCGTTTACCACCTACAGAGAGTTTTATATCAGCAGTTTGAAGTTTTGTTCTTGTCTAATGTCT  
CAATCTCAATCAATTTAGATCTTGCCCGATGTCGAAAAGGGCAACCCAATCGAACAATACCAAC  
CACATCAACTTTCCGTACAGATGATCAAAAATATCTACATCAACGATACTGCTATCCAAAAGAAGAGTT  
CATGTACGCTTTTGGGGGTACATTGACTACTGGTCGTCAATCTGTGCATCGTAGTTTTGGTAATCCTGTC  
CCTGCTGGTTTTGGCGCTTTTTCTTTGGGTACATTTTCACTAGGGCTTATCCTTCTGCAGGCCAGAGGT  
GTGACAGATCCAACGATACTTGTGGGATTGTTTTATTTGTTGCAGGGCTTGTGGAAATTATTGCAGGTA  
TATGGTGCATTGTCGTGGAGAATACTTGGGCCAGTGTAGTTTTCTTGGGATACGGTTCTTTTTGGATAGC  
TTATGGATGCATCGTGACCCCGGATTGGGACTTGTGCGGCCCTACGATACAGTTGAAGACTTCAATAA  
TGCTGTGGCAATGTTTTATGTCCATGGACCGTACTGACTTTCATCCTTTGGAGTTGTACCTGGAAATCC  
ACTTGGCCACTGACCGCCCTGGTTTTCTGTGGTGTCCATTGTTCTTAGTACTGACGATTGGATACTACA  
CCAGCTCAGCTGGTACTATCAAGGCTGGTGGAGCTGTATCGTTTGTGGAAAGTATACTTGGTTTTTACA  
ATATGTATGCTGGAATTGCAGACTCTACAAACAGTTACTTGGTTGTCAAACCATTGTTTATGCCTCATGC  
AGCAACTCCGAAGTATGAATGCACCAATCGTATAAAGTTTGGACTCGACGTCACGATCTCTATTTTC  
AATTAATCTGATTAATGTATAACTCAAATTCAGTTTTATAGCTGTATTCACTTAGCTGAGGCTCGTAAT

CACTTTGTGCAGGTTTCGGATTCAAATGCCATGAAGTATGCAGACACGGCTTCGCTAA  
ACCAGAATCCACCATCCACTGAATATAAGCCAAATGACCACCTAAATCTGAGGCCGCTAGATATAGAT  
ATGGGTTTGCTTTAACTTCATCCACTGGTAAATTACACGATACTACCGGATCATCTAAGGAGTTCAGCA  
GTACTGTTGGAGTGTAATATTTACTAGCTTGGTTAAGGGAGATCCAGCTTGATAGTAATATTTGGACT  
TGCAAATCCATACGCTGGGGCAGTATATTTTCGATCAAAGTCGACGATTCTCCGTACCAAGTAATCTTT  
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TTTGACAAGAATGAAGCCACTGCAGGATCGAATAGATACTTCCAGAGTAACTGTGGTTCAAATGATAA  
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TCGCCAAGATAATTGAGAACCAGTGCAGACCCTAAAGAAAATCCCAAAGATAGATAGGCTTGTGGG  
TATTTCTTATGTAGCTCATCAATAAAGTAACGGAGATCATCTGTTTCGACGGCCGAGTAAAGTCTTCCT  
GTTGACAATTTAGAGCGACAACATCCACGAGAGTTGAGTGTCAACAATTAACCAGCTTGATGAAG  
ATGCTGGCTCACATCTCGTATTCGGTTCGTGCGAGCCACCGCCAATCCGTGAATAATTACAACAAT  
TGATCCAGAGTTTGAGGCCAGTCAGCTTCCAGGTCTTGTCTTCTTGCACTGTTAGGAATCTAGCCG  
CGGATGCAATCGAGGCCATCCTTCAGGTAAAGTCTGATTGTATAAATAATTCCATTCATCGATTTCTGTT  
GGTGGATCAACTACAAAATCCGCAGTCGCTTGACCAAGATGTAAATGTGGATACTCATGATTTGCCTCA  
GGATATCGAACCATGCATCTTCCATAGTATATTTGAAATCTTTACTATAAGTCAGCTTTGGACGCGTATAA  
AGTCTGGAGAGCGCCATTGAACAGAAAAGGTGAAGGTTGAATTTGTAGCCATCGCTGAGTTCAGGT  
ACTAAAGTCTTAATAAATGTAGAAAAAGGAATGACATTCAAAGGTGCAAAGTCGTTCTTGCCAGACTT  
CAGCTTGAGTGCCACCGATTTATCGCTGTAGGTAGCATGAATCTGATTATGGAACCCCAAGATGGCAT  
GTAGGTGTAATTTTCGAAGAAAAGAAATCTAACGATTCGTCATAAAGGAAATGAAAAACGTTGTCCG  
AACCGAACAGAATTGAACCTTTATTGCCGCAACCAATTTATTTATCTGAAAATGTAAATATTGAAATTC  
TACACGATTAACACTAATTATAGGTGTTCTCTCTAATGGTACTACACCTGGAGTGGTTGTTCAACAATGAA  
TTCCTCCATTGCTTAAGAAATGAATTGAGCCATAGATGGTCCCTCCACACCACAGGTTGGCATGATT  
CTCATTTTCACATGCACAATTTTTGTTGTGCAAACCTAAGCTAACTTGACTCCATCCTTACACCATATTG  
CCTTGAATGTTGGCGCCCTATTGATTGTTGTTTCACTTGCCATCTCATATAACCCTAGGACTTGAATAT  
TCCTGTTCTAAACCCCCCAAAGCTCCTAATGGCTTCTAAGAGGACCTCAGTTCGAGGGGAATCAGAT  
TCAAATAACTATGCCTTATCAGACTCCTCCGATTTGAAGACAATGGGCATCCGAGATTGTCAAAAAGA  
CGATCTAGCCTGTTTTCTTTGAGTTCATTAAGCTCAGCCGAACCTTTACCACCTCCGGACGAAAAAGAT  
TTTATTGCGTTTACATCTGACGACAGACATTTCTCTTTGCTTCGAAATCTTCATATGGCAGATTTCATAAC  
GTTAATGAATGGTTTTCCGGTTTTATTCTATTGTTTCTGTCTTCGTTTACCTTGACTAAGCAAACCTC  
ACTACGTACAAAGGGCACACTTCTCATAATTTGGGTCTTTTTTTGACTTTTTTTGACGGTAAAGTTGC  
CAGATTGCGCAACAAAGCATCATTGATTGGTCAGGAGTTGGACTCTTTAGCCGATCTGATTTCAATTTGG  
GGTTGCTCCCGCTCTATTGCATTTCCCTAGGTTTACAACTACATTAGACGTCCATAGCCTAACATTT  
CTGGTTCTGTGTGGACTCACCAGATTAGCTAGGTTCAACGTAACAGTGGCCAACATAACAAAAGATAT  
CAAGGGTAAAGCTCACTATTTCGAAGGATTACCGATCCCTTCGACCCTGTGGCTAGTATTTAGTATGGC  
ATTTTGTGTTTATAAAGGATGGTATGAGTATGAGGATGGCCTTCCCTGGTCCATTATTATGGAAAGGAAAA  
TTTTTTGAATTCATTTGATTTCACTCTTTTTTGTCAATCAAGGATGTTGATGACCTCCAAAAGTCTCA  
AAATACCTAAACCTTGAATATGGATATGGTTGCATTATCAGAGTTCCGCCCATATTATTTCTGTGTAACC  
AAATTTACAGGTAGATATAATCTTCTATTACATATATTTACGAGCATGTGCAATTTTTTCATCGGAAG  
GATTAACGAATTTCTTACTTTTCAATAACAAAAGCAGCAAACTGTTGAAGCAAGAGACAACCCCCAC  
CAGGTTACGAGATAATTTAAATGGTTGTTTGTGTAATCAACTTTTGGTACTTTGCCAATAGGTACTCCAG  
CATTTACGAAGTGAATGGGCTGAATTCAGCGATGAGTCTTGATAAGCAGAAACCTTTGCCTCCCAA  
CCATGGGAGCCCGAAATGTTTCCAGCTGTGATTGATCATCGTTGTTTCACTTTCACTTTGCCACCGCC

ACCTCCACAAGTTCCATAAACCTCCTGATGATTCATGCCCGATTTGCTGGCCCTATCCTTAAGCCATTC  
 AGGATGATCCTTGAAATCTTGTACCACTTGGGCATATATTGGCTTGCAATTAGTCTCCTTGCACATTTCGC  
 TCCAAATCGACATATCTGAACATATTTGCCCTGGCTTATGTTCAATGTGAAACATTCCTTTTCGATGGTG  
 GTACTIONTCAAAGACATTCTAAAGTTATTGTACCCTCTGGCATAGATAAGTTTTTTAGCCTTCTTTTTCC  
 TTGGTTCACTTTCCCTTCAGATATCCAGCCTCTTCCACTAAAATCTTCCACCTCCATTAGATCTTGTG  
 AAAGGAGTGACAAGGATATAGCCCTTTCTGTCATCGTGCAATCGGGGACCTACTAACAGTTCCTTTTCG  
 TAGTCAAATCTACCAGTTATGAGCACCTTGCGATACTCGAAGTTCTCGACATCCTCTTCCCTGAAGTTC  
 TTGGGAAGAGGAAGTGGCTTGTATGTCAATCTGTCTTCCACTCAGCTACTAGGGAATTTTTCCATTTCC  
 AATCGCTGTAACTGCCAAAATCCGAGCCCCAAAGTTACTACTGGAGTTAGGCAAAGAAGGCCAAATAC  
 AACACTCCGCCAGTGCAGTGTGTTTCCATCTCTTCAAGGATTTGTTAGATGTAATTGGTTTCCAA  
 TCGATATCTAAGGTCTTGGGTGTTGCAGGGGGTTTAGGTGGTTCGAAGTGCAGAAATCTGCGGATCAA  
 AGAGATACTAGTTCATTTCTGAATTTTCGTACAAACATATGTCCTAACCAACACAAGAAGAACTTCT  
 AGCTGTGTTTGAAAAGTGCTCGAATTTCAAGTTGATGGATGCCGAAGGGAAGATAAGTTACCTTTGCT  
 AAATTCCTTAGTGCTTGACGAGGAATTGAGACGATAGAAAGTAACAAGTTGAAACAAGTATAAAATCA  
 AGACAAGATAATATAAACCAATTCATTGGGATTCACGTCCCATCTGTTTTATCAACTACCTGTCACCTC  
 TAAATTTCTCTCCACTGCGGGTTAGATCTTTACTCTAAATGCTATTAATAAATC

**The four primers below should be synthesized. Please note that there is a U in each primer.**

EHT1O1           GGTCTTAAU TGGAAAATACCTGGGCCAGT  
 EHT1O2           GGCATTAAU CTGTCAAACGAACCTGCACA  
 EHT1A3           GGACTTAAU GTTGGCGCCCCCTATTGATTT  
 EHT1A4           GGGTTTAAU CGGTGGCAAAGTGAAAGTGA

**Running PCR on Genomic DNA to amplify recombinant region**

PCR buffer 10X	1ul
dNTB 10mM	0.2 ul
Primer F 10uM	0.5 ul
Primer R 10uM	0.5 ul
Pol	0.2 ul
gDNA 10 ng tot (240 ng)	0.05 ul
H2O	7.55 ul
	-----
	10 ul

**Ligation of the PCR products with the vector**

Mix the following in a 0.2 ml PCR tube:

Tube 1 Tube 2

PCR product 1	75 ng/ul	5.3 ul
PCR product 2	~160ng/ul	2.5 ul
pH vector	315 ng/ul	0.6 ul

USER enzyme	1 ul
H2O	<u>0.6 ul</u>
Total	10 ul

Incubate at 37 C for 20 min followed by 25 C for 20 min.

### **Transformation of E. coli with the construct**

Transfer the ligation reaction mix to a precooled 1.5 ml Eppendorf tube.

Add 100 ul of chemically competent E. coli DH5 $\alpha$  cells ( $> 1 \times 10^6$  cfu) to the eppendorf tube.

Mix gently by tapping on the tube a couple of times and incubate for 30 min on ice.

Heat shock the cells by incubating them at 42 C for 45 sec, immediately afterwards return the cells to ice and incubate 2 min.

Add 900 ul of culture medium (SOC) preheated to 37 C, mix by inverting the tubes a couple of times and incubate for 1 hour at 37 C with shaking at 300 rpm.

Plate 100 ul directly on 1 plate LB + kanamycin.

Pellet the cells in a table top centrifuge for 1 min at 5000 rpm, discard about 9/10 of the supernatant and resuspend cells gently by pipetting up and down until cells have been separated. Plate approximately 50 ul on to single LB plate supplemented with 25 ug/ml kanamycin A.

Incubate plate overnight at 37 C.

### **Validating the presence of the inserts in E. coli transformants**

Pick up ~20 E. coli colonies from the kanamycin plates using sterile pipet tip fitted on the pipet itself and suspend in 40 ul water.

PCR:

Stock		1 rect.
Buffer	10x	1 ul
dNTPs	10mM	0.2 ul
Primer F	10 uM	0.4 ul
Primer R	10 uM	0.4 ul
Taq*	---	0.1 ul
H2O	-	7 ul
Colony	-	1 ul

Each clone has two reactions