



## MODIFIED FastTag® LABELING PROTOCOL FOR PCR PRIMERS

Nucleic acids exhibit some loss of hybridization efficiency after labeling with the FastTag® system. While this effect is negligible for larger nucleic acids, labeling of oligonucleotides may significantly affect the  $T_m$  of the target/probe hybrid. The more densely the primer is labeled, the greater the effect may be. Under stringent hybridization conditions, signal intensity may actually increase with decreased primer labeling density due to increased hybridization efficiency. Additionally, because the incorporation of labeled PCR primers is dependent on the overall amplification efficiency, presence of some unlabeled primer may improve the yield, thereby increasing the detection sensitivity of the PCR product. Therefore, some optimization of the labeling reaction and/or annealing conditions may be required to achieve the balance between label density and hybridization efficiency which results in maximum detection sensitivity.

The following is a supplemental modification of the FastTag® protocol for labeling oligonucleotides intended for use as PCR primers. We suggest that users start by labeling primers using reagent concentrations within the ranges specified in Steps 2 and 9 and assaying for optimal performance.

### Coupling of FastTag® reagent to oligonucleotides:

1. Reconstitute FastTag® reagent with 500  $\mu$ l deionized H<sub>2</sub>O giving a final concentration of 1 mg/ml. (Store the reconstituted FastTag® reagent at -20 °C to -80 °C in the dark.)
2. To a known volume (10 to 40  $\mu$ l at 0.5  $\mu$ g/ $\mu$ l) of primer in TE, add 0.2 to 0.5 volumes of FastTag® reagent. Proceed immediately to Step 3. *See Note A*
3. For photocoupling, place the reaction tube (cap open) into an ice bath and illuminate for 15 minutes with a mercury vapor lamp positioned 10 cm above the reaction tube or a 366 nm UV lamp positioned 1 cm above the reaction tube. For thermal coupling, overlay the reaction mixture with 50  $\mu$ l of mineral oil and heat at 95 °C for 10 minutes.
4. Following coupling, bring the FastTag®- primer solution to 80  $\mu$ l with deionized H<sub>2</sub>O and add 80  $\mu$ l of 0.1 M Tris, pH 9.5. *See Note B*
5. Add 160  $\mu$ l of 2-butanol (room temperature) to the FastTag®- primer solution, vortex vigorously, and centrifuge to separate the phases. Remove the upper butanol phase and discard. Re-extract the aqueous phase with an additional 160  $\mu$ l of 2-butanol, again discarding the butanol layer following centrifugation. *See Note C*
6. Proceed directly to the disulfide reduction reaction or store the FastTag®- primer at -20 °C to -80 °C for up to one year. *See Note D*

### Reduction of FastTag® disulfide linker arm:

7. To ~ 40  $\mu$ l ( 20  $\mu$ g) of FastTag®- primer from Step 6, add 5  $\mu$ l of reduction buffer (Reagent 1).
8. Add 5  $\mu$ l of reducing agent (Reagent 2) and incubate at room temperature (25 °C) for 10 minutes.

(over)

## Label-maleimide coupling to FastTag® thiols:

9. Add the following components, in the order shown, to the 50 µl reduction reaction from step 8 to give a final volume of 100 µl:
  - 1) 30 to 39 µl H<sub>2</sub>O
  - 2) 10 µl maleimide buffer (Reagent 3)
  - 3) 1 to 10 µl label-maleimide (Reagent 4)Mix.
10. Incubate the label-maleimide coupling reaction at 65 °C for 10 minutes or at room temperature (25 °C) for 30 minutes.
11. Precipitate the labeled primer away from unincorporated label-maleimide by adding, in order, the following components to the 100 µl label-maleimide coupling reaction: *See Note E*
  - 1) 100 µl H<sub>2</sub>O
  - 2) 50 µl of 10 M ammonium acetate
  - 3) 10 µl of 1 M MgCl<sub>2</sub>
  - 4) 1 µl of 20 mg/ml glycogen (or use a similar carrier)
  - 5) 625 µl of 95% ethanol (-20 °C)Mix.
12. Incubate at -20 °C for 15 minutes. Pellet the labeled oligonucleotide by centrifugation at 13,000 x g in a microcentrifuge for at least 15 minutes. Wash the pellet with 70% ethanol and centrifuge at 13,000 x g for 3 minutes. Resuspend the pellet in H<sub>2</sub>O or TE to give an appropriate working or stock concentration. *See Note F*

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### NOTES:

- A. For volumes of primer greater than 40 µl, labeling can be accomplished by adding proportionately larger volumes of mineral oil, 0.1 M Tris, and 2-butanol in steps 3 through 5. Following butanol extractions, the volume can be adjusted by ethanol precipitation (see note D). Resuspend in 40 µl of TE.
- B. Raising the pH of the FastTag®- primer solution facilitates the removal of unincorporated FastTag® reagent in step 5.
- C. The volume of the aqueous phase will be reduced to approximately 40 µl at this point.
- D. If desired, the FastTag®- primer can be further concentrated by ethanol precipitation according to the following procedure: Add 10 µl of 10 M ammonium acetate, 2 µl of 1 M MgCl<sub>2</sub>, 1 µl of 20 mg/ml glycogen, and 150 µl of cold 95% ethanol to 40 µl of FastTag®- probe. Pellet the FastTag®-primer by centrifugation and resuspend in the desired volume of TE.
- E. Texas Red®-labeled primers cannot be precipitated in ethanol. Remove unincorporated Texas Red® maleimide using spin column gel filtration.
- F. Unincorporated label-maleimide may inhibit enzyme activity in PCR. Further purification using spin column gel filtration (e.g., as per Sambrook, J., E. F. Fritsch and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., p. E.37 - E.38) may be required.
- G. For end labeling of oligonucleotides for PCR, also see EndTag™ Labeling Kits (for 5' end labeling, Cat. No. MB-9001; or 3' end labeling, Cat. No. MB-9002).

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