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Enzymatic Labeling of DNA with Fluorescent Triphosphates

Fluorescently labeled triphosphates are used to incorporate a fluorescent label into a growing DNA chain during the polymerase chain reaction (PCR). When DNA is amplified, the addition of labeled deoxynucleotides to the reaction mixture results in their enzymatic incorporation and the production of a labeled PCR product by Taq polymerase. Introducing fluorescent labels directly during PCR has advantages in situations with limited DNA template or when amplifying a specific DNA fragment.

The current protocol provides general recommendations for performing PCR with fluorescently labeled triphosphates and subsequent detection of the resulting amplification product in an agarose gel. The parameters listed below, including the amplification program, the concentration of DNA template, primers, and fluorescent deoxynucleotide, as well as the amount of PCR product loaded onto the agarose gel, should be optimized for specific experimental conditions.

For DNA amplification with fluorescent triphosphates, a 5× ProbeMaster® Lyo UNI reaction mixture, which includes HS Taq DNA polymerase, reaction buffer with Mg^{2+}, and a mixture of unlabeled triphosphates, can be used.

Preparation of Fluorescent Triphosphate Solution

1. Dissolve the lyophilized fluorescent triphosphate in deionized water to a stock concentration of 1 mM (for example, add 50 µL of water to 50 nmol of lyophilized powder).*
2. Mix the solution thoroughly and centrifuge it to remove droplets.

   Important! The resulting fluorescent triphosphate solution should be stored in the dark, and further experimental work should be carried out under low-light conditions.

* Diluting the lyophilized product to a stock concentration of 1 mM and adding a conveniently measurable volume of the triphosphate solution to the PCR mixture (3.5–7 µL per 35 µL reaction volume, see «DNA Labeling» section, step 1) increases the accuracy of adding the required amount of modified triphosphate to the reaction.

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DNA Labeling

1. Mix the components for the required number of labeling reactions according to the table below. Vortex the prepared mixture and centrifuge briefly to collect the droplets.

   Calculation for 1 reaction with a volume of 35 µL:**

Component	Volume	Note
ProbeMaster Lyo UNI, 5× reconstituted reaction mixture	7 µL	—
Direct primer	0.7–2.1 µL of a 10 µM solution	Final concentration: 200–600 nM
Reverse primer	0.7–2.1 µL of a 10 µM solution	
Fluorescently labeled triphosphate	3.5–7 µL of a 1 mM solution	Final concentration: 0.1–0.2 mM ***
Deionized water	To the total volume of the reaction solution 35 µL**	—
DNA	The sample volume is calculated depending on the type and concentration of the DNA template. Plasmid DNA (1–100 pg), genomic DNA (50–100 ng), etc., can be used as a template	Add separately to each PCR tube in step 2.3
Total reaction volume	**35 µL****	

** When using a different reaction volume, the volumes of the reaction components should be recalculated while maintaining the given proportions.

*** The quantity of fluorescently labeled triphosphate may affect the yield of the enzymatic reaction and the labeling degree. In most cases, it is recommended to add fluorescent triphosphates to the PCR mixture at a final concentration of 0.1 to 0.2 mM (i.e., in ratios of 0.5:1 to 1:1 to the corresponding unlabeled triphosphate); however, for each type of labeled triphosphate, an optimal concentration in the reaction mixture should be determined separately. Note that increasing the concentration of labeled deoxynucleotides may lead to PCR inhibition.

Important! When adding fluorescent triphosphates, the yield of PCR product may be lower than with unlabeled nucleotides alone.

2. Add the prepared mixture to the PCR tubes, excluding the volume of the DNA sample.
3. Separately add the missing volume of the DNA sample to each tube. Close the tube caps and centrifuge to remove droplets.

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4. Perform standard DNA amplification using the program indicated below.

Stage	Temperature	Time	Number of cycles
Primary denaturation, activation of HS Taq polymerase	95 °C	5 min	1
Denaturation	95 °C	10–30 sec	25–35
Annealing of primers	Should be determined individually for each pair of primers	10–30 sec	
Elongation	72 °C	15–60 sec (depends on the length of the amplicons)	
Completion of the chain	72 °C	5 min	1

5. The fluorescently labeled PCR product can be visualized on an agarose gel without the addition of an intercalating dye to the sample.
6. Since the fluorescence of unreacted labeled nucleotides can complicate the visual detection of the resulting PCR product on gel electrophoresis, the additional purification of the DNA from unincorporated triphosphates is recommended.

DNA purification from triphosphates

To obtain bright, visible bands on the agarose gel and remove most of the free fluorescent triphosphates, it is recommended to concentrate the DNA by reprecipitating the PCR product with ethanol from one or more tubes containing amplification products (after combining them into a single tube).

For complete removal of fluorescent deoxynucleotides, the resulting PCR product should be further purified by gel filtration on columns or ultrafiltration.

1. Add 1/2 volume of 5 M ammonium acetate solution and 2.5–3 volumes of 96% ethanol to the PCR product (from one or more tubes). For example, for every 35 µL of solution containing the PCR product, add 17.5 µL of 5 M ammonium acetate and 87.5–105 µL of 96% ethanol. To better visualize the DNA precipitate at this stage, we recommend adding a co-precipitant (e.g., linear polyacrylamide).
2. For effective DNA precipitation, incubate the mixture at low temperatures (e.g., for 30–60 minutes at -20 °C).
3. Centrifuge the mixture for at least 15 minutes at 10,000–12,000× *g*, then carefully remove the supernatant without disturbing the precipitate.
4. Wash the precipitate with cold 70% ethanol, and carefully remove the supernatant.
5. Dissolve the precipitate in 10–20 µL of water or elution buffer (depending on the desired concentration of labeled DNA after purification and, accordingly, the brightness of the bands of the final product on the agarose gel).
6. If necessary, the amplification products can be stored at -20 °C.

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Electrophoresis

1. Mix the samples with the loading buffer for agarose gel and load 5 µL of the sample into the gel wells, then perform electrophoresis. For better visualization of the fluorescently labeled PCR product, it may be necessary to load a larger sample volume into the well or to dissolve the DNA in a smaller volume of elution buffer.
2. After gel electrophoresis, detect the fluorescence of the labeled DNA using a UV transilluminator or a gel documentation system with a suitable filter, depending on the emission spectrum of the fluorescent triphosphate used.

   Important! DNA labeling with modified triphosphates may alter the electrophoretic mobility of the PCR product in agarose gels.
3. After detecting the labeled DNA, incubate the gel in a dye solution (e.g., dsGreen, dsGold, ethidium bromide) to visualize the unlabeled control PCR product and DNA size marker.

   Important! After staining the gel, the fluorescence of the labeled PCR product may overlap with the fluorescence of the gel stain, so it is recommended to record the DNA fluorescence before incubating the agarose gel in the dye solution.

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