

# Benchmarks

this ratio increases. In a degenerate primer pair, the degeneracy of the upstream and downstream primer are often different from each other (1,3,4). In this situation, a concentration ratio for a degenerate primer pair of 1:1 does not result in an effective concentration ratio of 1:1, since the effective concentration of the primer with the higher degeneracy is lower. In this case, increasing the total concentration of the more degenerate primer with respect to the less degenerate primer will reduce the unbalance in their effective concentrations and increase the specificity and sensitivity of the degenerate primer reaction.

Although relative levels of degeneracy between two members of a degenerate primer pair provide useful information for optimizing the concentration ratio of the primer pair, our results suggest that there is not a linear relationship between relative degeneracy and optimal primer pair ratios. One reason may be that degeneracy is not the only factor that influences the ratio of the effective concentration to total concentration of a degenerate primer. The complexity of the template DNA (i.e., how many different targets are present in the template mixture) may also affect the optimization of primer ratios used in degenerate PCR. If the template mixture contains a greater number of members of a gene family, then it can be amplified by a greater number of primer species in the degenerate primer mixture, resulting in an increase in the effective concentration. The result is that the ratio of the effective concentration to total concentration of a degenerate primer may change from sample to sample.

Mismatches between template and primer that generally occur in degenerate PCR will also influence the effective primer concentration (5). We have observed that when using degenerate primers in a reaction, multiple gene fragments are amplified that are identical in sequence except in the primer region, indicating that primers with a limited mismatch to the cDNA sequence can prime the same cDNA sequence. These results confirm that mismatches are a common occurrence when using degenerate primers for PCR amplification. By providing several different primer sequences that can effectively amplify a particular template sequence,

mismatches actually increase the effective concentration of the degenerate primer. However, it is not possible to calculate the magnitude of their influences; therefore, determination of the optimal concentration ratio for a degenerate primer pair must be achieved empirically, using relative primer degeneracy as a guideline for optimization.

## REFERENCES

1. Bovenkamp, D.E. and P.A. Greer. 2001. Degenerate PCR-based cloning method for Eph receptors and analysis of their expression in the developing murine central nervous system and vasculature. *DNA Cell Biol.* 20:203-213.
2. Buck, L. and R. Axel. 1991. A novel multi-gene family may encode odorant receptors: a molecular basis for odor recognition. *Cell* 65:175-187.
3. Chen, F. and C.A. Suttle. 1995. Nested PCR with three highly degenerate primers for amplification and identification of DNA from related organisms. *BioTechniques* 18:609-612.
4. Harwood, C.A., P.J. Spink, T. Suretheran, I.M. Leigh, E.M. de Villiers, J.M. McGregor, C.M. Proby, and J. Breuer. 1999. Degenerate and nested PCR: a highly sensitive and specific method for detection of human papillomavirus infection in cutaneous warts. *J. Clin. Microbiol.* 37:3545-3555.
5. Huang, M.M, N. Arnhem, and M.F. Goodman. 1992. Extension of base mispairs by *Taq* DNA polymerase: implications for single nucleotide discrimination in PCR. *Nucleic Acids Res.* 20:4567-4573.
6. Knittel, T. and D. Picard. 1993. PCR with degenerate primers containing deoxyinosine fails with *Pfu* DNA polymerase. *PCR Methods Appl.* 2:346-347.

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## Modified Rapid Expansion Detection Method to Analyze CAG/CTG Repeat Expansions

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Trinucleotide repeat expansions (TREs) have been associated with several genetic neurological and neuromuscular disorders including Huntington disease, Fragile X syndrome, myotonic dystrophy, and Friedreich ataxia (1, 11,12). Among trinucleotide repeats, the expansion of CAG/CTG has been studied most extensively because the expansion of this repeat is found to be associated very frequently in neurological and neuromuscular disorders.

Repeat expansion detection (RED) has been used widely to identify and locate TREs in the human genome. The RED technique was introduced by Schalling et al. (8) and modified by other investigators (5,13,14). This method allows the detection of expanded repeats without prior knowledge of the location of the repeats or the flanking sequences. In a RED reaction, adjacent phosphorylated short oligonucleotides that anneal to TREs containing genomic DNA template are ligated with a thermostable DNA ligase (Ampligase<sup>®</sup>; Epicentre, Madison, WI, USA). These ligated oligonucleotides are then electrophoresed on a gel, transferred to nylon membrane, and visualized by hybridization with a radiolabeled probe. The details of the published protocol are as follows. Phosphorylation of oligonucleotides is carried out using ATP in the presence of T4 polynucleotide kinase. The ligation reactions are performed in 400–500 cycles of 20 s ligation at 65°C–75°C, according to the length of oligonucleotides used, and 10 s denaturing at 95°C. This ligation reaction is linear compared to an exponential PCR. In each cycle of the ligation reaction, only one copy of the ligated oligonucleotides is produced; thus, the RED product yield is very low. Therefore, compared to PCR-based methods, a large amount of starting genomic DNA template is required in a RED analysis. Published protocols suggest the use of 1–10 µg genomic DNA (2,3,8,10,14). After

# Benchmarks

performing the ligation reaction, the product is electrophoresed on a denaturing 6% polyacrylamide (19:1 acrylamide bis-acrylamide), transferred onto a nylon membrane by capillary action, and hybridized with radiolabeled repeat-oligonucleotide that is complementary to the oligonucleotide used in ligation reaction. The membrane is then exposed to X-ray film.

One of the main limitations of the RED method has been the need for large quantities of genomic DNA for each analysis. The amount of available patient specimen is a limiting factor for several research areas, including cancer genetics. For example, most tissue samples obtained from cancer patients are small and yield very small quantities of DNA, which in turn limits their use for research. To increase the efficiency of the RED method (by using smaller quantities of DNA), we have modified the protocol as described below. For optimization, we have used the human leukemia cell line, HL-60 (ATCC, Manassas, VA, USA) as the source for DNA template. In a previous screen using the RED method, we have shown that HL-60 carries a relatively large CAG repeat (approximately 80 CAGs) compared to other cell lines.

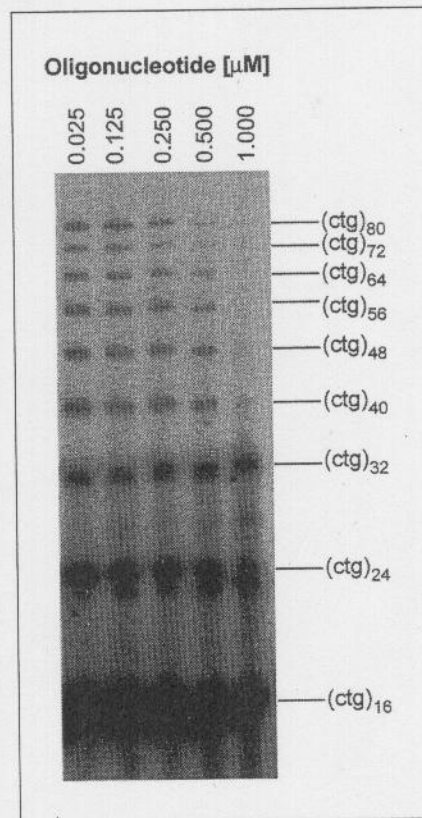
The following changes were made to the traditional protocol: (i) before the ligation reaction, the oligonucleotides are phosphorylated using  $\gamma$ - $^{32}\text{P}$  ATP, (ii) the gel transfer and hybridization steps are eliminated, and (iii) the amount of genomic DNA required is reduced to a great extent. The labeling reaction is carried out in a 20- $\mu\text{L}$  total volume containing 0.125  $\mu\text{M}$  (CTG) $_8$  oligonucleotide, purified by PAGE (Invitrogen Canada, Burlington, Ontario, Canada), 15  $\mu\text{Ci}$   $\gamma$ - $^{32}\text{P}$  ATP, 3000 Ci/mmol (Perkin Elmer Life Sciences, Boston, MA, USA), 0.175  $\mu\text{M}$  ATP, 1 $\times$  T4 polynucleotide kinase buffer and 10 U T4 polynucleotide kinase (Invitrogen Canada). The mixture is incubated at 37°C for 30 min, and the reaction is then stopped by heating to 65°C for 5 min. The labeled oligonucleotides can be used immediately or stored at -20°C for future use. The ligation reaction is carried out in a 20- $\mu\text{L}$  total volume containing 0.1–0.5  $\mu\text{g}$  genomic DNA template, 5  $\mu\text{L}$  labeling mixture, 1 $\times$  ligase buffer, and 15 U Ampligase. The reaction is performed in

a PTC-100<sup>TM</sup> thermal cycler (MJ Research, Waltham, MA, USA) by applying an initial denaturation for 5 min at 95°C, 500 cycles of ligation at 65°C for 30 s, and denaturing at 95°C for 10 s. Ligation products are mixed with a 1:1 ratio of loading buffer containing 98% formamide, 10 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol. A 10- $\mu\text{L}$  aliquot from each ligation reaction is run on a 6% denaturing polyacrylamide gel (1 mm thick) at 500 V for 2 h. The gel is dried and exposed to X-ray film for 12–24 h.

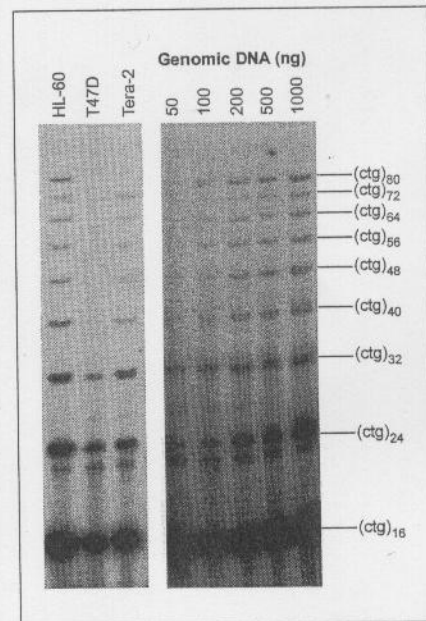
The amount of oligonucleotide used in the labeling reaction has a significant influence on the efficiency of the ligation reaction. The use of a large amount of oligonucleotide would reduce the total labeling efficiency, resulting in excess unlabeled oligonucleotide in the labeling reaction. During the ligation reaction, unlabeled oligonucleotides compete with labeled oligonucleotides in binding to the genomic DNA tem-

plate. An excess of unlabeled oligonucleotides will influence the production of ligation products, specifically those made using the low copy number expanded repeats as a template. Titration of different concentrations of (CTG) $_8$  oligonucleotide has demonstrated that the efficient ligation for large repeats can be performed using oligonucleotide concentrations between 0.025 and 0.5  $\mu\text{M}$  in the labeling reaction (Figure 1). As seen in Figure 1, there is an inverse relationship between the oligonucleotide concentration used in the labeling reaction and the quality of the ligation products obtained. The ligation products were successfully detected at an oligonucleotide concentration of 0.025  $\mu\text{M}$ , whereas they are almost undetectable at 1.0  $\mu\text{M}$ .

This modification has also shortened the protocol to a great extent. The introduction of labeled oligonucleotides into the ligation reaction has eliminated the need for two cumbersome steps. These steps involve the transfer of the ligation



**Figure 1. Titration of oligonucleotide concentration in the labeling reaction.** The labeling reactions were carried out using different oligonucleotide concentrations ranging from 0.025 to 1.0  $\mu\text{M}$ . A 10- $\mu\text{L}$  aliquot of the reaction was loaded in each lane.



**Figure 2. RED analysis using varying amounts of genomic DNA as a template.** RED results were obtained using 500 ng genomic DNA from different cell lines with different CAG repeat lengths: Tera-2-(CTG) $_{92}$ , T47D-(CTG) $_{32}$ , and HL-60-(CTG) $_{80}$  (left panel). Genomic DNA from HL-60 cell line was used to determine the sensitivity of the RED method in terms of DNA concentration. The ligation reaction was carried out in a 20- $\mu\text{L}$  volume (as described in the text) using various amounts of starting genomic DNA ranging from 50 ng to 1  $\mu\text{g}$  (right panel).

# Benchmarks

products on the gels to nylon membranes and hybridization of the nylon membranes using radiolabeled probes. The elimination of these steps in the modified method successfully reduced the labor and the time involved in performing the traditional RED analysis.

A significant advantage of the modified RED analysis is that it allows the use of a small amount of genomic DNA as a starting material compared to the traditional method. In fact, the necessity for large amounts of genomic DNA may have been one of the reasons for the limited application of this technique in diseases other than neurological disorders. In this study, we have shown that the modified RED method can be used to detect genomic repeat expansions using as little as 50–100 ng genomic DNA (Figure 2). This is comparable to the amount of genomic DNA used in standard PCR. As seen in Figure 2, the modified RED method was able to detect sufficiently an intense ligation signal using 200 ng genomic

DNA. The signal was also visible at lower concentrations (50 and 100 ng), although it was not as intense.

The number of neurological diseases associated with TREs has been increasing extensively. The RED method constitutes a powerful tool to identify other diseases in addition to neurological and neuromuscular disorders, caused by the same mechanism. The application of this method is not restricted to the study of trinucleotide repeats only. Several other diseases have also been reported to be associated with the expansion of other repeat motifs, including tetranucleotides, pentanucleotides, hexanucleotides, and even dodecanucleotides (4,6,7,9). The modified RED method described here allows a rapid and sensitive screening for TREs that can be efficiently applied to various diseases in studies where limited amounts of patient genomic DNA are available.

## REFERENCES

1. Cummings, C.J. and H.Y. Zoghbi. 2000. Fourteen and counting: unraveling trinucleotide repeat diseases. *Hum. Mol. Genet.* 9:909-916.
2. Hofferbert, S., N.C. Schanen, F. Chehab, and U. Francke. 1997. Trinucleotide repeats in the human genome: size distributions for all possible triplets and detection of expanded disease alleles in a group of Huntington disease individuals by the repeat expansion detection method. *Hum. Mol. Genet.* 6:77-83.
3. King, B.L., H.Q. Peng, P. Goss, S. Huan, D. Bronson, B.M. Kacinski, and D. Hogg. 1997. Repeat expansion detection analysis of (CAG)<sub>n</sub> tracts in tumor cell lines, testicular tumors, and testicular cancer families. *Cancer Res.* 57:209-214.
4. Lalioti, M.D., H.S. Scott, C. Buresi, C. Rossier, A. Bottani, M.A. Morris, A. Malafosse, and S.E. Antonarakis. 1997. Dodecamer repeat expansion in cystatin B gene in progressive myoclonus epilepsy. *Nature* 386:847-851.
5. Lindblad, K., M.L. Savontaus, G. Stevanin, M. Holmberg, K. Digre, C. Zander, H. Ehrsson, G. David et al. 1996. An expanded CAG repeat sequence in spinocerebellar ataxia type 7. *Genome Res.* 6:965-971.
6. Liquori, C.L., K. Ricker, M.L. Moseley, J.F. Jacobsen, W. Kress, S.L. Naylor, J.W. Day, and L.P. Ranum. 2001. Myotonic dystrophy type 2 caused by a CCTG expansion in intron 1 of ZNF9. *Science* 293:864-867.
7. Matsuura, T., T. Yamagata, D.L. Burgess, A. Rasmussen, R.P. Grewal, K. Watase, M. Khajavi, A.E. McCall et al. 2000. Large expansion of the ATTCT pentanucleotide repeat in spinocerebellar ataxia type 10. *Nat. Genet.* 26:191-194.

8. Schalling, M., T.J. Hudson, K.H. Buetow, and D.E. Housman. 1993. Direct detection of novel expanded trinucleotide repeats in the human genome. *Nat. Genet.* 4:135-139.
9. Sirugo, G. and K.K. Kidd. 1998. Repeat expansion-detection analysis of telomeric uninterrupted (TTAGGG)<sub>n</sub> arrays. *Am. J. Hum. Genet.* 63:648-651.
10. Sirugo, G., A.S. Deinard, J.R. Kidd, and K.K. Kidd. 1997. Survey of maximum CTG/CAG repeat lengths in humans and non-human primates: total genome scan in populations using the repeat expansion detection method. *Hum. Mol. Genet.* 6:403-408.
11. Usdin, K. and E. Grabczyk. 2000. DNA repeat expansions and human disease. *Cell Mol. Life Sci.* 57:914-931.
12. Vincent, J.B., A.D. Paterson, E. Strong, A. Petronis, and J.L. Kennedy. 2000. The unstable trinucleotide repeat story of major psychosis. *Am. J. Med. Genet.* 97:77-97.
13. Vincent, J.B., T. Klempan, S.S. Parikh, T. Sasaki, H.Y. Meltzer, G. Sirugo, P. Cola, A. Petronis, and J.L. Kennedy. 1996. Frequency analysis of large CAG/CTG trinucleotide repeats in schizophrenia and bipolar affective disorder. *Mol. Psychiatry* 1:141-148.
14. Zander, C., J. Thelaus, K. Lindblad, M. Karlsson, K. Sjoberg, and M. Schalling. 1998. Multivariate analysis of factors influencing repeat expansion detection. *Genome Res.* 8:1085-1094.

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