Table 1, minimal overlap of signals was observed, thus confirming that CFP can be used as an internal control for transcription efficiency when measuring LEF/TCF-dependent YFP expression. To validate the LEF/TCF-dependent, fluorescence-based reporter gene assay, transfections were performed using various stimulators and inhibitors of the canonical Wnt pathway (Figure 1). Co-expression of Wnt1 resulted in a 9-fold stimulation of LEF-EYFP reporter gene activity, which was dependent on LEF expression. Likewise, β-catenin co-expression resulted in an 8-fold LEF-dependent increase in activity. Consistent with luciferase-based LEF-dependent reporter gene assays, the stimulation of LEF-EYFP reporter gene expression by Wnt1 was inhibited by co-expression of either Axin or glycogen synthase kinase-3β (GSK-3). Similar levels of Wnt activation of LEF-EYFP reporter gene expression were observed using SuperFect™ (Qiagen, Valencia, CA, USA) transfected COS-1 cells (data not shown). The use of a destabilized YFP (p2EYFP-1, with a 2-h half-life) as the reporter gene did not significantly alter the results of the assay with respect to the levels of stimulation and inhibition (data not shown).

The results demonstrate that the LEF/TCF-dependent, fluorescence-based assay is comparable to luciferase-based assays with respect to the levels of activation by Wnt1 and inhibition by Axin and GSK-3 (5). The advantages of the fluorescence-based assay include ease of lysate preparation, as well as the rapidity and ease of fluorescent measurement of reporter gene and internal control gene expression. In addition, unlike luciferase, the fluorescence-based assay does not require the addition of expensive substrates; thus, the cost per assay is low.

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Optimal Ratio of Degenerate Primer Pairs Improves Specificity and Sensitivity of PCR

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PCR utilizing degenerate primers is a powerful tool to search for new sequences related to a known gene family or to amplify simultaneously several members of a gene family. Despite the power of the technique, it is often limited by a lack of specificity and sensitivity. Since degenerate primers are a mixture of several to thousands of related sequences, they inevitably yield non-specific products in addition to the specific one. This problem becomes more serious when the available PCR template is in low abundance. To increase sensitivity, nested PCR is often used, and protocols using as many as three rounds of degenerate primer PCR have been described (3,4). In many circumstances, these additional sets of degenerate primers may not be available because of the constraints of primer design. In this case, optimization of degenerate primer PCR may be the only choice. Although optimizing methods for general PCR are well documented, there are few reports describing the specific parameters for degenerate primer PCR. It has been shown that proofreading polymerase causes failure of degenerate primer PCR because of its 3′→5′ exonuclease activity that may degrade degenerate primers (6). The importance of primer design and the necessity of using relatively high degenerate primer concentrations are well accepted (1). Annealing temperature is also an important factor in degenerate PCR, as it is in general PCR. We have used degenerate primers extensively to PCR-amplify and clone members of the very large family of olfactory receptors (2). In the process of utilizing this technique, we have identified another factor important for optimizing degenerate primer PCR. The ratio of the concentrations of upstream to downstream degenerate primers is critical for maximizing sensitivity and specificity of the PCR, especially when (i) the amount of available PCR template is low, (ii) high
concentrations of degenerate primers are used, and (iii) the degeneracy of upstream and downstream degenerate primers is different.

The following degenerate primers and PCR conditions were used to establish optimal conditions for specificity and sensitivity in the amplification of olfactory receptors from cDNA derived from mRNA extracted from olfactory epithelium of the tiger salamander, *Ambystoma tigrinum* Upstream (sense) primer is 5'-GAYMGGTWYGTCGGATGTG-3', and downstream (antisense) primer is 5'-TVRBCYRTAR TRADNGGRRTT-3'. The upstream primer has four degenerate positions, each coding for two bases (Y, M, W, and Y), and the downstream primer has eleven degenerate positions, seven of which code for two bases (Y, M, W, and Y), three of which code for three bases (V, B, and D), and one of which codes for four bases (N). The degeneracy of the upstream primer is 16, whereas that of the downstream primer is 13,824. Thus, the degeneracy of the downstream primer is significantly greater than that of the upstream primer, and this difference is a key factor affecting PCR amplification and is addressed in this procedure. Primer pair A was prepared in accordance with current degenerate PCR protocols (3,4) that use the same concentration of upstream and downstream primer (1). It contains 20 μM upstream primer and 20 μM downstream primer; therefore, its concentration ratio is 1:1. Primer pair B is designed using a different ratio, with 2 μM upstream and 20 μM downstream primers, resulting in a concentration ratio of 1:10. PCR template was prepared by reverse transcription of 10 μg total RNA extracted and purified from salamander olfactory epithelium. After reverse transcription, the cDNA was divided into three serial dilutions: 1:1, 1:100, and 1:1000, which were used for testing PCR sensitivity. A 5x PCR master mixture was made to minimize the manipulation differences when preparing the reactions. This mixture contains 1 mM dNTPs, 200 mM Tricine-KOH, 80 mM KCl, 17.5 mM MgCl2, 18.75 μg/mL BSA, and 5× TITANIUM® Taq DNA polymerase (BD Biosciences Clontech, Palo Alto, CA, USA). Reactions were prepared by mixing 13 μL double-distilled water, 4 μL 5x PCR master mixture, 2 μL primer pair A or B, and 1 μL serially diluted cDNA PCR template. For template mixtures using 1:1 and 1:100 dilutions, the following PCR conditions were used: 94°C for 3 min, followed by 35 cycles of 30 s at 94°C, 45 s at 56°C, and 60 s at 72°C. The last cycle was followed by a final elongation step at 72°C for 10 min. For templates using a 1:1000 dilution, PCR conditions were identical to the above, except that the number of cycles was increased from 35 to 42. After the reaction was completed, 2 μL PCR product were loaded on a 1.5% TAE agarose gel.

Figure 1 shows an image of the agarose gel on which the PCR products were run. When a 1:1 template dilution is used, primer pair A generates a band of the expected size (approximately 500 bp), as well as several nonspecific bands, both longer and shorter in length (Figure 1, lane 2). However, when primer pair B (1:10 ratio) is substituted for primer pair A (1:1 ratio), most of the nonspecific bands are not amplified and the predominant product is a band of the expected size (Figure 1, lane 3). This difference becomes more prominent when lower amounts of template (1:100 dilution) are present (Figure 1, lanes 4 and 5). If the available template is further limited (1:1000 dilution) and more PCR cycles are added, then primer pair A generates so much nonspecific product that the desired band is totally overwhelmed and is not evident in the gel (Figure 1, lane 6). However, primer pair B yields useful product in these conditions, producing one weak but specific band of the expected size (Figure 1, lane 7). Since the buffer system and the reaction conditions, including annealing temperature, are all the same for the two groups, the enhanced specificity and sensitivity resulting from use of primer pair B is due to the different concentration ratios of the two primer pairs, demonstrating that increasing the relative concentration of the more degenerate primer produces the optimal results. In addition to using a 1:1 and a 1:10 primer ratio, we also tested a 1:100 ratio (0.2 μM upstream primer and 20 μM downstream primer) and a 1:800 ratio (0.025 μM upstream primer and 20 μM downstream primer), as well as 2 μM for both the upstream and downstream primers, to test a 1:1 ratio with lower concentrations. Under all of these conditions, the reactions failed even after several rounds of optimization (data not shown).

An understanding of the characteristics of degenerate primers is important for interpretation of these results. Unlike homologous primers, degenerate primers contain sequences that are both specific sequences that are complements to sequences contained within the PCR template as well as nonspecific sequences that do not match any of the desired template sequences. Therefore, the concentration of a degenerate primer mixture is actually the combined concentration of the specific and the nonspecific primer sequences. Since only the specific primer sequence(s) will effectively amplify the desired product(s), the effective concentration of a degenerate primer mixture is more important than the total concentration and should be considered separately. The ratio of effective concentration to total concentration is related to the degree of primer degeneracy. As degeneracy increases,
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.

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


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®, 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