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RAPID COMMUNICATION

# A color-based FHGC approach facilities DNA-based clinical molecular diagnostics

The flow-through hybridization and gene chip (FHGC) was developed to used in clinical molecular diagnostics for thalassemia, human papillomavirus (HPV), and other diseases. FHGC could improve hybridization efficiency and reduce hands-on time, thus improving precision, reproducibility, and traceability. Multiple genotypes can be detected simultaneously without incurring high costs.[1] During the experiment, the polymerase chain reaction (PCR) product or buffer forced through the membrane matrix, PCR products are apprehended by a probe attached to the membrane, and molecular typing of the disease or microorganism was determined after subsequent amplification by the streptomycin avidin (the details are given in the Supplementary File).[2,3] However, the steps prior to hybridization, such as DNA extraction, PCR amplification, and sample loading into the hybridization wells, require a significant amount of manual operation. Each batch involves handling dozens of samples, and Eppendorf (EP) tubes used to load the amplification mixture are typically packed tightly together. Furthermore, the reagent is colorless and transparent, making it difficult to determine whether the sample was loaded into EP tube or not with a very small sample size. Operator carelessness or distraction will result in errors and unnecessary trouble.

To avoid the problems mentioned above, we attempted to add special dyes to the double-distilled water ($ddH_2O$) that dissolves the DNA and help to track which reagent was added to which well (Fig. 1A), and diagnostic reagent kits designed to detect mutations of common thalassemia gene were used in subsequent studies. The addition of dyes to $ddH_2O$ that dissolves DNA provides some advantages for manual or semi-automatic DNA extraction, as the final step of collecting DNA requires careful inspection by the operator. If $ddH_2O$ for dissolving DNA is colored, it becomes easy to observe DNA collection from the sample. The following requirements must be met to screen the appropriate dyes for FHGC: (1) Dyes color development in $ddH_2O$ for DNA dissolution; (2) PCR to amplify the samples that were not affected by dyes; (3) The dyes did not stain the nitrocellulose membrane used for hybridization; (4) The dyes could not leave a distinct background on the nitrocellulose film to avoid interference with the result interpretation.

Thirty-eight candidate dyes were selected to identify a suitable dye (Table S1). First, all these candidate dyes were added to DNA solution respectively to identify whether they colored DNA solution or not. We set two-three different concentrations for each dye to prevent the effect of dye concentration on the experimental reaction in the above conditions. We used an EP tube as a reference because some dyes led to precipitate or did not produce any colour when added to DNA solution. Typically, seven candidate dyes, such as numbers 9 and 18, do not produce any color in DNA solutions and are thus eliminated (Fig. 1B, C). The colored DNA solution was then tested to identify if it remained colored after mixing with PCR reagent. Some dyes, such as 6 and 8, became light, even illegible or transparent. We reserved the dyes with light colors for subsequent screening to screening as many appropriate dyes as possible, excluding completely transparent dyes (Fig. 1B, D). The dyes and reagents capable of rendering color in PCR mix were placed into PCR instrument for amplification and hybridization. After hybridization, as showed in Figure 1B, E, and Figure S1, some color reagents inhibited nucleic acid amplification of PCR or hybridization reaction, and some dyes stained the hybrid nitrocellulose film. Because hybridization is the final presentation of the experimental results, all dyes that do not meet the requirements in this step, such as dyes 1, 19, and 33, are excluded.

We chose dye number 26, methyl red sodium salt ($C_{15}H_{14}N_3NaO_2$), from the dyes that meet the above criteria for further investigation. To investigate the effects of different concentrations of this dye on PCR amplification and flow-through hybridization, we prepared a DNA solution of methyl red sodium salt with concentrations ranging from 0.01% to 10%. Methyl red sodium salt produces color whether added to DNA or a premixed PCR amplification solution.

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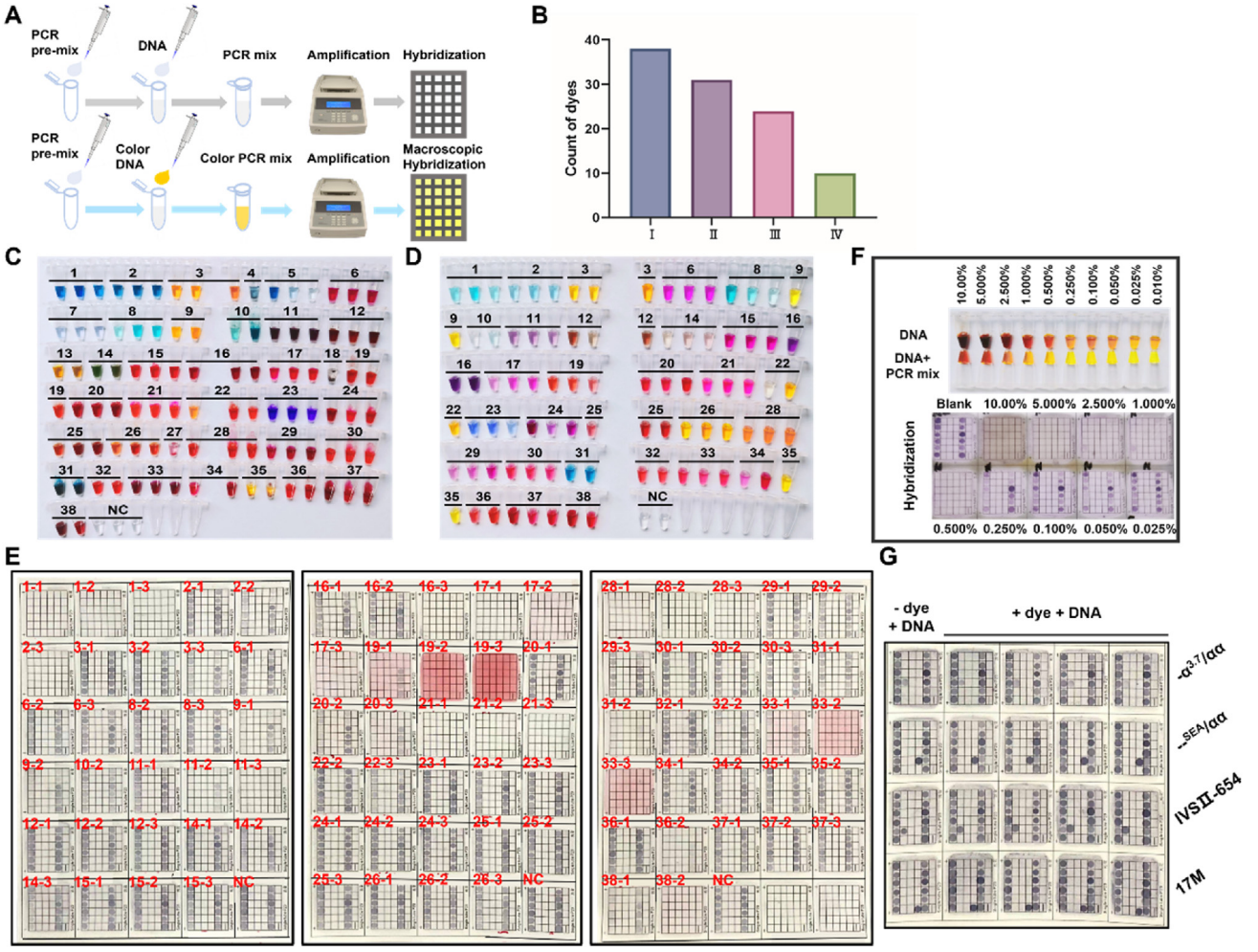


**Figure 1** Screening suitable dyes. **(A)** Flow-through hybridization diagram. **(B)** Schematic diagram of dye screening (I: Thirty-eight types of dyes. II: Screening candidate dyes added to DNA solution. III: Screening candidate dyes added to premix of PCR. IV: Screening candidate dyes added to hybridization solution). **(C)** Color rendering of various dyes in DNA solution. **(D)** Color rendering after adding PCR reaction solution. **(E)** Effects of different dyes on flow-through hybridization. The blue dots in each of the squares are due to DNA binding to the probe and is colored by a cascade amplification system. The first and fifth columns are wild gene sites, and each of the other squares represents a mutation type. **(F)** The upper graph shows the concentration gradient of prefabricated PCR reaction solution; the under graph shows the membrane's color rendering after flow-through hybridization. **(G)** Reagent specificity analysis after dye addition [from left to right: (-dye + DNA) × 1, (+dye + DNA) × 4]. Each row contained the same mutant sample, with mutation of -α3.7, -SEA, IVSII-654 and 17M, running from top to bottom.

When the concentration of methyl red sodium salt in dissolved DNA was 0.01%, the dye could not color the hybridization well after diluting by the PCR amplification reagent or hybridization reagent. However, high methyl red sodium salt concentrations inhibited PCR amplification or hybridization. Following the comparison, we concluded that the optimal methyl red sodium salt concentration for FHGC is 0.1%—0.025%, which did not interfere with PCR amplification and flow-through hybridization (Fig. 1F; Fig. S2).

The modified reagent's performance was scrutinized. According to industry standards, the minimum nucleic acid concentration for FHGC is 25 ng. A sample with an initial concentration of about 100 ng/μL was diluted four times to produce a sample with a concentration of 25 ng/μL. The diluted DNA amplification for chip hybridization did not affect the final gene chip color production, and the corresponding gene squares showed clear blue dots (Fig. S3). The colorful DNA solution was kept at 4 °C for one week for PCR amplification and hybridization. The results revealed that dye addition had no significant effect on the color of hybridized gene chip. It demonstrated that the modified reagent is stable (Fig. S4). The specificity of modified reagent was examined. Four different types of thalassemia mutations were collected, two of which were α-thalassemia mutations ($-\alpha^{3.7}$, $^{-SEA}$) and two were β-thalassemia mutations (IVSII-654, 17M). It was identified that the improved reagent did not affect PCR amplification, flow-through hybridization, and color rendering. There is no additional background on the nitrocellulose film, and the interpretation of results does not interfere (Fig. 1G).

A variety of dye-adding reagents have been developed based on the different properties of reagents, some to aid in the interpretation of results and others to simplify the operation process.[4,5] we tried to dissolve dyes in water, which helped track the moving direction of reagents and reduce the error of loading samples, Among all the 38 dyes tested, few were matched with our screening conditions, such as blue dye (Acid violet 17, No. 23) and red dye (Ponceau S, No. 32). Some dyes, such as No. 23, 24, and 25, did not affect FHGC reaction in our screening concentration range. However, for further performance verification, we chose methyl red sodium salt. According to internal discussions, using this modified reagent will reduce our costs by about one-fifth, lower the sampling error rate, and improve the operator's experience. We believe that this is a reagent worth popularizing.

## Conflict of interests

The authors declare no competing interests.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.07.007.

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