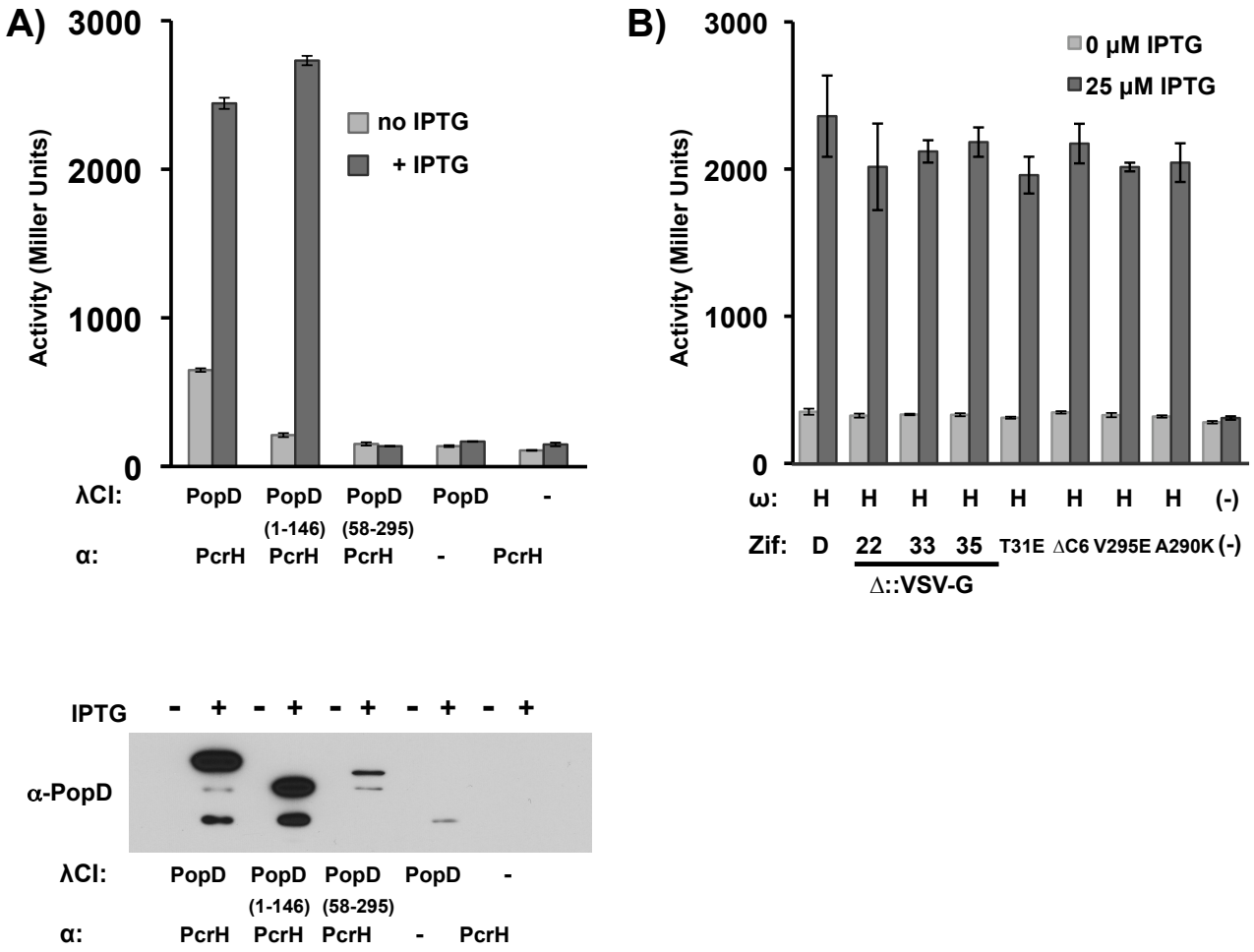

Fig. S1. PopD N-TES and C-TES mutants retain the ability to bind to PcrH.
The interaction between PcrH and PopD was assayed by two-hybrid analysis. (A) PcrH was expressed as a fusion to RNA polymerase subunit alpha, and the indicated portion of PopD was fused to lambda cI [full length, the amino-terminal half of PopD (1-146) or the C-terminus excluding the chaperone binding domain (58-295)]. Expression of the fusion proteins was induced with 50μM IPTG, with the exception of the cI-PopD(58-295) fusion protein, which was induced with 250μM IPTG. Cell extracts of the assayed strains were separated by SDS-PAGE and probed for the presence of PopD by Western blot (panel below the beta-galactosidase assay results). As in *P. aeruginosa*, PopD was unstable in the absence of PcrH or when the fragment fused to cI was unable to bind to PcrH (PopD 58-295). (B) PcrH was expressed as a fusion to RNA polymerase subunit ω and PopD TES mutants were expressed as fusions to the monomeric DNA binding protein Zif. Expression was induced with 25 μM IPTG. Protein interaction was measured in *E. coli* KDZif1 by virtue of their ability to activate *lacZ* expression from a test promoter.

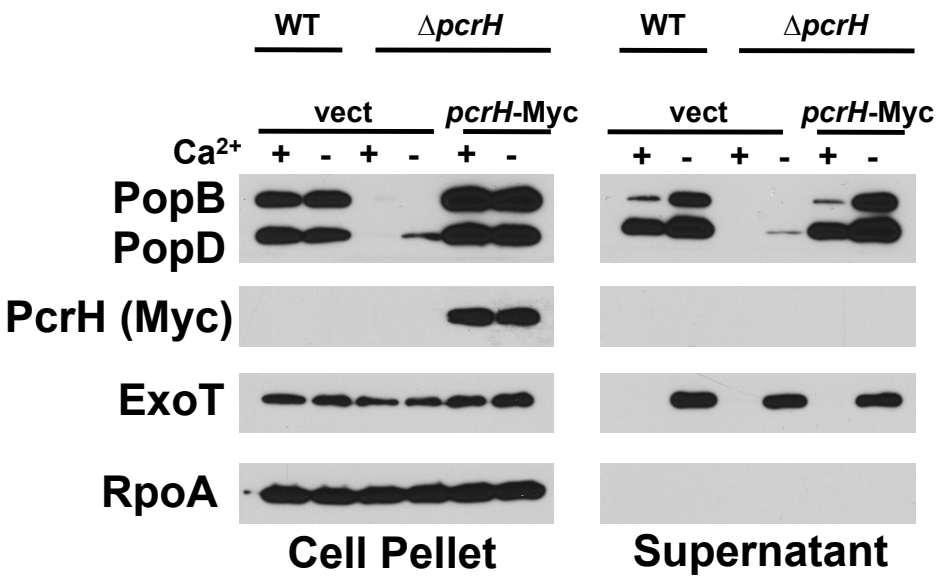

Fig. S2. PcrH stabilizes PopB and PopD in the cytoplasm of *P. aeruginosa* PAO1. Expression and secretion of PopB and PopD was assayed by RECC assay in a *pcrH* null mutant complemented with a plasmid expressing a Myc-tagged version of PcrH or vector alone. The expression and secretion of PopB and PopD was compared to the parental strain (PAO1F Δ*fleQ*). Expression of the Myc-tagged PcrH was detected by Western blot. The RNA-polymerase alpha subunit served as loading control and ExoT export was detected to illustrate that the type III secretion machinery is intact and otherwise functioning normally.

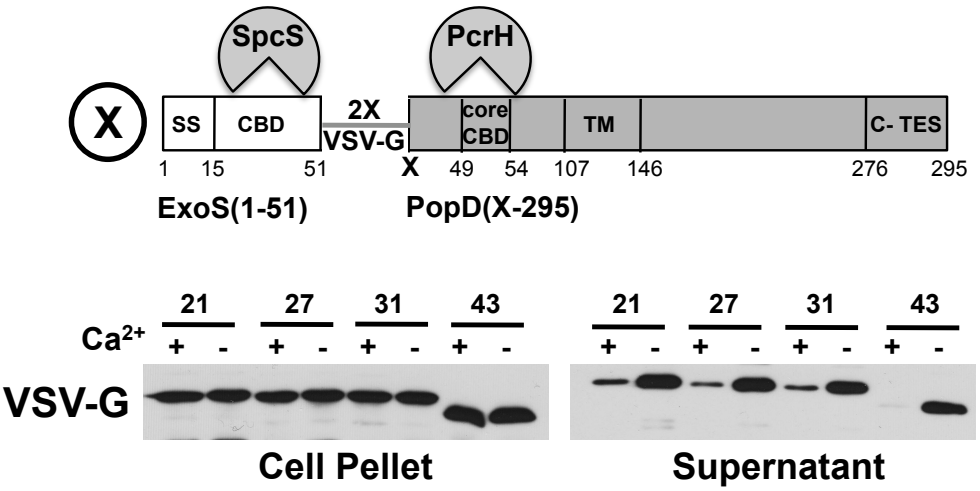

Fig. S3. A chaperone-adjacent domain of PopD is required for export of the translocator before effectors.

Fusions of ExoS(1-51) to amino-acids 21-295(21), 27-295(27), 31-295(31) or 43-295(43) [ExoS(1-51)-VSV-G-PopD(**X**-295)] were expressed in PAO1F Δ*fleQ* Δ*exoS* Δ*popBD* and their export was monitored in the presence or absence of calcium. The presence of the fusion protein in the cell pellet or supernatant fractions was determined by Western blot directed against the common VSV-G epitope.

Table S1. Primers used in this study

Primer Name	Sequence (5' to 3')	Description
HisMBP-5R	ATATAgaattcTAAGGAGGCGCCCCCATGCACCAC CATCACCATCACCATCACAAAATCGAAGAAGGT AAACTGG	5' primer to create N-terminally His tagged malE (MBP) without signal sequence for fusions with EcoRI site
malE-3K	ATATAggtaccTgcggccgcCTTGGTGATACGAGTCT GCGC	3' primer to amplify malE, inserts NotI site in polylinker, with KpnI site
popD5R	AAAAAgaattcTTAGGAGGCGCCCCCATGATCGAC ACGCAATATTCCCT	5' primer for popD ORF with EcoRI site
popDEX3	AAAAAaagcttCGCGCGGAGACGGCTCAGACCACT	3' popD primer with HindIII site
popDd2-10-Eco-5	AAAAAgaattcTTAGGAGGCGCCCCCATGACCCAG GCCGCGATCCCCTCCGA	5' primer to delete codons 2-10 from popD with EcoRI site
popDd11-21-Eco-5	AAAAAGAATTCTTAGGAGGCGCCCCCATGATCG ACACGCAATATTCCCTGGCGGCTCCCGGCGCC GCCGGGCGTTCC	5' primer to delete codons 11-20 from popD with EcoRI site
S20-D21-eco-5	AAAAgaattcTTAGGAGGCGCCCCCATGCATATTC AATCGCTTCAGCAGAGTCCGTCTTTCGCCGTCG AATTGCACCAGGCCGCCGCTCCCGGCGCCGCC	5' primer to fuse codons 1-20 of exoS to popD codon 21+ with EcoRI site
DS-5R	AAAAAgaattcATCAGGAGAAGGCAACCATCATGA TCGACACGCAATATTCCCTGGCGGCTACCCAGG CCGCGATCCCCT	5' primer to fuse codons 1-20 of popD to exoS codon 21+ with EcoRI site
DS-5	GCGGCTACCCAGGCCGCGATCCCCTCCGAGCC GATCAGTGGGCGTTTGGGACAGATTGAGG	5' primer to fuse codons 1-20 of popD to exoS codon 21+
exoS3H	AAAAAaagcttGGTCAGGCCAGATCAAGGCCGCG CAT	3' exoS primer with HindIII site
popDdC20-3H	AAAAAaagcttTCACTGCAGGACGTCCTTCATGAA GCCGGCA	3' primer to delete the last 20 codons of popD with HindIII site
popB5R	AAAAAgaattcTTAGGAGGCGCCCCCATGAATCCG ATAACGCTTGAA	5' primer for popB ORF with EcoRI site
popBEX3	AAAAAaagcttGACGTCTCCTCAGATCGCTGCCGG T	3' popB primer with HindIII site
popBdC20-3H	AAAAAaagcttTCAGAAGATCCGCTCCATGATTTCC TGGAAT	3' primer to delete the last 20 codons of popB with HindIII site
popDdC6-3-H	AAAAAaagcttTCAACGCCAGGCCTGGTTATGGCT C	3' primer to delete the last 6 codons of popD with HindIII site
popDA290K-3H	AAAAAaagcttTCAGACCACTCCGGCCGCCTTACG CCAGGCCTGGTTATGGCTCTGGGT	3' primer to make popD A290K mutation with HindIII site

popDV295E-3H	AAAAAaagcttCGCGCGGAGACGGCTCACTCCACT CCGGCCGCCGCA	3’ primer to make popD V295E mutation with HindIII site
popDA290C-3H	AAAAAaagcttTCAGACCACTCCGGCCGCGCAACG CCAGGCCTGGTTATG	3’ primer to make popD A290C mutation with HindIII site
popDV295C-3H	AAAAAaagcttTCAGCACACTCCGGCCGCCGCACG CCAGGCCTGGTTATG	3’ primer to make popD V295C mutation with HindIII site
popDT31E-3-1	CGGCGCCGCCGGGCGTTCCGTCGGCGAGCCG CAAGCGGCTGCGGACCTGCCGC	5’ primer to make popD T31E mutation
popDT31E-5-2	GCGGCAGGTCCGCAGCCGCTTGCGGCTCGCCG ACGGAACGCCCGGCGGCGCCG	3’ primer to make popD T31E mutation
popDd100-170-3-1	TCCAGCGCGACAACGAGAACCAGGCGCTTGGC AAGACCT	5’ primer to delete codons 100-170 of popD
popDd100-170-5-2	AGGTCTTGCCAAGCGCCTGGTTCTCGTTGTCGC GCTGGA	3’ primer to delete codons 100-170 of popD
popDd150-220-3-1	TGAAGAACGGCAAGGCCATCCAGTCCTTCGTCC AGATGGCCAA	5’ primer to delete codons 150-220 of popD
popDd150-220-5-2	TTGGCCATCTGGACGAAGGACTGGATGGCCTTG CCGTTCTTCA	3’ primer to delete codons 150-220 of popD
popDd190-270-3-1	TCGTCGGCAAGGTCTGGGCGAAGGACGTCCTG CAGCTCATC	5’ primer to delete codons 190-270 of popD
popDd190-270-5-2	GCAGGACGTCCTTCGCCCAGACCTTGCCGACG ATCTTGCGA	3’ primer to delete codons 190-270 of popD
popDd190-280-3H	AAAAAaagcttCAGACCACTCCGGCCGCCGCACG CCAGGCCTGGTTATGGCTCTGGGTCGCCCAGA CCTTGCCGACGATCTTG	3’ primer to delete codons 190-280 of popD with HindIII site
popD(22-32)VG-5Pvu	AAAAAcgatcgCTTATACAGATATTGAAATGAATAG ATTAGGAAAACAAGCGGCTGCGGACCTGCCGC AGGT	5’ primer to replace codons 22-32 of popD with a (1X) VSV-G tag with PvuI site
popD(33-43)VG-3-1	TATACAGATATTGAAATGAATAGATTAGGAAAAG CGCGGGCCGACCGGGTCGAACTGAA	5’ primer to replace codons 33-43 of popD with a (1X) VSV-G tag
popD(33-43)VG-5-2	TTTTCCTAATCTATTCATTTCAATATCTGTATACG GCGTGCCGACGGAACGCCCGGCGGC	3’ primer to replace codons 33-43 of popD with a (1X) VSV-G tag
popD(35-45)VG-3-1	TATACAGATATTGAAATGAATAGATTAGGAAAAG CCGACCGGGTCGAACTGAACGCTC	5’ primer to replace codons 35-45 of popD with a (1X) VSV-G tag
popD(35-45)VG-5-2	TTTTCCTAATCTATTCATTTCAATATCTGTATACG CTTGCGGCGTGCCGACGGAACG	3’ primer to replace codons 35-45 of popD with a (1X) VSV-G tag
popD(58-68)VG-3-1	TATACAGATATTGAAATGAATAGATTAGGAAAAG AGCTGGACAGCAGCGTCGAGCTT	5’ primer to replace codons 58-68 of popD with a (1X) VSV-G tag
popD(58-68)VG-5-2	TTTTCCTAATCTATTCATTTCAATATCTGTATACA CCTGGCGCGGAGCGTTCAGTTC	3’ primer to replace codons 35-45 of popD with a (1X) VSV-G tag

exoS5R	AAAAAgaattcATCAGGAGAAGGCAACCATCATGCATA	5’ exoS primer with EcoRI site
exoS51-2VG-Not	AAAAAgcggccgcTTTTCCTAATCTATTCATTTCAATATCTGTATATTTTCCTAATCTATTCATTTCAATATCTGTATACTCACCCTTCGGCGCGTCCTG	3’ primer to amplify exoS codons 1-51 with (2x) VSV-G tag and NotI site
popD21-5-Not	AAAAAgcggccgcAGCTCCCGGCGCCGCCGGGCGTTCCGT	5’ primer to amplify codons 21+ of popD with NotI site
popD27-5Not	AAAAAgcggccgcACGTTCCGTCGGCACGCCGCAAGCGGCT	5’ primer to amplify codons 27+ of popD with NotI site
popD31-5Not	AAAAAgcggccgcAACGCCGCAAGCGGCTGCGGACCTGCCGCA	5’ primer to amplify codons 31+ of popD with NotI site
popD43-5Not	AAAAAgcggccgcAGCCGCGCGGGCCGACCGGGTCGAACT	5’ primer to amplify codons 43+ of popD with NotI site
popD96-5-Not	AAAAAgcggccgcAGACAACGAGAACCAGTCGATCATCCA	5’ primer to amplify codons 96+ of popD with NotI site
pcrH5R-opt	AAAAAGAATTCGAAACATCAGGAGAAGGCAACCATCATGAACCAGCCGACCCCTTCCGAC	optimized pcrH5R; contains region upstream of exoS
pcrH2Myc-3-1	ATTTCTGAAGAAGATTTGGGCGAGCAGAAACTGATCTCGGAGGAGGACCTGCAATGAATCCGATAACGCTTGAAC	5’ primer that introduces a (2X) Myc tag at the C-terminus of pcrH with HindIII site
pcrH2Myc-5-2	CTCGCCCAAATCTTCTTCAGAAATCAACTTTTGTTCAGCGTTATCGGATTCATATGCTCGATC	3’ primer that introduces a (2X) Myc tag at the C-terminus of pcrH
popD-5Not	AAAAAgcggccgcAATCGACACGCAATATT	5’ primer to fuse popD to 3’ end of MBP, Zif or ω with NotI site
pcrH-5Not	AAAAAgcggccgcAAACCAGCCGACCCCTTCCGAC	primer to fuse pcrH to 3' end of Zif or omega
pcrH3H	AAAAAaagcttTCAAGCGTTATCGGATTCATAT	pcrH 3’ primer with HindIII site
fleQ-5-1	AAAAAgaattcTACAACGATATGCTCAGCGCTT	5’ external fleQ primer with EcoRI site
fleQ-5-2	AACTCGAGCCGCAAGCATGCTGAAGCGCCACATTTTGATCAGCTGCCTT	3’ fleQ deletion primer, deletes codons 4-487 of fleQ
fleQ-3-1	TTCAGCATGCTTGCGGCTCGAGTTTCGGATGATTGACAGGTCGTTT	5’ fleQ deletion primer, deletes codons 4-487 of fleQ
fleQ-3-2	AAAAAaagcttGATGACGATGACGCCGCCGGGA	3’ external fleQ primer with HindIII site
1699-5-1	AAAAAgaattcATGCTGGCGCTGGTCGACCAGGCGT	5’ external pcr1 primer with EcoRI site
1699-3-2	AAAAAaagcttCCGACCCGGCTCATGCGGCGAGCACT	3’ external pcr1 primer with HindIII site
1699-5-2	AACTCGAGCCGCAAGCATGCTGAAGGTCAATTCAGAAGGCCCGTATGCCA	3’ pcr1 deletion primer, removes codons 10-81

1699-3-1	TTCAGCATGCTTGCGGCTCGAGTTGAGCGCGAA GAGGAGCAGCAGCATGGA	5’ pcr1 deletion primer, removes codons 10-81
pcrH-5-1	AAAAAgaattcGACGCTGGCGGTATCGATCTGGT	5’ pcrH external primer with EcoRI site
pcrH-5-2	AACTCGAGCCGCAAGCATGCTGAACGGCTGGTT CATGGATACCTCTAGAT	3’ pcrH null primer
popD3-1n	TTCAGCATGCTTGCGGCTCGAGTTTTCATGAAG GACGTCCTGCAGCTCA	5’ popD null primer
popD-3-2	AAAAAaagcttAGGGTCAGTTGCGCTGCGAGAAT	3’ popD external primer with HindIII site
pcrH5R	AAAAAgaattcGGAGGGGTATCCATGAACCAGCCG ACCCCT	5’ pcrH primer with EcoRI restriction site
ED21popD-5-1	GAGGCGCGCACGGTCCGGCGCAGGGCTCCCG GCGCCGCCGGGCGTTCC	5' primer to amplify popD21-295 and fuse to C-terminus of exsE
ED21exsE-5-2	GGAACGCCCGGCGGCGCCGGGAGCCCTGCGC CGGACCGTGCGCGCCTC	3' primer for 5' flank to fuse exsE to popD(21-295)
EDexsE-3-1	CGTGCGGCGGCCGGAGTGGTCTGAGGTGCTGG ATGCTGTTGCC	5' primer to amplify region downstream of exsE and fuse to popD
EDpopD-3-2	GGCAACAGCATCCAGCACCTCAGACCACTCCG GCCGCCGCACG	3' primer to amplify popD and fuse to region downstream of exsE
1711-5-1	AAAAAgaattcGCCGAGTTCGCAGGCCGTATCGGT	5’ flanking primer for exsE with EcoRI site
1711-3-2	AAAAAaagcttCAATCGTTGCCAGATCTTTCTT	3’ flanking primer for exsE with HindIII site
EinsD-5-1	CGGCATGAGGGTTTGAGCGGCCCCATGATCGA CACGCAATATTCCCTG	5’ primer, amplifies 5' end of popD, replace codons 108-143 of popD with exsE (-ATG and TGA), exsE 5'flank (to cross into exsE locus)
EinsDflank-5-2	CAGGGAATATTGCGTGTCGATCATGGGGCCGCT CAAACCCTCATGCCG	3’ primer to amplify 5' flank next to exsE (X-over partner is EinsD-5-1)
EinsD-E5	CAGTCGATCATCCACGCGCAGAAGAAAATCGAA TCGATTTCGCCGGTG	5’ primer, amplifies exsE, fuse to 5' end of popD
EinsD-D5-2	CACCGGCGAAATCGATTCGATTTTCTTCTGCGC GTGGATGATCGACTG	3’ primer, amplifies 5' end of popD, pair with EinsD-5-1
EinsDflank-3-1	GAGGCGCGCACGGTCCGGCGCAGGAAGAACG GCAAGGCCATCAGTCAA	5’ primer, amplifies 3' end of popD
EinsD-E3	TTGACTGATGGCCTTGCCGTTCTTCCTGCGCCG GACCGTGCGCGCCTC	3’ primer, amplifies exsE
EinsDflank-3-2	AAAAAaagcttACGCCAGGCCTGGTTATGGCTCTG	3’ primer, amplifies 3' end of popD, before dC6 start, with HindIII site

pcrG2-5-1	AAAAAgaattcCTGCCGGTGCTGTCCTACCAGGAG	5' primer for pcrG 5' flank with EcoRI site
pcrG2-3-2	AAAAAaagcttCCGCGGTCAGCGCCTTGAGCTCGT	3’ primer for pcrG 3’ flank with HindIII site
Gd30-40-5-2	CAGCAGCTCGCCGGCGTCCGCCAGGCGGCCG CGTTCCTCGCTG	3’ primer to delete codons 30-40 of pcrG
Gd30-40-3-1	CAGCGAGGAACGCGGCCGCCTGGCGGACGCC GGCGAGCTGCTG	5’ primer to delete codons 30-40 of pcrG
pcrGd60-70-5-2	CTGCGTCGGCTGGGAACTGCGGGCGGCTCGCG CCAGCTCTCG	3’ primer to delete codons 60-70 of pcrG
pcrGd60-70-3-1	CGAGAGCTGGCGCGAGCCGCCCGCAGTTCCCA GCCGACGCAG	5’ primer to delete codons 60-70 of pcrG
pcrH-3-2	AAAAAaagcttTCTCGACTTCGCCGGTGATTTT	3’ external pcrH primer with HindIII site
H-MfeI-5-2	GAAGCCCAGCGCATAcaattgCTCCAGGGTGTCCT C	3’ primer, introduces silent MfeI site in pcrH at codon 38-39
H-MfeI-3-1	GAGGACACCCTGGAGcaattgTATGCGCTGGGCTT C	5’ primer, introduces silent MfeI site in pcrH at codon 38-39
CMyc2-3H	AAAAAaagcttTCACAGGTCCTCCTCCGAGAT	3’ primer to amplify genes tagged at C-terminus with 2xMyc tag
Hmut45	CTGGAGcaattgTATGCGCTGGGCTTCNNNCAGTA CCAGGCGGGCAAGTGG	5’ pcrH mutagenesis primer, Asn45 mutated with silent MfeI site in pcrH at codon 38-39
Hmut46	CTGGAGcaattgTATGCGCTGGGCTTCAACNNNTA CCAGGCGGGCAAGTGGGAC	5’ pcrH mutagenesis primer, Gln46 mutated with silent MfeI site in pcrH at codon 38-39
Hmut49	CTGGAGcaattgTATGCGCTGGGCTTCAACCAGTA CCAGNNNGGCAAGTGGGACGACGCGCAG	5’ pcrH mutagenesis primer, Ala49 mutated with silent MfeI site in pcrH at codon 38-39
Hmut50	CTGGAGcaattgTATGCGCTGGGCTTCAACCAGTA CCAGGCGNNNAAGTGGGACGACGCGCAGAAG	5’ pcrH mutagenesis primer, Gly50 mutated with silent MfeI site in pcrH at codon 38-39
Hmut51	CTGGAGcaattgTATGCGCTGGGCTTCAACCAGTA CCAGGCGGGCNNNTGGGACGACGCGCAGAAGA TC	5’ pcrH mutagenesis primer, Lys51 mutated with silent MfeI site in pcrH at codon 38-39
Hmut53	CTGGAGcaattgTATGCGCTGGGCTTCAACCAGTA CCAGGCGGGCAAGTGGNNNGACGCGCAGAAGA TCTTCCAG	5’ pcrH mutagenesis primer, Asp53 mutated with silent MfeI site in pcrH at codon 38-39

Hmut54	CTGGAGcaattgTATGCGCTGGGCTTCAACCAGTACCAGGCGGGCAAGTGGGACNNNGCGCAGAAGATCTTCCAGGCAC	5’ pcrH mutagenesis primer, Asp54 mutated with silent MfeI site in pcrH at codon 38-39
pcrHd46-71-5-2	GCAGGCGCCCAGGCCGAGAAAGTAGTTGAAGCCCAGCGCATAGAGCTG	3’ primer to delete pcrH codons 46-71
pcrHd46-71-3-1	CAGCTCTATGCGCTGGGCTTCAACTACTTTCTCGGCCTGGGCGCCTGC	5’ primer to delete pcrH codons 46-71
popD-3Asc	AAAAAGGCGCGCCTCAGACCACTCCGGCCGCCGCA	3' primer to clone PopD ORF into pACλCI35
popD1-146-3Asc	TAATATggcgcgccTCAGCCGTTCTTCAACGCGCCGAGGCTGCCGACCAC	3' primer to clone PopD(1-146) into pACλCI35
popD58-5Not	AAAAAgcggccgcACTCGACCCGGTGCGCATGGAAGCGGCCGG	5' primer to clone PopD beginning at residue 58 into pACλCI35
pcrH-3Asc	AAAAAggcgcgccTCAAGCGTTATCGGATTCATAT	3' primer to clone pcrH ORF into pBRα35