General Guide for RPA Primer & Probe Design

★ **Primer and Probe Design Software:** It is recommended to use *Beacon Designer* or *Oligo 7* to design primers and probes. The design requirements for primers and probes differ from those for conventional PCR. Below are the specific requirements and recommendations for RPA primer and probe design:

1. Primer Design Requirements

1. **Primer Length:** Recommended length is between 28–35 nucleotides (nt).
2. **Sequence Characteristics:**
    - Avoid palindromic sequences, homopolymeric runs of a single nucleotide, and regions with strong internal secondary structures.
    - The Tm value (melting temperature) is not a primary consideration in RPA primer design.
3. **Screening Test:**
    - It is recommended to perform a primer screening test before conducting the RPA amplification reaction to achieve higher detection sensitivity.

2. Probe Design Requirements

1. **Probe Length:** Recommended length is between 46–52 nucleotides (nt).
2. **Sequence Characteristics:**
    - Avoid palindromic sequences, internal secondary structures, and consecutive identical nucleotides.
    - The probe sequence may include nucleotide analog substitutions (e.g., tetrahydrofuran residue, THF — sometimes called *dSpacer*) to improve hybridization stability.
3. **Labeling (see Figure 1):**
    - **Lateral Flow Strip Method:** The 5′ end of the probe should be labeled with an antigen tag, typically a FAM group or carboxyfluorescein.
    - **Fluorescence Method:** Place a fluorophore and a quencher group upstream and downstream of the THF site, respectively.

- The 3′ end of the probe should contain a polymerase extension blocking group (e.g., C3-spacer, phosphate group, or dideoxynucleotide) to prevent nonspecific extension.

4. **Notes**

- Before starting the experiment, check whether the 5′-end label of the probe and the 5′-end label of the downstream primer are compatible with the lateral flow strip or detection platform being used.
- Before performing RPA lateral flow strip amplification, conduct a primer screening test to optimize detection sensitivity and specificity.

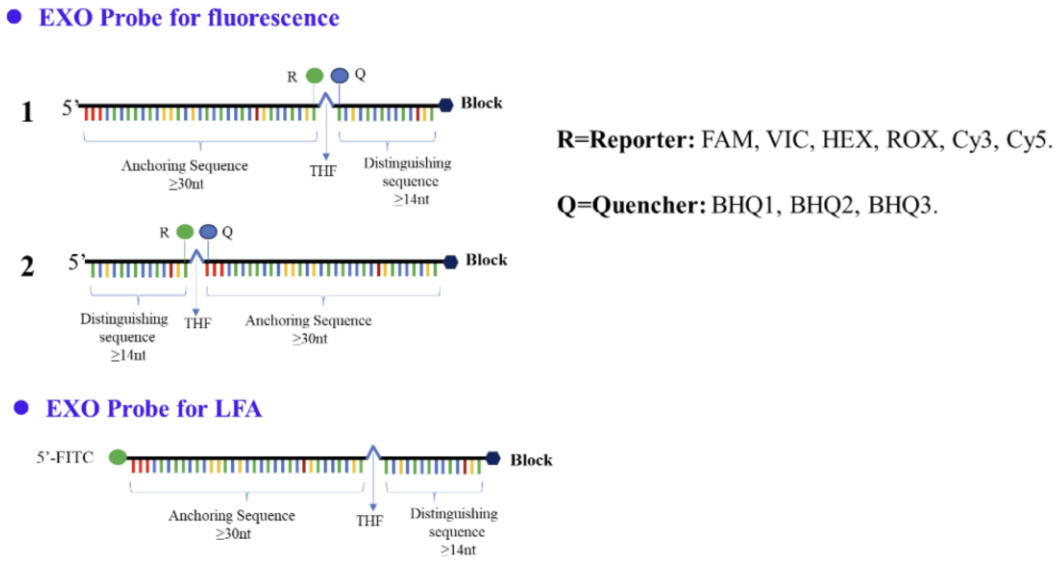

Figure 1. Formats of RPA Probes