 

# Simple and efficient system for synthesis of non-radioactive nucleic acid hybridization probes using PCR

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Non-radioactive probes for library screening, solution hybridization, blot analysis and *in situ* message detection have several important advantages for safety, stability and convenience of use. Multiple labeling can be done by co-hybridization of probes synthesized with differently substituted nucleotides or by sequential hybridization and staining (1) and the sensitivity of detection now approaches that of $^{32}P$ when LumiPhos$^{530TM}$ (Boehringer Mannheim, Indianapolis, IN) is used as a reporter for phosphatase-conjugated antibodies or streptavidin. I will describe a flexible and convenient method for systematically making nonradioactive probes which is aimed at labs that are small in size and/or work minimally with molecular tools.

The system involves utilization of the pGEM vector family (Promega Corp., Madison, WI) and primers that recognize the SP6 and T7 promotor sequences to direct PCR-mediated nonradioactive labeling of cDNA inserts. The combined polylinker regions of these plasmids contain approximately 30 restriction sites for subcloning cDNAs, and a number of unique sites (NheI, SspI, etc.) allowing remote linearization of the plasmid. Inserts of 200−1000 bp can be easily amplified to produce **full-length** probes.

Template DNA for probe synthesis may be crude bacterial lysate, miniprep or purified plasmid, as long as the sample is introduced into the reaction mix as ~1−10 ng of **linear** DNA in 10 μl TE, pH 8.0. This requires a somewhat higher than normal $Mg^{++}$ concentration in the 10× reaction buffer (25−30 mM) to accommodate the EDTA, however, it also promotes standardization of the amplification system. Digoxigenin-11-dUTP or biotin-dUTP (Boehringer Mannheim) is incorporated with high efficiency if the labeling mixture contains each nucleotide equivalent at 50−200 μM final concentration, and the ratio of substituted-dUTP to dTTP is 1:2.

A typical 100 μl reaction mix that will generate up to 10 μg of labeled probe contains the following:

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|---|---|
| 10.0 μl | 10× reaction buffer (100 mM Tris, pH 8.4; 500 mM KCl; 0.1% Triton X100; 1.0% gelatin; **25 mM $MgCl_2$**); |
| 10.0 μl | linear plasmid DNA (1.0−10.0 ng) in TE, pH 8.0; |
| 10.0 μl | dNTP/labeled-dUTP mix (1.0 mM @ dATP, dCTP, dGTP; 0.67 mM dTTP; 0.33 mM dig-dUTP or biotin-dUTP) |
| 1.0 μl | 10 μM SP6 promotor primer [5′-d(GATTTAGGTGACACTATAG)-3′] |
| 1.0 μl | 10 μM T7 promotor primer [5′-d(TAATACGACTCACTATAGGG)-3′] |
| 0.4 μl | 2.0U DNA Taq DNA polymerase. |

Thirty to thirty five cycles [(94°C/15 sec)(55°C/15 sec)(72°C/15 sec)] produced optimal amplification in a Biosycler thermal oven [Bios Corp., New Haven, CT]. These parameters may be adjusted to accommodate temperature block format. This system can also be used (data not shown) to generate single stranded DNA probes by using one primer at 0.1−1.0% of normal concentration. Probe may be purified to facilitate quantitation and to exchange buffer for long-term storage at 4°C. Quantitation is best achieved by fluorometry using Hoechst dye (2) to detect only dsDNA. Alternatively, yield may be estimated by comparison of 5 μl of reaction mix with ethidium bromide stained standards on a gel. Figure 1 compares the yield of amplification in an unlabeled reaction mixture with biotin-dUTP and digoxigenin-dUTP labeled reactions.

Due to the nature of an amplification reaction, less than 1% of DNA in the probe is unlabeled. Additionally, there is little need to 'clean' the probe prior to hybridization since unincorporated 'label' does not interfere with hybridization and primers may, in fact, act as non-specific blocking agents. This technique is the easiest way to generate probes that are short (<400 bp), but uniformly full-length. It is particularly useful for *in situ* hybridization and for studying members of multigene families that are highly homologous and require short probes for optimal discrimination between related sequences. One reaction can provide enough probe for 20−50 blot hybridizations (10−50 ng of probe/ml) or 10−20 ml of *in situ* hybridization solution (200 ng of probe/ml). Utilization of this system is also less costly than random priming reaction labeled with $^{32}P$-dCTP and it provides probe that is stable for up to one year at 4°C.

## REFERENCES

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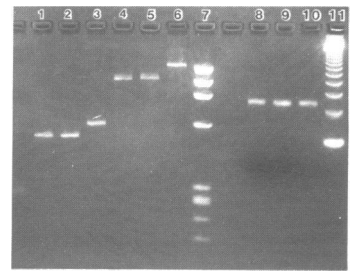


**Figure 1.** Agarose gel analysis of nonradioactive probe amplification from pGEM vector. 100 μl reactions were performed and 8 μl was run on a 3% NuSieve/1% GTG agarose gel (FMC Bioproducts, Rockland, ME) in TAE buffer at 100 V (Lanes 1−7 were run for 2 hours; lanes 8−11, for 1 hour). **Lane 7** is HaeIII cut ΦX173 DNA, and **lane 11** is a 123 bp ladder (BRL/Life Technologies, Inc., Gaithersburg, MD). **Lanes 1−3** is the product from a 573 bp insert; **Lanes 4−6**, a 1018 bp insert; and **Lanes 8−10**, a 257 bp insert. **Lanes 1, 4** and **8** contain products synthesized with unlabeled nucleotide mix; **Lanes 2, 5** and **9**, with biotin-dUTP nucleotide mix; and **Lanes 3, 6** and **10**, with digoxigenin-dUTP nucleotide mix. Note that all yields are roughly equivalent and that the relative mobility of the amplified DNA is shifted ~10% when it contains digoxigenin-dUTP.