Itt1p, a novel protein inhibiting translation termination in \textit{Saccharomyces cerevisiae}

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Abstract

**Background:** Termination of translation in eukaryotes is controlled by two interacting polypeptide chain release factors, eRF1 and eRF3. eRF1 recognizes nonsense codons UAA, UAG and UGA, while eRF3 stimulates polypeptide release from the ribosome in a GTP- and eRF1-dependent manner. Recent studies have shown that proteins interacting with these release factors can modulate the efficiency of nonsense codon readthrough.

**Results:** We have isolated a nonessential yeast gene, which causes suppression of nonsense mutations, being in a multicopy state. This gene encodes a protein designated Itt1p, possessing a zinc finger domain characteristic of the TRIAD proteins of higher eukaryotes. Overexpression of Itt1p decreases the efficiency of translation termination, resulting in the readthrough of all three types of nonsense codons. Itt1p interacts \textit{in vitro} with both eRF1 and eRF3. Overexpression of eRF1, but not of eRF3, abolishes the nonsense suppressor effect of overexpressed Itt1p.

**Conclusions:** The data obtained demonstrate that Itt1p can modulate the efficiency of translation termination in yeast. This protein possesses a zinc finger domain characteristic of the TRIAD proteins of higher eukaryotes, and this is a first observation of such protein being involved in translation.

**Background**

The final step of protein biosynthesis represents the termination codon-dependent release of a nascent completed peptide chain from the ribosome. In eukaryotes, this process is controlled by two protein factors: eRF1, recognizing all three types of nonsense codons, and eRF3, which stimulates polypeptide release in a GTP- and eRF1-dependent manner [1–3]. In the yeast \textit{Saccharomyces cerevisiae}, the eRF1 and eRF3 release factors are encoded by the SUP45 and SUP35 genes, respectively, and are often designated as the Sup45 and Sup35 proteins [4]. Partial inactivation of these release factors by
mutations results in enhanced nonsense codon readthrough, which can be revealed in yeast by suppression of nonsense mutations, while deletions of the corresponding genes are lethal. It was shown for vertebrates and yeast that eRF3 and eRFI interact with each other to form a heterodimeric complex both in vivo and in vitro [2,4–6]. Yeast eRF3 has a complex structure and is composed of the amino-terminal region and carboxy-terminal (C) domain of 253 and 432 amino acids, respectively [7–9]. The conserved C domain of Sup35p is responsible for its function in translation termination and is essential for cell viability, while the N-terminal region is neither conserved, nor essential. This region may be further subdivided into the middle (M) domain of unknown function and N-terminal (N) domain of 123 amino acids, which is responsible for the prion properties of eRF3.

In human cells, eRF1, being in excess, enhances the efficiency of translation termination, which is consistent with its function in translation [10]. However, in yeast only simultaneous overexpression of both release factors is required for the antisuppressor effect [4], suggesting that a complex of these factors is active in vivo in translation termination.

Premature termination of translation is not the only consequence of the occurrence of nonsense mutations: they can also enhance decay rate of the corresponding mRNA (Nonsense Mediated Decay; NMD). This phenomenon has been observed in both prokaryotic and eukaryotic cells. Several factors involved in NMD have been identified in yeast S. cerevisiae. Among them, the UPF1, UPF2, and UPF3 genes are characterized best [for review, see [11]]. Mutations in these genes selectively stabilize mRNAs containing early nonsense codons without affecting the decay rate of most wild-type mRNAs. These mutations manifest themselves as nonsense suppressors and initially it was concluded that the suppression was solely due to the increase of abundance of nonsense-containing mRNAs and corresponding readthrough proteins. However, later it was demonstrated that it is not so and nonsense suppression was due to the impaired ability of Upf1 protein to enhance translation termination at nonsense codons. The upf1 mutations were identified that suppress nonsense mutations, but do not stabilize nonsense codon-containing mRNAs [12,13]. Another member of this protein superfamily, Mtt1p, whose overexpression enhances the level of nonsense codon readthrough, was recently identified [16]. This protein also interacts with the translation termination factors, but in contrast to Upf1p is not involved in the control of NMD and exerts an opposite effect on translation termination.

Here we present a study directed for identification of additional proteins involved in translation termination in yeast. A screen for multicopy nonsense suppressors revealed a novel protein that inhibits translation termination by binding to polypeptide chain release factors. Interestingly, this protein belongs to a recently described class of TRIAD zinc finger proteins, many of which are presumed to be involved in transcription.

### Results

**Isolation and characterization of the ITT1 gene**

*S. cerevisiae* strain 2G-DM8, carrying the non-suppressible ura3-52 mutation and nonsense mutations his7-1 (UAA), trp1-289 (UAG) and lys2-187 (UGA) was transformed with the yeast genomic library. The transformants were then replica plated on the media selective for the plasmid marker *URA3* and lacking histidine, tryptophane or lysine. Fifty transformants able to grow on either one of these media were selected. Only one of them grew on all media. The plasmid-less *Ura*`' colonies appearing after streaking this transformant on YPD medium, became His`, Trp` and Lys`, which confirmed that suppressor phenotype of the transformant depended on the presence of plasmid.

Plasmid DNA recovered from this transformant carried a genomic DNA insert of approximately 5 kb. The sequence responsible for multicopy suppression was delimited to a *XbaI-HindIII* region of 2.7 kb. Sequencing of this fragment showed that it contains the open reading frame (ORF) YML068w (GenBank # CAA86252.1), which encodes a polypeptide of 464 amino acids with estimated molecular mass of 54 kDa. No function was ascribed to this ORF, which we designated as *ITT1* (Inhibitor of Translation Termination). The codon adaptation index (CAI) of the *ITT1* gene (Yeast Protein Database) is 0.127, which suggests that this gene is expressed at low levels. The deduced Itt1 protein possesses a zinc finger domain. This domain starts from residue 180 and includes three double zinc fingers: a RING C4HC domain. This domain starts from residue 180 and includes three double zinc fingers: a RING C4HC element followed by two elements of C4HC and C4HC structure. A search for Itt1p homologues revealed 8 proteins in *Caenorhabditis elegans* and 8 proteins in man with similarity ranging from 20 to 35% (Figure 1). *S. cerevisiae* has one more TRIAD protein, YKR017c, but its similarity to Itt1p is lower than that of some TRIAD proteins from...
To quantify the suppressor effect of overexpressed Itt1p, unsuccessful. Our attempts to disrupt it in a haploid strain were essential, for it is necessary. Curiously, despite this gene being essential for viability. Curiously, despite this gene being essential for viability, the obtained strain was sporulated for the strain with enhanced level of nonsense suppression due to deletion of UPF1 gene. Similar data were obtained for the strain with enhanced level of nonsense suppression due to deletion of UPF1 (data not shown).

To characterize the Itt1p function, the diploid strain H8 was sporulated, and tetrads were dissected. The 2:2 segregation for the LEU2 disruption marker observed for 39 tetrads analyzed demonstrated that the Itt1p gene is not essential for viability. Curiously, despite this gene being essential, our attempts to disrupt it in a haploid strain were unsuccessful.

To quantify the suppressor effect of overexpressed Itt1p, the 1A-H8-B2 psi strain, carrying the itt1::LEU2 disruption, was transformed with either multicopy YEplac122-ITT1 or centromeric YCplac22-ITT1 plasmids. Northern blot analysis revealed that amount of ITT1 mRNA in the transformant with multicopy plasmid increased approximately 10-fold compared to the transformant with centromeric one (data not shown). Overexpression of Itt1p increased the readthrough levels of all types of stop codons, which confirms omnipotence of the ITT1 multicopy suppression (Figure 2). The levels of nonsense suppression did not noticeably depend on the presence of wild-type ITT1 gene. Similar data were obtained for the strain with enhanced level of nonsense suppression due to deletion of UPF1 (data not shown).

Itt1p interacts with eRF1 and eRF3

The inhibition of translation termination by excess Itt1p could be due to decreased levels of release factors. However, neither extra copies, nor deletion of the ITT1 gene affected the abundance of eRF1 and eRF3 (data not shown). Itt1p could also inhibit termination by binding to the eRF1 and eRF3 release factors. To examine the interaction of Itt1p with eRF1 and eRF3, we studied the ability of immobilized Itt1p to bind purified eRF1 and eRF3. These proteins were individually expressed in E. coli as fusions with either 6-histidine or GST tags. Also, we used eRF3 expressed in yeast as GST-eRF3 fusion, since it was observed earlier that eRF3 N-terminal part may be folded incorrectly in bacteria [18] and our unpublished data. The purified GST-Itt1p associated with the glutathione agarose beads was incubated with the purified bacterially-expressed His6-eRF3, His6-eRF3N2 (eRF3 amino acids 1–153), eRF3C (amino acids 254–685) and eRF1, as well as with eRF3 isolated from yeast.
Then unbound proteins were removed by washing, and GST-Itt1p with associated proteins were eluted and analyzed by Western blotting using polyclonal antibodies against eRF3 and eRF1. Itt1p specifically bound eRF1, eRF3 and eRF3N2, but did not bind the eRF3C fragment and GST protein (Figure 3). The interaction of Itt1p with eRF1 and eRF3 was also studied using yeast lysates as a source of these proteins. Immobilized GST-Itt1p precipitated eRF3 and eRF1 from lysates of cells with the multicopy $SUP35$ and $SUP45$ plasmids, but not from wild-type lysates (data not shown). This probably indicates that interaction between the studied proteins is relatively weak.

**The suppressor effect of excess Itt1p depends on both eRF1 and eRF3 release factors**

To test the role of eRF1 in the $ITT1$ suppressor effect, the multicopy plasmids with $ITT1$ and $SUP45$ were simultaneously introduced into the strain 5V-H19. These transformants did not express the suppressor phenotype (Figure 4). In contrast to eRF1, the overexpression of eRF3 did not abolish the suppressor effect of the Itt1p overexpression. It is noteworthy that overexpression of eRF1 alone does not cause antisuppressor effect [4] and overproduction of eRF3 does not suppress the ade2-1 UAA mutation in the 5V-H19 strain (data not shown).
These data suggest that eRF1 is a primary target for the Itt1p inhibition, while the Itt1p interaction with eRF3 may not play an important role in the suppressor effect. To check the latter, we examined the effect of excess Itt1p in the strain 1-5V-H19, which encodes only the eRF3 C-terminal domain unable to interact with Itt1p. This strain and 5V-H19 were transformed with the multicopy ITT1 plasmid, which carried both ITT1 and SUP45, revealed that the overexpression of eRF1 completely abolished suppression caused by overexpression of the Itt1 protein.

**Discussion**

This paper describes a novel protein, Itt1p, involved in the control of translation termination in yeast. Two lines of evidence support this conclusion: (i) overexpression of Itt1p enhances the readthrough of UAA, UAG and UGA nonsense codons; (ii) Itt1p interacts with both eRF1 and eRF3 polypeptide chain release factors. It is noteworthy that in contrast to deletions of SUP45 and SUP35 the knockout of ITT1 is not lethal and does not noticeably influence the nonsense codon readthrough. This suggests that Itt1p is not essential for the release of completed polypeptide chains from the ribosome.

The suppressor effect of Itt1p was observed at increased Itt1p to eRF1 ratio, but did not occur when this ratio was normal, including the case when both proteins were overexpressed. This suggests that eRF1 experiences quantitative, rather than qualitative alteration. The simplest explanation of these and other data is that the binding of Itt1p to eRF1 makes it inactive in translation termination. It is important that Itt1p is expressed at lower level than eRF1. According to published estimates [19], the difference in CAI of eRF1 (0.334) and Itt1p (0.127) could mean that eRF1 is expressed at about 3-fold higher level than Itt1p. The role of eRF3 in the suppressor effect of Itt1p is less clear. The suppressor effect of excess Itt1p was not affected by overexpression of eRF3, but was reduced in the presence of N-terminally truncated eRF3, which does not interact with Itt1p. To explain this, it is possible to suggest that the interaction of Itt1p with eRF3 is weaker than with eRF1, but it strengthens the binding of Itt1p to eRF1/eRF3 complex, thus enhancing the suppressor effect of Itt1p. However, the effect of the N-terminally truncated eRF3 can also be explained by observation that it promotes translation termination better than complete protein [8]. It may be difficult to distinguish these two mechanisms and we consider it likely that both take place.

It is not clear whether the inhibitory effect of Itt1p represents its main function, or whether it is a consequence of recruiting eRFs for some function different from the

![Figure 3](image-url)

Itt1p interacts with both eRF3 and eRF1. All proteins were isolated from *E. coli*, except eRF3 (Sac), isolated from *S. cerevisiae*. The indicated proteins were incubated with GST-Itt1p or GST proteins immobilized on glutathione-Sepharose 4B. Following washing, bound proteins were eluted and analyzed by Western blotting with polyclonal antibodies against eRF3 and eRF1.
translation termination. The second opportunity looks more appealing, and it is supported by some structural features of Itt1p. Itt1p belongs to a unique family of zinc finger proteins called TRIAD. It contains three double zinc finger elements, one of which belongs to a RING class [17]. The similarity of Itt1p with its homologues from other eukaryotes is not high (20–30%), but it spans the whole length of Itt1p with the cysteines and histidines of zinc finger elements being highly conserved. This suggests a functional similarity of Itt1p to at least some TRIAD proteins.

The alignment of Itt1p with its closest homologues (Figure 1) and other TRIAD proteins (not shown) reveals notable similarity of the second and third double zinc finger elements, manifested in similar spacing of cysteines and some conserved residues. This is in contrast to the earlier assignment of the third finger as belonging to a RING class $\text{C}_3\text{HC}_4$[17]. One cause of this difference is that the histidine of the proposed RING signature is poorly conserved (marked $\text{s}$ in Figure 1). On the other hand, highly conserved histidine and cysteine residues (marked 7 and 8) were disregarded previously [17]. Intriguingly, in our version the third element contains an odd number of conserved residues, nine. Either one of the residues is unimportant for binding zinc (marked $\&$), or there could be alternative configurations for zinc binding by this element.

Many of RING finger-containing proteins bind ubiquitin-conjugating enzymes and are the substrates for E2-dependent ubiquitination [20]. It was proposed that this
mechanism can be used to target the RING-containing protein or associated proteins for degradation in a regulated manner. If so, the excess Itt1p could cause suppression by accelerating degradation of eRF3 and eRF1. However, this is not the case, since the overexpression of Itt1p did not affect the levels of eRF3 and eRF1. It is also known that RING finger proteins can be transcriptional factors [21] and nuclear localization was predicted for many of the TRIAD proteins [17]. However, only few of these proteins were functionally characterized. Two of them are human androgen receptor activator ARA54 (Figure 1) and rat protein kinase C-associated protein [22,23]. For both proteins it may be suggested that they function in cytoplasm and nucleus and play a role in transcription. Itt1p may also be involved in transcription regulation since two-hybrid analysis has shown that it interacts with the Snf1p transcription factor [24]. This, together with the fact that Itt1p contains a putative nuclear import signal (Figure 1), allows to speculate that Itt1p may perform coupling of translation termination with transcription of certain genes.

Itt1p is not the only protein that could link translation termination with other cellular processes in yeast. At present, two such proteins are known. The first is Upf1p, which is involved in the control of NMD pathway and stimulates translation termination probably by binding to eRF3 and eRF1 release factors [13,14]. Strikingly, this protein and its partners, Upt2p and Upf3p, are also required to control the total accumulation of large number of mRNAs in addition to their role in RNA surveillance, though mechanisms of such control are unknown [25]. The second is Mtt1p, a homologue of Upf1p, which interacts with both release factors, but is not involved in the NMD control and inhibits translation termination [16]. This protein has 5’→3’ DNA-dependent helicase activity and is thought to be involved in chromosome replication [26–28]. However, the question is still open, whether such proteins can mediate the interdependence of these cellular processes.

Conclusions
The data presented in this work show that the increased expression of the ITT1 (YML068w) gene reduces efficiency of translation termination. The Itt1 protein can bind to the translation termination factors eRF1 and eRF3 and we propose that the resulting complex(es) are incompetent for termination. eRF1 appears to be a primary target for the inhibition by Itt1p, since the suppressor effect of the excess Itt1p is reverted by overexpression of eRF1, but not of eRF3. Itt1p possesses a zinc finger domain characteristic of the TRIAD proteins of higher eukaryotes, and this is a first observation of such protein being involved in translation. However, the role of this protein is probably not restricted by translation. Itt1p interacts with the transcriptional factor Snf11 and therefore could function in transcription, similarly to some of its homologues from other species.

Materials and Methods
Genetic methods
We used standard organic (YEPD) and synthetic complete (SC) media for yeast [29] and LB medium for bacteria [30]. Appropriate amounts of amino acids, bases, and antibiotics were added when necessary. The 5’-fluoroorotic acid (5FOA) medium was prepared according to [31]. The final concentration of 5FOA was 400 μg/ml. All solid media contained 2.0% agar. Yeast cells were grown at 30°C, and bacteria at 37°C. Standard yeast genetic procedures for mating, sporulation, and tetrad analysis were used [29]. DNA transformation of yeast and Escherichia coli cells was performed as described previously [32,33].

Strains and plasmids
The yeast strains 2G-DM8 (MATα ade2-144,717 pheA10 his7-1 lys2-l87 trp1-1 ura3-52) and 5V-H19 (MATα ade2-1 SUP55 can1-100 leu2-3,112 ura3-52) were described in [8]. The strain 1-5V-H19 was obtained by replacing the wild-type SUP35 gene of 5V-H19 with the SUP35-C deletion allele, which encodes amino acids 254–685 of eRF3 [9]. The diploid H8 strain (MATα/MATa trp1-289/ trp1-289 trp1-289 leu2-3,112/ leu2-3,112 ura3-52/ ura3-52 his3-Δ1/ his3-Δ1) was described in [34]. Construction of other strains is described below. All strains were [psi].

DNA manipulations were carried out by standard protocols [30]. The DH5α E. coli strain [supE44 lacZΔ16 Φ80 lacZΔ1 M15] hsdR17 recA1 endA1 gyrA96 thi-1 relA1 was used for plasmid construction [30]. To create yeast genomic library, chromosomal DNA of the 5V-H19 strain was partially digested with Sna3A and fractionated on agarose gel. DNA fragments ranging from 6 to 12 kb were isolated. The ends of chromosomal DNA fragments were partially filled in with Klenow enzyme and ligated to partially filled in Sall site of the YEplac195 plasmid [35]. The ligation products were used for E. coli transformation.

The plasmids containing ITT1, SUP45 and SUP35 were constructed as follows. The 2.7 kb Xhol-HindIII genomic fragment carrying the ITT1 gene was cloned into the same sites of pBluescript II KS(+) (Stratagene, USA), and the resulting plasmid was designated as pITT1. The ITT1 SacI-HindIII fragment of this plasmid was inserted into the multicopy vectors YEplac195, YEplac81, YEplac112, with URA3, LEU2 and TRP1 selectable markers, respectively, and into the TRP1-carrying centromeric vector YCpplac22 [35]. The 2.5 kb Xhol-SalI SUP45-csarymg fragment of pEMBLYex4-SUP45 [4] was cloned
into the SalI site of YEplac195 to yield YEplac195-SUP45. The 2.7 kb SalI-SalIITI fragment of the plasmid pITTI was inserted into the same sites of YEplac195-SUP45 to obtain the pITTI-SUP45 plasmid. The 3.6 kb XhoI-XbaI SUP35-carrying fragment of pEMBLyex4-SUP35 [8] was inserted into the SalIand XbaI sites of YEplac195 which resulted in the plasmid YEplac195-SUP35.

The construction of the hybrid genes encoding N-terminal fusions of GST (glutathione S-transferase) to eRF1, eRF3 and eRF3C was described earlier [18,36]. To construct the pGST-ITTI plasmid encoding GST-Itt1p fusion protein, the 1.7 kb BamHI-HindIII fragment carrying the entire ITTI gene (BamHI site was engineered at the 5’ end of the ITTI ORF) was cloned into the same sites of pGEX-2TH, which was obtained from the pGEX-2T plasmid (Pharmacia, Sweden) by EcoRI to HindIII replacement. To obtain plasmids expressing His6-eRF3N2 and His6-eRF3, the SUP35 coding sequence from engineered BglII site at position-12 before the start codon to BalI site at codon 154 or XbaI site in the 3’ non-coding region, which was filled-in by Klenow, was cloned into BamHI and HindIII sites of pQE-10 (Qiagen) in-frame and downstream of the 6-histidine tag sequence. Both proteins have N-terminal extension MRGSHHHHHHHTDLAT. All fusion proteins were expressed in the E. coli strain TG1 {supE HsdA5 thiA (lac-proAB) F’ [traD36 proAB + lacI5 lacZ M15]}. The ITTI gene was disrupted as follows. The 1.0 kb EcoRI-XbaI fragment internal to the ITTI ORF was replaced with the 2.0 kb LEU2-carrying SalI-XbaI fragment of the plasmid pJJ283 [37]. The EcoRI and SalI ends of these fragments were filled in with Klenow enzyme prior to ligation. The obtained plasmid was cleaved with BglII and HindIII and the obtained 3.6 kb fragment carrying the itti::LEU2 disruption allele was used to transform the strain H8. The Ura+ transformant was designated as H8-B2 and confirmed to be heterozygous for the ITTI disruption by Southern blot analysis (data not shown). The strain 1A-H8-B2 carrying the itti::LEU2 allele was obtained as a meiotic segregant of the diploid H8 heterozygous for the ITTI disruption allele.

The strain 1A-H8-B2-R was obtained from 1A-H8-B2 by replacing the wild-type SUP35 gene with the SUP35-C deletion allele using the integration/excision method [38]. The pPL4455eC-SUP35C integrative plasmid (lacking the Clal fragment with 2 μm replication origin) bearing a SUP35-C allele [8] was linearized by SalI site internal to the SUP35 ORF and integrated into the SUP35 gene of the strain 1A-H8-B2 by selecting of transformants on uracil omission medium. The Ura+ integrants expressing both eRF3 and eRF3C were selected by Western blotting and placed onto the 5-FOA containing medium to select for the alternative URA3-SUP35 or URA3-SUP35-C excision. The obtained Ura+ clones of one integrant were screened by Western blotting to select a clone expressing only the eRF3C protein (data not shown).

β-Galactosidase assays of yeast strains transformed with the pUKC815/817/818/819 series vectors

The URA3-carrying plasmid pUKC815 encodes a PGK1-lacZ gene fusion, while the pUKC817, pUKC818 and pUKC819 plasmids are identical to pUKC815 except that one of the three termination codons, UAA, UAG and UGA, respectively, is present in-frame at the junction of the PGK1 and lacZ genes [39]. Suppression of the in-frame premature stop codons will result in β-galactosidase activity and the levels of β-galactosidase activity can therefore be used to quantify the readthrough of nonsense codons. The nonsense suppression levels were determined as ratio of β-galactosidase activities in the cells transformed with plasmids pUKC817, pUKC818 and pUKC819 to that of the transformant with pUKC815. Individual transformants were grown selectively in SC supplemented with the required amino acids and bases to mid-exponential phase at 30°C.

Preparation of yeast cell lysates

Yeast cultures were grown to ODp600 of 1.5, harvested, washed in water and lysed by vortexing with glass beads in buffer A (25 mM Tris-HCl [pH 7.4], 100 mM NaCl, 5 mM MgCl2, 1 mM dithiothreitol) containing 1 mM phenylmethylsulfonylfluoride to limit proteolytic degradation. Cell debris was removed by centrifugation at 15,000 g for 10 min.

Purification of eRF1, eRF3 and eRF3 fragments and assay for interaction with immobilized GST-Itt1p

The GST-Itt1p, GST-eRF1, GST-eRF3C fusion proteins expressed in E. coli and GST-eRF3 expressed in yeast were isolated by affinity chromatography on glutathione-Sepharose 4B (Pharmacia). The GST extension from GST-eRF1 and GST-eRF3 was removed with thrombin (Sigma), and from GST-eRF3C with factor Xa (Promega), as described [40]. The His6-eRF3 and His6-eRF3N2 proteins expressed in E. coli were isolated using TALON resin (Clontech) according to manufacturer's instructions. The glutathione-Sepharose 4B resin with immobilized GST-ITT1p was incubated with yeast lysates or with the purified eRF1, eRF3, eRF3N2 and eRF3C proteins for 2 h at 4°C and then washed with a 40-fold resin volume of buffer A. Bound proteins were eluted with 2% SDS and analyzed by Western blotting.
Protein gel electrophoresis and Western blot analysis

Protein samples were separated on an SDS polyacrylamide gel as described [41] and electrophoretically transferred to nitrocellulose sheets [42]. Western blots were probed with polyclonal rabbit anti-eRF3, anti-eRF3C or anti-eRF1 (a gift of K. M. Jones, University of Kent) antibody. Bound antibodies were detected with the Amer sham ECL system as instructed by manufacturer.

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