

# The Protein Truncation Test (PTT)

By Frans B.L. Hogervorst<sup>(a)</sup>

The Netherlands Cancer Institute, Amsterdam

<sup>(a)</sup>Address correspondence to Dr. Frans B.L. Hogervorst, Department of Pathology, Antoni van Leeuwenhoekhuis/The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands, Telephone: 31 20 512 2753, FAX: 31 20 512 2759 or e-mail: [fransh@hermes.nki.nl](mailto:fransh@hermes.nki.nl)

## Introduction

An increasing number of disease genes have been identified in which the majority of mutations result in premature termination of translation. Standard techniques to detect minor changes in DNA [e.g., SSCP (Single-Strand Conformation Polymorphism) analysis, heteroduplex analysis and DGGE (Denaturing Gradient Gel Electrophoresis) analysis] reveal all sequence changes, including those which are not pathogenic (polymorphisms) or are of questionable pathogenicity (missense mutations). We have developed a rapid and efficient test, designated the Protein Truncation Test (PTT), which specifically uncovers certain disease-causing mutations (i.e., only those causing premature translation termination). Although initially developed for use with Duchenne Muscular Dystrophy (DMD; 1), PTT is now used for screening many different disease genes (Table 1).

**Table 1. Applications of PTT in Human Molecular Genetics.**

| Disease                                    | % Truncating Mutations** | Gene         | References          |
|--|--------------------------|--------------|---------------------|
| Familial Adenomatous Polyposis             | 95%                      | <i>APC</i>   | 5,6                 |
| Hereditary desmoid disease                 | 100%                     | <i>APC</i>   | 9                   |
| Ataxia telangiectasia                      | 90%                      | <i>ATM</i>   | 10                  |
| Hereditary breast and ovarian cancer       | 90%                      | <i>BRCA1</i> | 3                   |
|  | 90%                      | <i>BRCA2</i> | 4                   |
| Cystic Fibrosis                            | 15%                      | <i>CFTR</i>  | 11                  |
| Duchenne Muscular Dystrophy                | 95%                      | <i>DMD</i>   | 1,12                |
| Emery-Dreifuss Muscular Dystrophy          | 80%                      | <i>EMD</i>   | Leiden, unpublished |
| Fanconi anaemia                            | 80%                      | <i>FAA</i>   | 13                  |
| Hunter Syndrome                            | ~50%                     | <i>IDS</i>   | 14                  |
| Hereditary non-polyposis colorectal cancer | ~80%                     | <i>hMSH2</i> | 15                  |
|  | ~70%                     | <i>hMLH1</i> | 16                  |
| Neurofibromatosis type 1                   | 50%                      | <i>NF1</i>   | 17                  |
| Neurofibromatosis type 2                   | 65%                      | <i>NF2</i>   | 18                  |
| Polycystic Kidney Disease                  | 95%                      | <i>PKD1</i>  | 8                   |
| Rubinstein-Taybi Syndrome                  | 10%                      | <i>RTS</i>   | 19                  |

\*\*The percentage of truncating mutations reported which should be detectable using PTT.

## Principle of the Protein Truncation Test

The Protein Truncation Test procedure is outlined in Figure 1. In summary, the coding region of a gene is screened for the presence of translation terminating mutations using *de novo* protein synthesis from amplified copy. The procedure includes three important steps. The first step involves the isolation of genomic DNA and amplification of the target gene coding sequences using PCR<sup>(b)</sup> or, alternatively, isolation of RNA and amplification of the target sequence using Reverse Transcription PCR (RT-PCR). The resulting PCR products are then used as a template for the *in vitro* synthesis of RNA, which is subsequently translated into protein. The final step is the SDS-PAGE analysis of the synthesized protein. The shorter protein products of mutated alleles are easily distinguished from the full length protein products of normal alleles.

<sup>(b)</sup>The PCR process is covered by patents issued and applicable in certain countries. Promega does not encourage or support the unauthorized or unlicensed use of the PCR process.

The key feature of PTT is a specifically designed, tailed, sense forward primer used in the PCR amplification step (Table 2). This primer contains four specific regions. A T7 (or SP6 or T3) promoter sequence at the 5'-end directs the production of RNA, using the corresponding phage RNA Polymerase. The promoter sequence is separated from a eukaryotic translation initiation sequence (Kozak sequence; 2) by a spacer sequence of 3-6bp. The 3'-end contains the target gene sequence (approximately 17-20bp) in-frame with the ATG codon from the Kozak sequence. To facilitate cloning of the amplified product, a restriction site sequence is frequently engineered into the 5'-end of the primer. This restriction site should be chosen from those known to cut efficiently when positioned at the end of a linear DNA molecule.

Although there are a few examples in which genomic DNA is used as the template for PTT [e.g., *BRCA1* (3), *BRCA2* (4) and *FAP* (5,6)], mRNA is more commonly used. The mRNA is reverse transcribed to cDNA, which then functions as a template for amplification. The best source of RNA is cells in which the target gene is abundantly expressed, but for practical reasons RNA is generally isolated from freshly drawn peripheral blood lymphocytes or cultured skin fibroblasts. Depending upon RNA transcription levels, nested PCR is often necessary to amplify minute amounts of truncated transcripts.

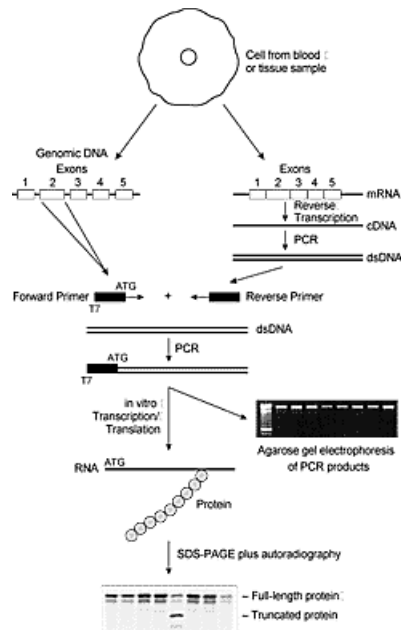


Figure 1. Schematic diagram of Protein Truncation Test.

Table 2. Sequences of Different T7-Modified Oligonucleotide Primers for *in vitro* Transcription and Translation.

| Restriction Site Sequence | Bacteriophage Promoter T7 Sequence | Spacer | Eukaryotic Translation Initiation Sequence | Target Sequence | References |
|---------------------------|------------------------------------|--------|--|-----------------|------------|
| GGATCC                    | TAATACGACTCACTATAGGG               | AG     | CCACC ATG                                  | NNN NNN NNN     | 4,20       |
| GGATCC                    | TAATACGACTCACTATAGGG               | AG     | CCACC ATG G                                | NN NNN NNN      | 15,16      |
| GGATCC                    | TAATACGACTCACTATAGG                | AACAG  | CCACC ATG                                  | NNN NNN NNN     | 1,6        |
| nnn                       | TAATACGACTCACTATAGG                | AACAG  | CCACC ATG G                                | NN NNN NNN      | 3,13       |

"N" and "n" = any base

## Designing primer pairs

Several factors are important for the design of the PTT primer pairs. If a disease gene contains several kilobases of coding sequence, it is necessary to amplify several 1-2kb fragments. The choice of primers ultimately determines the sensitivity obtained; they should be designed with care. To ensure that truncation mutations in the 5'- or 3'-end of a fragment are not missed, flanking segments should have an overlap of 350-500bp. The ATG-initiation codon in the tailed sense forward primer must be in-frame with the coding sequence. If the AUG is not in-frame, translation will start at the first internal translation initiation site and an unexpected, shorter translation product will be produced. If possible, the tailed forward primer of the first segment should be located upstream of the natural translation initiation site; otherwise, mutations affecting this site will not be detected. A reverse primer should **not** be selected near the end of a region where a large open reading frame is present in one or both of the alternative reading frames; in such cases a frameshift mutation to an alternative reading frame will not cause a truncation and it would thus be missed. It is important to place the forward primer in a region where the sequence contains a codon for the labeled amino acid to be incorporated (e.g., cysteine and methionine are infrequently encoded amino acids while leucine and lysine are more common).

When PTT is used for RNA-based mutation detection, primers from overlapping segments should be located in different exons. This should avoid the failure of PCR to amplify both segments of one allele, due to an intragenic deletion of a single exon or to a splicing defect. Similarly, primers covering both the end of one exon and the beginning of the next (i.e., spanning an exon junction) should be avoided.

Successful amplification of the product to be analyzed via transcription/translation should not be too demanding. Although transcription/translation of PCR products up to 4-5kb is not difficult, best results are obtained when transcribing/translating amplification fragments of up to 2kb from genomic DNA and fragments of 1.3-1.6kb from cDNA.

## Mutation detection

After RT-PCR or PCR amplification, agarose gel electrophoresis is necessary to determine yield and size of the products. Usually 10-40ng/ $\mu$ l of PCR product is sufficient for PTT detection. The presence of abnormally sized products is indicative of splicing mutations or genetic rearrangements such as deletions or duplications. The next step in PTT is the *in vitro* transcription/translation reaction. The development of the TNT<sup>®</sup> T7 Quick Coupled Transcription/Translation System<sup>(c,d,e)</sup> has greatly simplified this step (7). In a single tube, both *in vitro* transcription and translation are performed. The TNT<sup>®</sup> T7 Quick System (Cat.# L1170) has further reduced sample handling by offering a master mix to which only label and PCR products have to be added.

<sup>(c)</sup>U.S. Pat. Nos. 5,324,637 and 5,492,817 and European Pat. No. 566 714 B1 have been issued to Promega Corporation for coupled transcription/translation systems that use RNA polymerase and eukaryotic lysates.

<sup>(d)</sup>U.S. Pat. No. 5,552,302 has been issued to Promega Corporation for the methods and compositions for production of human recombinant placental ribonuclease inhibitor (PRI). Inhibitors of Angiogenin, which comprises a segment of human PRI, is the subject of U.S. Pat. Nos. 4,966,964, 5,019,556 and 5,266,687 assigned to the President and Fellows of Harvard College and exclusively licensed to Promega Corporation.

<sup>(e)</sup>For research use only; not for use in diagnostic procedure.

## Protein detection

There are several options for the detection of newly synthesized proteins. The most commonly used procedure is incorporation of radiolabeled amino acids during synthesis, using [<sup>35</sup>S]methionine, [<sup>35</sup>S]cysteine or [<sup>3</sup>H]leucine. The TNT<sup>®</sup> T7 Quick Coupled System is, at present, only available for [<sup>35</sup>S]methionine. For separation of the translation products, appropriate SDS-PAGE conditions must be chosen for simultaneous detection of nearly full length products as well as small products. Usually a 15% polyacrylamide gel is used for PTT products of up to 60-75kDa. After electrophoresis, the SDS-PAGE gels can be treated with chemiluminescent solutions to enhance the signals; the gels are then dried. Generally a 2-18 hour exposure to X-ray film results in a clear signal. The use of a beta-emission detector [PhosphorImager<sup>™</sup> (Molecular Dynamics)] reduces the exposure time to less than 1-2 hours and preserves the results in a digitized manner. In general, analysis of the translation products demonstrates strong signals or bands for the desired translation products, occasionally accompanied by several smaller, weaker molecular weight bands. These weaker products often derive from internal, weak translation initiation sites (AUG) and, in exceptional cases, might obscure the analysis and/or detection of truncated fragments. Repositioning of primers and/or overlapping regions can be used to correct this.

Abnormal, short protein products indicate the presence of truncation mutations. Whether or not both truncated and wild-type translation products are detected in the same sample depends upon the disease. In X-linked syndromes (e.g., DMD and Hunter Syndrome), the analysis of male patients is simplified by the presence of only one allele which either contains a mutation or does not.

## Non-radioactive detection technique

In many laboratories the use of non-radioactive detection methods is becoming an increasingly important issue. For this purpose, systems like Promega's Transcend<sup>™</sup> Non-Radioactive Translation Detection Systems can be used. Newly synthesized proteins are labeled by direct incorporation of biotin-charged lysines. After SDS-PAGE separation and transfer to a membrane, the biotinylated proteins are detected by a streptavidin-conjugate and visualized using a chemiluminescent substrate. One clear advantage of the non-radioactive procedure is that it can be used by laboratories not approved for isotopic experiments. Furthermore, although the non-radioactive procedure requires more handling (i.e., blotting to membranes), the final results are obtained more rapidly due to much shorter exposure times (minutes versus hours).

An additional advantage of PTT over conventional assays is that the length of the truncated protein pinpoints the position of the mutation, thereby facilitating its confirmation by sequence analysis. Difficulties can be expected in the case of in-frame deletions comprising 4kDa or less, for example those due to exon skipping, caused by splice site mutations. In such events, the truncated protein is only slightly smaller than the wild-type product and thus difficult to detect by SDS-PAGE. Fortunately, such mutations are accompanied by smaller RT-PCR products which can be easily detected by agarose gel analysis.

The boundaries of the translated fragments represent the most critical regions for PTT analysis and are primarily determined by the resolution of SDS-PAGE analysis. Early (N-terminal) mutations may result in products which have too little or no label incorporated, or

their electrophoretic migration might fall outside of the resolution range. Late (C-terminal) mutations might result in a size difference which is not resolved near the top of the gel. Finally, mutations at translation initiation and termination sites represent a special case. Here, an internal control of the overlapping segment is not available; special attention (e.g., sequence analysis or SSCP) is required to reveal these mutations.

## Limitations

Mutation detection by PTT may be limited in two ways: 1) failure to amplify the mutated allele and, 2) failure to detect very small in-frame deletions/insertions or missense mutations. Amplification failure can have several explanations including a mutation of the primer binding site (an altered or deleted sequence), large insertions, translocations and inversions which go beyond amplifiable lengths, or duplications when one or both primer binding sites lie within the duplicated segment. Mutations which affect the amount of RNA produced or which render the mutated mRNA unstable may also not be detected. PTT will fail to detect very small in-frame deletions/insertions because the mobility shifts are too small to detect. Missense mutations will go undetected.

## Disease genes analyzed by PTT

Initially, PTT was developed for detecting mutations in the large dystrophin gene (1) which causes the disease state of Duchenne Muscular Dystrophy. Subsequently, additional genes have been identified which contain nonsense and frameshift mutations corresponding to a major fraction of patients exhibiting either the disease or carrier state of the disease. PTT is now widely used to identify and characterize these mutations (Table 1). Clinically important tumor suppressor genes such as those involved in breast cancer [*BRCA1* (3) and *BRCA2* (4)] and colon cancer (*APC*; 5,6) are ideally studied through the use of PTT. These genes contain one or two large exons, within which a majority of the known truncation mutations lie, thus a DNA-based diagnostic protocol can be used. Although most laboratories currently use established DNA-based PCR protocols, some have switched to the more demanding RNA-based PCR protocols (i.e., RT-PCR). However, one of the disadvantages of the RNA-based approaches is the necessity of recontacting specimen donors for collection of fresh samples for RNA isolation.

Other disease characteristics make PTT the method of choice for mutation analysis. In diseases where the majority of cases are caused by a single mutation or a small number of specific mutations, PTT may present an attractive method with which to reveal the remaining mutations. For example, in Cystic Fibrosis one mutant type (delF508) comprises 70% of disease mutations; of the remaining 30% of mutations, half cause premature termination of translation. PTT can be useful in ascertaining the relationship between genotype and disease severity because truncating mutations are usually associated with a severe phenotype. For example, Hunter Syndrome and Limb-Girdle Muscular Dystrophy patients with a severe phenotype usually carry truncating and not missense mutations. Missense mutations are more frequently found in less severe phenotypes but this greatly depends on the disease gene and the location of the missense mutations (whether or not a functional domain is affected). Furthermore, PTT can be useful for detecting the disease gene when it is present in multiple copies, which can complicate mutation analysis since PCR simultaneously amplifies all gene copies. We have successfully used PTT to detect truncating mutations in a mixture of PCR fragments derived from the many gene copies of the *PKDI* gene (8), which causes Polycystic Kidney Disease.

## Future of PTT

PTT has clear advantages over other screening methods when a large portion of the mutations are due to small genetic changes, causing truncation mutations. The ability to use mRNA instead of genomic DNA significantly reduces the number of reactions to be performed, simplifies the procedure and speeds the analysis. For example, the complete coding region of the DMD gene is among the largest known genes, encompassing 79 exons. However, a complete analysis of the gene can be performed by amplifying 10 fragments from mRNA. In addition, by only identifying disease causing truncation mutations, PTT analysis is not hampered by the screening to remove phenotypically silent mutations such as polymorphisms. Polymorphisms are more frequent in the noncoding region of a gene and are not detected by PTT. Finally, the size of the truncated product pinpoints the DNA mutation, and thus enhances sequence analysis for confirmation.

Since the first publication regarding the use of PTT for DMD four years ago, there has been a tremendous increase in PTT use and applications. The identification of mutations in tumor suppressor genes has greatly benefited from PTT screening, as most of these genes are multi-exonic, encompassing several thousand base pairs (up to 10kb) of coding region, and germline mutations are predominantly truncation mutations. In the near future it is expected that methods will be developed to: 1) non-isotopically label the proteins (e.g., fluorescent amino acids), which will minimize gel handling and eliminate the need to blot the gel and, 2) use tailed primers which add a N- or C-terminal tag to the translation target. This should eliminate the detection of internal translation initiation products and, ultimately, this type of tag may enable the automation and quantitative measurement of full length and truncated products. In a research setting, PTT provides a rapid and versatile mutation detection technique for the direct testing of candidate disease genes. This facilitates early analysis when the candidate gene's sequence is still incomplete but sufficient for the generation of a primer pair.

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**Editor's note:** *Promega is the leading supplier of reagents for the PTT. The TNT<sup>®</sup> Coupled Transcription/Translation Systems have been cited in over 20 publications investigating mutations related to disease, including breast cancer, colon cancer and Duchenne's Muscular Dystrophy. To facilitate performing PTT assays Promega has developed quality, innovative reagents. The TNT<sup>®</sup> T7 Quick Transcription/Translation System provides all the reagents for the transcription/translation step of the PTT in one tube. Unpurified PCR products can be added directly to the optimized TNT<sup>®</sup> T7 Quick Master Mix. Throughput is improved as only one pipetting step of the TNT<sup>®</sup> T7 Quick Master Mix is required.*

*The Access RT-PCR system has been successfully used for the RT-PCR amplification of mRNA prior to transcription/translation in the PTT assay (NF1 gene, personal communication). The Access RT-PCR System is a sensitive one-tube amplification method.*

*An attractive alternative to radioactive detection of PTT products is non-radioactive detection of translation products using the Transcend<sup>™</sup> Colorimetric (Cat.# L5070) or Chemiluminescent Detection Systems (Cat.# L5080). A biotinylated lysine is incorporated into the protein produced and the protein is detected with a streptavidin conjugate. Labeled lysines are particularly useful to detect proteins produced in the PTT assay, as lysines are one of the most common amino acids. Many gene segments may not contain methionine which is commonly used as a radioactive label. For more information on these products, see page 27 of this issue of Promega Notes. Please contact Promega for your copy of the PTT Bibliography, Part# BL002.*

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