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Screening for Unknown Mutations by a Bioluminescent Protein Truncation Test with Homogeneous Detection

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Abstract: The protein truncation test (PTT) is important in screening for unknown mutations that cause premature termination of mRNA translation. PTT involves amplification of the interrogated sequence, *in vitro* transcription/translation, separation of the generated polypeptides, and detection. In this article, we report a bioluminescent protein truncation test, in which the detection of the nascent protein is performed directly in the expression mixture, within seconds, without the need for separation and purification. A DNA fragment encoding apoaequorin is fused, in-frame, downstream of the interrogated sequence. The fusion product is subjected to *in vitro*, coupled transcription and translation in the presence of coelenterazine. A wild-type DNA template allows translation to continue after the 3′ end of the interrogated sequence, producing a chimeric protein whose C-terminal domain is the photoprotein aequorin. Aequorin is detected, with a high sensitivity, by its characteristic Ca^{2+}-triggered, flash-type bioluminescent reaction. Active photoprotein is not produced when a truncating mutation is present in the interrogated sequence. As a model, the method was applied to the detection of truncating mutations in the APC gene (adenomatous polyposis coli).

Introduction

The protein truncation test (PTT) enables the screening for unknown mutations that cause premature termination of mRNA translation, i.e., nonsense, frameshift, and splice site mutations.[1] The attractive characteristic of PTT is that it does not detect amino acid substitutions or deletions, which usually do not cause disease. On the contrary, truncating mutations are almost always associated with a severe phenotype. A large number of disease-related genes have been identified in which the majority of the mutations responsible for the disease cause premature termination of translation, leading to a truncated and nonfunctional protein, for instance, genes involved in Duchenne muscular dystrophy (DMD gene), breast cancer (BRCA1 and BRCA2 genes), colon cancer (APC gene), neurofibromatosis (NF1 and NF2 genes), tuberous sclerosis (TSC1 and TSC2 genes), a significant proportion of patients with cystic fibrosis, Tay-Sachs, beta thalassemia, and many other diseases.[2,3]

The PTT consists of the following steps: (a) amplification of the target DNA or RNA sequence by PCR or RT-PCR, respectively; (b) *in vitro* transcription and translation of the amplified fragments; and (c) analysis of the products. In conventional PTT, the expression products are labeled with radioactive amino acids, such as [^{35}S]methionine or [^{14}C]leucine. The products are then separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) and detected by autoradiography. The presence of a truncating mutation is denoted by the detection of shorter proteins than the expected full-length product.

The main limitations of conventional PTT, which restrict its widespread use, are (i) the health hazards and inconvenience associated with the use and disposal of radioisotopes; (ii) SDS-PAGE is the most laborious step and prevents automation; (iii) mutations that are located close to the 3′ end of the target are not detectable because the size of the product is almost equal to the full-length wild-type protein; (iv) mutations located near the 5′ end of the target sequence are also not detectable because the translation product is too short.

In recent years, a number of modifications were reported in order to address the limitations of the original PTT, mainly to improve the detection of the newly synthesized protein, i.e., to eliminate the radioisotopes and/or SDS-PAGE.[2−9] The incorporation of fluorescent dyes into the synthesized protein via modified lys-tRNA enables the detection of the expression products directly in the gel, using a laser-induced fluorescence scanner. Alternatively, an epitope tag may be introduced, by PCR, into the synthesized protein. Following SDS-PAGE and western blot, the immobilized protein is detected by using a specific antibody.[5,6] Introduction of two epitope tags, or a biotin moiety and an epitope tag, allows the capture of the nascent protein by immobilized antibody or streptavidin, washing out the other components of the expression mixture, and detection

(1) Roest, P. A.; Roberts, R. G.; Sugino, S.; Van Ommen, G.-J.; den Dunnen, J. T. *Hum. Mol. Genet.* **1993**, *2*, 1719–1721.
(2) Den Dunnen, J. T.; Van Ommen, G. J. *Hum. Mutat.* **1999**, *14*, 95–102.
(3) Hauss, O.; Müller, O. *Methods Mol. Biol.* **2007**, *375*, 151–164.
(4) Traverso, G.; Diehl, F.; Hurst, R.; Shuber, A.; Whitney, D.; Johnson, C.; Levin, B.; Kinzler, K. W.; Vogelstein, B. *Nat. Biotechnol.* **2003**, *21*, 1093–1097.
(5) Rowan, A. J.; Bodmer, W. F. *Hum. Mutat.* **1997**, *9*, 172–176.
(6) Kahmann, S.; Herter, P.; Kuhnen, C.; Müller, K.-M.; Muhr, G.; Martin, D.; Soddemann, M.; Müller, O. *Hum. Mutat.* **2002**, *19*, 165–172.
(7) Gite, S.; Lim, M.; Carlson, R.; Olejnik, J.; Zehnbauer, B.; Rothschild, K. *Nat. Biotechnol.* **2003**, *21*, 194–197.
(8) Du, L.; Lai, C.-H.; Concannon, P.; Gatti, R. A. *Mutat. Res.* **2008**, *640*, 139–144.
(9) Garvin, A. M.; Parker, K. C.; Haff, L. *Nat. Biotechnol.* **2000**, *18*, 95–97.

by using an antitag antibody.[7,8] In another approach the tag-labeled protein is immunopurified and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF).[9]

In all the above methods, detection of the newly synthesized polypeptide relies on one or more separation steps (e.g., electrophoresis, western blot, or immunochemical capture onto a solid surface). We here report a protein truncation test, in which the detection of the nascent protein is performed directly in the expression mixture, in 3 s, by a homogeneous assay without the need for electrophoresis or solid-phase separation and purification. A DNA fragment coding for the apoprotein of the photoprotein aequorin (apoaequorin) is fused, in-frame, downstream of the interrogated sequence. The fusion DNA is expressed by *in vitro*, coupled (one-tube) transcription and translation in a mixture supplemented with the chromophore coelenterazine. A wild-type DNA template allows translation to continue after the 3′ end of the interrogated sequence, producing a chimeric protein whose C-terminal domain is apoaequorin. Upon synthesis, apoaequorin is converted into fully active photoprotein aequorin in the expression mixture and then detected, with high sensitivity, by exploiting its characteristic Ca^{2+}-triggered, flash-type bioluminescent reaction. Aequorin is not produced when a truncating mutation is present in the interrogated sequence. Because our method does not depend on size analysis of the translation products, it is suitable for rapid screening of mutations that are located close to the ends of the target sequence.

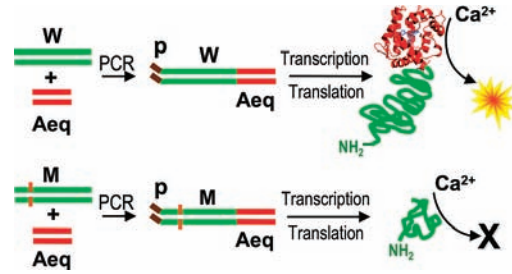
Experimental Section

Isolation of Genomic DNA from Whole Blood. Genomic DNA from whole blood was isolated by using the Blood XL Nucleospin kit (Macherey-Nagel). A 7.5 mL volume of each sample was used. The DNA concentration was determined spectrophotometrically at 260 nm.

Amplification of Apoaequorin-Coding Sequence (Aeq). PCR amplification of the apoaequorin coding sequence (PCR2) was carried out in a 50 μL reaction in the presence of 500 nM of each of the forward and reverse primer (5′-GTCAAGCTTACATCAGACTTCGACAAC-3′ and 5′-AAGGGGTTATGCTAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTAGGGGACAGCTCC-3′, respectively), 2 mM $MgCl_2$, 250 μM dNTP, and 1 U Phusion high fidelity DNA polymerase (Finnzymes). A 35 fmol amount of the pBAeqbirA plasmid[10] was used as template. The cycling program included an initial cycle of denaturation (98 °C, 40 s) followed by 30 cycles of denaturation (98 °C, 15 s), annealing (56 °C, 30 s), and extension (72 °C, 25 s), and a final extension step at 72 °C for 7 min. The PCR product was purified from the excess of primers by using the QIAquick PCR purification kit (Qiagen). The concentration of purified DNA was determined by agarose gel electrophoresis (2%) and densitometry of gels stained with ethidium bromide.

Amplification of APC DNA. PCR amplification of a selected region (amino acids 1138−1382) of the APC gene (PCR1) was carried out using ∼150 ng of genomic DNA in a 25 μL reaction mixture containing 250 nM of each of the forward and reverse primer (5′-GGATCCTAATACGACTCACTATAGGGAGACCACCATGAAGCCTACCAATTATAGTGAACGTTACTCT-3′ and 5′-GTTGTCGAAGTCTGATGTAAGCTTGACGAGTGGGGTCTCCTGAACATAGTGTTCAGGTGGACTTTTGGGTGTC-3′, respectively), 2 mM $MgCl_2$, 100 μM dNTP, and 0.5 U Phusion DNA polymerase. Amplification was performed by touch-down PCR as follows: an initial cycle of denaturation at 98 °C for 40 s, followed by 30 cycles of denaturation at (98 °C, 15 s), annealing (70 °C, 30 s, reduced by 0.4 °C per cycle), and extension at 72 °C for 90 s, and a final extension step at 72 °C for 7 min. The reaction product was confirmed by agarose gel electrophoresis (1.5%). The band corresponding to the desired size was excised from the gel, and the DNA was eluted with the QIAquick gel extraction kit. The concentration of purified DNA was determined as above.

Scheme 1. Principle of the Bioluminescent Protein Truncation Test Based on a Homogeneous Detection of the Nascent Protein[a]



[a] W: wild-type sequence; M: mutant; Aeq: apoaequorin-coding sequence; p: promoter.

Preparation of APC-Aeq Fusion DNA. The overlap extension PCR was carried out in a 25 μL mixture containing 250 nM each of 5′-GGATCCTAATACGACTCACTATAGGGAGACCACCATGAAGCCTACCAATTATAGTGAACGTTACTCT-3′ and 5′-AAGGGGTTATGCTAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTAGGGGACAGCTCC-3′, 2 mM $MgCl_2$, 200 μM dNTP, 0.5 U Phusion DNA polymerase, 1.7 fmol of APC DNA, and 16.6 amol of Aeq-DNA. The thermal cycling program included an initial denaturation step (98 °C, 40 s) followed by 30 cycles of denaturation (98 °C, 15 s), annealing (70 °C, 30 s, reduced by 0.5 °C per cycle), and extension (72 °C, 90 s), and a final extension step at 72 °C for 7 min. The size and concentration of the final product, APC-Aeq DNA (1386 bp), were determined as above.

Coupled *in Vitro* Transcription and Translation of APC-Aeq DNA. The APC-Aeq fusion product (2−1000 amol) was transcribed and translated *in vitro* (a 3 μL reaction) using the TnT T7 Coupled Wheat Germ Extract System (Promega) and incubated for 90 min at 30 °C. The expression reaction mixture was supplemented with coelenterazine (Nanolight Technology) at a final concentration of 8 μM. PCR products were diluted at least 20 times prior to transcription/translation in order to eliminate inhibition of the expression from the components of the PCR mixture.

Determination of the Photoprotein Aequorin. A 2 μL aliquot of the expression reaction mixture was pipetted in opaque polystyrene microtiter wells (Microlite 2+, Thermo Labsystems). The bioluminescence of aequorin was measured in the microplate luminometer (PhL from Mediators) by injecting 50 μL of triggering solution (100 mM $CaCl_2$, 100 mM Tris-HCl, pH 7.5) into each well and integrating the signal for 3 s.

Results and Discussion

Assay Principle. The principle of the proposed PTT method that is based on a direct homogeneous assay of the generated polypeptide is illustrated in Scheme 1.

The interrogated DNA (or mRNA) sequence is amplified by PCR. The upstream PCR primer contains the T7 RNA polymerase promoter sequence, the eukaryotic translation initiation sequence (Kozak consensus), and the start codon, thereby enabling the incorporation of these sequences upstream of the interrogated DNA. Through PCR, the 3′ end of the amplified DNA is fused, in-frame, with a DNA fragment comprising the apoaequorin-coding sequence, a translation-termination codon, and a $(dA/dT)_{30}$ segment. The fusion DNA is subjected to a coupled, cell-free transcription and translation in the presence of coelenterazine, an imidazopyrazine chromophore. If the

(10) Verhaegen, M.; Christopoulos, T. K. *Anal. Biochem.* **2002**, *306*, 314–322.

interrogated sequence does not contain mutations causing premature termination of protein synthesis, then translation continues along the fusion transcript and a chimeric polypeptide chain is generated whose C-terminal domain consists of apoaequorin. Once formed, apoaequorin reacts with coelenterazine to give fully active aequorin, a Ca^{2+}-dependent photoprotein. If a nonsense or frameshift mutation exists in the interrogated sequence, then protein synthesis stops and active aequorin is not produced.

Aequorin is a complex of apoaequorin, coelenterazine, and molecular oxygen (attached to coelenterazine as peroxide).[11] Addition of Ca^{2+} induces a conformational change in aequorin that causes oxidative decarboxylation of coelenterazine by the bound oxygen to produce coelenteramide and light ($\lambda_{max} = 470$ nm). This characteristic flash-type bioluminescent reaction allows detection of aequorin down to one attomole (10^{-18} mol) within 3 s.[10,12]

Aequorin was chosen in this work due to a number of unique features that facilitate *in vitro* expression and detection: (a) It consists of a single and relatively short polypeptide chain of 189 amino acids (MW 21000); (b) it does not require post-translational modifications in order to obtain functional conformation; (c) apoaequorin is able to form fully active aequorin in the *in vitro* coupled transcription/translation reaction mixture supplemented with coelenterazine; (d) aequorin can be measured readily in the expression mixture without prior separation (a homogeneous assay), thus avoiding the time-consuming steps of western blotting or affinity capture with antibodies.

Fusion of APC DNA with Aeq DNA. Studies in familial adenomatous polyposis have shown that 95% of germ-line mutations are missense and frameshift mutations that lead to the formation of premature stop codons and give rise to truncated inactive proteins. Most of these mutations are clustered between codons 1286 and 1513 (exon 15) of the APC gene.[13] The part of the APC gene that we chose to study, as a model, spans the region between codons 1138 and 1382 and therefore belongs to the area where most truncating mutations are clustered.

The APC fragment of interest was PCR amplified from genomic DNA isolated from whole blood. The forward primer contained, at the 5′ end, a 37-nt extension comprising the T7 RNA polymerase promoter, Kozak sequence, and start codon (ATG). The reverse primer also carried a 5′ extension that introduced the first 27 bp of the apoaequorin-coding sequence into the PCR product while maintaining the same reading frame with the APC sequence. The amplified APC DNA (796 bp) was fused by overlap extension PCR to a 617-bp DNA fragment consisting of the apoaequorin-coding sequence, the stop codon (TAA), and a 30-bp (dA/dT) tail. Fusion was achieved through the homologous 27-bp sequence at the 3′ end of the APC PCR product and the 5′ end of the apoaequorin DNA. The generated APC-Aeq fusion DNA (1386 bp) is shown in Figure 1.

Optimization of Reaction Conditions for *in Vitro* Expression of APC-Aeq Fusion DNA. Optimization studies were performed by using APC-Aeq fusion DNA fragments prepared from a wild-type (normal) sample and two cell lines, CaCo-2 human epithelial colorectal adenocarcinoma cells and SW480 human colon carcinoma cells (Interlab Cell Line Collection). CaCo-2 and SW480 contain homozygous mutations at codons 1367 (C→T) and 1338 (C→T), respectively, that lead to premature stop codons.

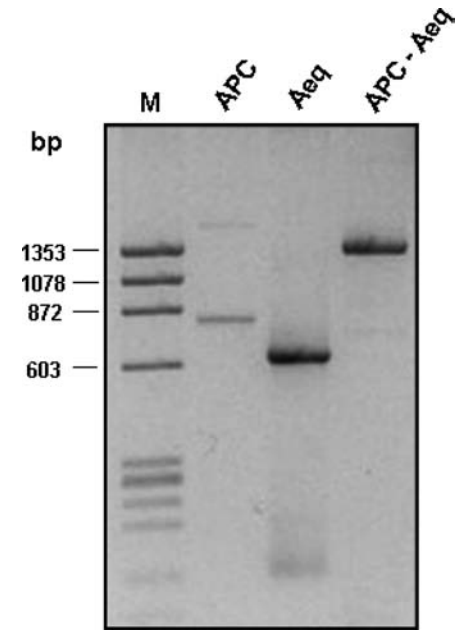


Figure 1. Electropherograms (1.5% agarose gel with ethidium bromide staining). M: DNA markers; APC: amplified DNA (interrogated sequence) from adenomatous polyposis coli; Aeq: aequorin-coding sequence.

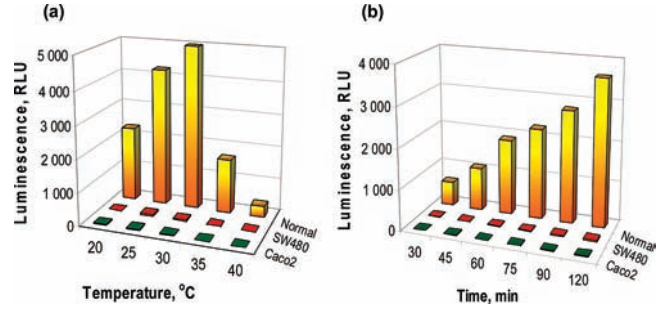


Figure 2. (a) Effect of the temperature on the coupled *in vitro* transcription and translation reaction. APC-Aeq fusion DNA was prepared from a wild-type (normal) sample and from the SW480 and CaCo-2 cell lines that carry truncating mutations. APC-Aeq DNA was expressed *in vitro* for 90 min. The activity of the generated photoprotein aequorin was determined by the addition of a Ca^{2+}-containing triggering solution. (b) Time course of the *in vitro* expression of APC-Aeq fusion DNA prepared from a normal sample and the SW480 and CaCo-2 cell lines. Expression was performed at 30 °C. In both studies the APC-Aeq DNA concentration was 333 pM. A 2 μL aliquot of the expression reaction mixture was used for the measurement of the generated aequorin.

The effect of the temperature of the *in vitro* coupled transcription/translation reaction was studied in the range 20 to 40 °C. The reaction was allowed to proceed for 90 min, and the bioluminescence of the synthesized chimeric protein was measured. The results are presented in Figure 2.

The luminescence obtained from the wild-type sample increases with the temperature, and the maximum signal is achieved at 30 °C. At higher temperatures the signal drops, due to inactivation of various factors of the expression mixture. On the other hand, the signals obtained from the CaCo-2 and SW480 cell lines were very low (≤1% of the signal of the wild-type sample) throughout the entire range of temperatures.

The time course of the synthesis of APC-Aeq chimeric protein was studied at 30 °C, and the reaction was allowed to proceed

(11) Head, J. F.; Inouye, S.; Teranishi, K.; Shimomura, O. *Nature* **2000**, *405*, 372–376.
(12) Glynou, K.; Ioannou, P. C.; Christopoulos, T. K. *Protein Expr. Purif.* **2003**, *27*, 384–390.
(13) Kinzler, K. W.; et al. *Science* **1991**, *251*, 1366–1370.

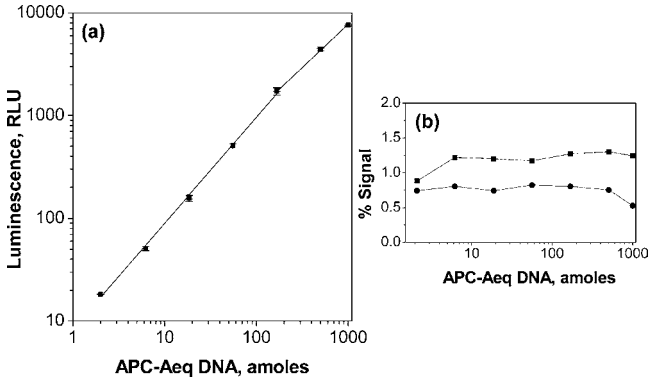


Figure 3. (a) Influence of the input APC-Aeq DNA on the amount of photoprotein. APC-Aeq fusion DNA was from a wild-type sample. Each point represents the mean value of 5 measurements. The error bars correspond to ±1 standard deviation of the signal. (b) Dependence of the translation leaking on the amount of mutant APC-Aeq DNA from SW480 (squares) and CaCo-2 (circles) cell lines.

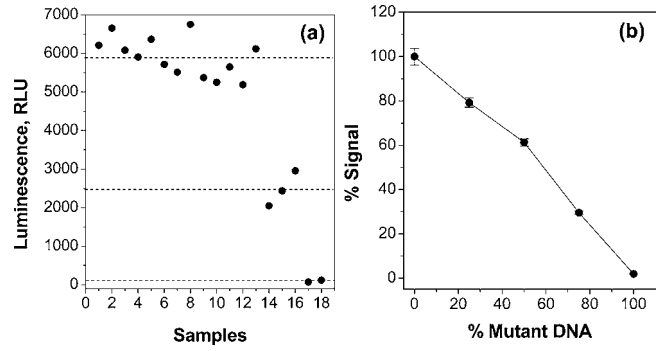


Figure 4. (a) Analysis of clinical samples. Genomic DNA was isolated from 13 normal (wild-type) samples, two mutant cell lines, a patient who was found with multiple clinically suspicious polyps after colonoscopy, and two synthetic heterozygotes. The concentration of APC-Aeq DNA was 333 pM. All expression mixtures (3 μL) were incubated for 90 min at 30 °C. (b) Detection of mutant DNA at various percentages. The wild-type and mutant DNA were mixed at various ratios prior to the expression. The signal from the wild-type was normalized to 100, and the signal of the mixtures was expressed as a percentage of the normal. Each point represents the mean value of 3 measurements. The error bars correspond to ±1 standard deviation.

for time intervals ranging from 30 to 120 min prior to the measurement of the bioluminescence of generated aequorin (Figure 2). We observed a continuous increase of the luminescence of the wild-type sample with the incubation time. Since the signal after 90 min incubation was 76% of the maximum, we chose the 90 min interval for subsequent experiments to ensure a high yield of the synthesized aequorin without compromising the practicality of the method. The signals obtained from the CaCo-2 and SW480 cell lines remained ≤1.4% of the signal of the wild-type sample throughout the range of incubation times. The "illegitimate" synthesis of very low but measurable photoprotein levels even from the mutant APC-Aeq DNA templates was attributed to translation initiation downstream from the point of the mutation. However, the signal due to this translation "leaking" is ≤1.4% of the signal generated by the normal APC-Aeq DNA and therefore does not interfere with the discrimination of the mutant from the wild-type DNA fragments.

The dependence of the luminescence on the amount of APC-Aeq fusion DNA in the expression mixture was studied by performing a series of expression reactions in a final volume of 3 μL containing 2 to 1000 amol (1.8−915 pg) of APC-Aeq DNA. The mixtures were incubated at 30 °C for 90 min, and then the generated chimeric protein was measured via the bioluminescent reaction of aequorin. In Figure 3, the luminescence signal is plotted against the amount (amol) of APC-Aeq DNA in the expression mixture. As low as 2 amol (1.8 pg) of APC-Aeq DNA is easily detectable with a signal-to-background ratio greater than 300. This corresponds to 1.2×10^6 copies of fusion DNA. The background is defined as the luminescence signal obtained when no APC-Aeq DNA is present in the expression mixture.

It is worth emphasizing that although transcription/translation is a complex process requiring the concerted action of numerous factors, there is a simple linear relation between the luminescence of the synthesized protein and the amount of the APC-Aeq DNA template introduced in the expression mixture. The linear range extends up to 200 amol of fusion DNA.

The high sensitivity is a crucial feature for the wide applicability of the method to a variety of target sequences. First, it allows the use of small amounts of fusion DNA (down to pg) as a template for the *in vitro* coupled transcription/translation reaction. This is particularly useful in cases where the yield of overlap extension PCR might be very low and the fusion DNA is undetectable by electrophoresis. Second, because of the high detectability, the proposed method tolerates large losses of photoprotein activity that might be due to inhibition of aequorin activity by the fused polypeptide chains.

We have investigated the dependence of translation leaking on the amount of mutant APC-Aeq DNA (CaCo-2 and SW480) present in the expression mixture. As the amount of APC-Aeq DNA increased from 2 amol to 1000 amol, there was a 190-fold and a 373-fold increase of the signal from CaCo-2 and SW480, respectively. However, as it is clearly shown in Figure 3 for a broad range of the amount of DNA templates, the levels of translation leaks remained in the range 0.5−1.3% of the signal obtained from equal amounts of wild-type APC-Aeq DNA template.

Analysis of Samples. Genomic DNA was isolated from 13 normal (wild-type) samples, two mutant cell lines, a patient who was found with multiple clinically suspicious polyps after colonoscopy (this patient was a heterozygote), and two synthetic heterozygotes (prepared by mixing equimolar quantities, 1 fmol, of the fusion DNA from a wild-type and each of the mutant samples). The APC gene was amplified by PCR and fused, in frame, with the Aeq DNA. The fusion products were subjected to coupled *in vitro* transcription and translation under optimized conditions, and the luminescence of the synthesized aequorin was measured. The results, presented in Figure 4, show the clear distinction between normal, homozygote for the mutation, and heterozygote samples. Heterozygotes give signals around 2500 RLU. The homozygote mutants give signals at the level of 100 RLU, whereas the signals obtained from the normal samples are around 5900 RLU.

To evaluate the sensitivity of the method to homozygote and heterozygote DNA, various amounts of wild-type and mutant amplified DNA were mixed and subjected to *in vitro* transcription/translation. In Figure 4 it is observed that the luminescence from samples containing higher than 25% mutant DNA was significantly lower than that of the wild-type.

Reproducibility. The reproducibility of the method was assessed by expressing the fusion DNA obtained from a wild-type, a homozygote mutant, and a heterozygote sample. The CVs were 8, 11, and 1.8%, respectively ($n = 3$).

Conclusions

The in-frame fusion of the apoaequorin-coding DNA downstream of the interrogated sequence enables the direct, rapid, nonradioactive, and homogeneous assay of the synthesized chimeric protein in the expression mixture, thereby avoiding the laborious steps of SDS-PAGE, western blotting, or affinity purification of the expression product by capture onto a solid surface. Besides the homogeneous detection, the proposed method does not require nested PCR for the introduction of two epitopes, as in PTT-ELISA.[8] The low signal of the mutant cell lines minimizes the probability of false positive results. False negatives could arise if the mutant DNA is lower than 25%. Because our method does not depend on size analysis of the translation products, it is suitable for rapid screening of mutations that are located close to the ends of the target sequence. The CaCo-2 mutation is near the 3′ end of the interrogated sequence. In fact, the C→T base change is located only two nucleotides away from the position of the downstream PCR primer for the APC gene. The signal obtained from the CaCo-2 cell line is consistently about 1.5% of that obtained from the wild-type DNA.

Current research efforts in our laboratory aim at (a) development of multiplex PTT assays capable of simultaneous interrogation of several target sequences for unknown mutations (this goal will be accomplished through the exploitation of color variants of photoproteins and/or variants with different emission kinetics)[14−16] and (b) interrogation of much larger DNA sequences. Although in the present work the fusion of the target DNA with the apoaequorin sequence was achieved via overlap extension PCR, we are working on the development of an alternative protocol, which employs DNA ligase to join directly (avoiding the overlap extension PCR step) the amplified target DNA and the aequorin DNA.

Supporting Information Available: Complete ref 13. This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) Rowe, L.; Combs, K.; Deo, S.; Ensor, C.; Daunert, S.; Qu, X. *Anal. Chem.* **2008**, *80*, 8470–8476.
(15) Maguire, C. A. ; Deliolanis, N. C.; Pike, L.; Niers, J. M.; Tjon-Kon-Fat, L. A.; Sena-Esteves, M.; Tannous, B. A. *Anal. Chem.* **2009**, *81*, 7102−7106.
(16) Frank, L. A.; Borisova, V. V.; Markova, S. V.; Malikova, N. P.; Stepanyuk, G. A.; Vysotski., E. S. *Anal. Bioanal. Chem.* **2008**, *391*, 2891–2896.