Chapter 7

Differentiation of Fungi Using Hybridization Probes on the LightCycler®

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Abstract

The polymerase chain reaction is a powerful molecular tool for the detection and analysis of very small amounts of DNA. Today, hybridization probes are often used in real-time PCR for more sensitive and specific detection of pathogens and for determination of gene regulation or mutation analysis instead of intercalating dyes like SYBR Green. Here, we describe how to generate suitable primers and hybridization probes for the specific detection of fungal DNA. Furthermore, we show the advantages of hybridization probes using the LightCycler-PCR for the detection of different *Candida* spp. and *Aspergillus* spp. in patient blood samples. The methods used to develop such PCR assays will also be presented in the following protocol.

Key words: Real-time PCR, Hybridization probes, Primer design, Databases, Aspergillus, Candida

1. Introduction

The most critical factor in medicine is the analysis of a wide range of parameters in small volume patient samples. Often immunosuppressed patients, e.g., after solid organ transplantation or hematopoietic stem cell transplantation, are affected by a number of complications like infections with bacteria, viruses, and especially fungi (*Candida* spp. or *Aspergillus* spp.) that also increase mortality (1–3). An early diagnosis of invasive fungal infections is essential for the initiation of specific antifungal therapies and to avoid the unnecessary administration of baseline therapy especially in cancer patients undergoing chemotherapy. Therefore, methods for an early detection of fungal infections are still warranted since current diagnostic tools lack sufficient sensitivity for the early diagnosis.

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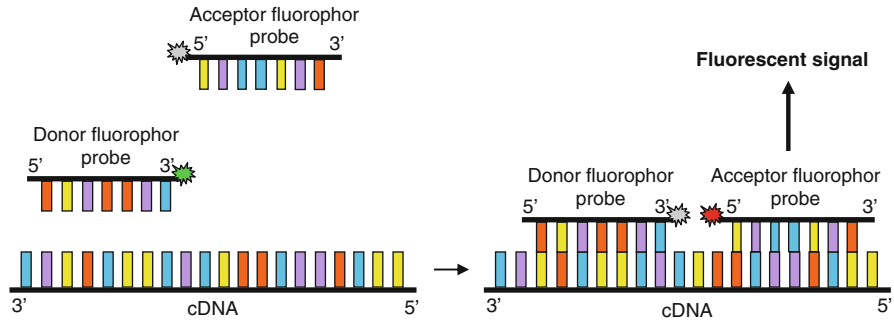

Fig. 1. Principle of FRET using hybridization probes.

Real-time PCR is a powerful and reliable tool for the detection of pathogens in serum, tissue, or blood and allows additional quantification of the amplified DNA in real-time by using fluorescent dyes (4, 5). The basic principle of such PCR assays is so-called fluorescence resonance energy transfer (FRET, Fig. 1). A fluorescent donor molecule transfers its energy to a nearby acceptor molecule by changing the fluorescence intensity. There are different types of fluorescent probes, e.g., TaqMan probes or hybridization probes. If the fluorescent donor probe is close to the acceptor probe and both hybridization probes bind to the target gene sequence (e.g., DNA of fungi), the acceptor probe emits a fluorescent signal proportional to the amplified target DNA. The generation and analysis of melting curves after DNA amplification allows the differentiation of *Aspergillus* spp. and *Candida* spp. (6).

We describe the development of primers and hybridization probes for detection of different *Candida* spp. and *Aspergillus* spp. in one sample.

2. Materials

2.1. Databases for Design of Primers and Hybridization Probes

1. Gene and sequence database: http://www.ncbi.nlm.nih.gov/guide/.
2. Database for intron and exon research: http://www.genome.ucsc.edu/.
3. Free online tool for design of primers, e.g., http://www.frodo.wi.mit.edu/primer3/.
4. Free online tool for proofing of primers: http://www.premierbiosoft.com/netprimer/index.html.
5. Roche UniversalProbeLibary: https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp.
6. Alignment software; VectorNTI (not free): http://www.invitrogen.com.

2.2. PCR

1. The LightCycler® 480 Real-Time-PCR System, LightCycler® 480 Instrument, and Data station and software (Roche Applied Science, Mannheim, Germany).
2. LightCycler® 480 Probes Master Kit (Roche Applied Science, Mannheim, Germany) for real-time PCR. Store at –20°C.
3. Fungal DNA (50 ng/µl): For isolation of DNA from *Candida* spp. and *Aspergillus* spp. the QiaAmp DNA Mini Kit (QIAGEN, Düsseldorf, Germany) or equivalent can be used according to the manufactures protocol (see Note 1). Store at –20°C.
4. 18SrRNA Primers (7) (100 pmol/µl): sense primer 5 µM (5′-ATT GGA GGG CAA GTC TGG TG-3′) and anti-sense primer 20 µM (5′-CCG ATC CCT AGT CGG CAT AG-3′) for amplification of 18SrRNA of fungi (TIB MOLBIOL, Berlin, Germany). Store at –20°C.
5. Hybridization probes (7) (1 nmol) for *Candida* spp.: (1) probe (5′-AGC CTT TCC TTC TGG GTA GCC ATT-3′) labeled with fluorescein (FL1) at 3′ end and (2) probe (5′-TGG CGA ACC AGG ACT TTT ACT TTG A-3′) labeled with LightCyler 640 Red (LC1) (TIB MOLBIOL, Berlin, Germany). Store at –20°C.
6. Hybridization probes (1 nmol) for differentiation between *Candida* spp. and *Aspergillus* spp., probe 1 (5′-CCA AGG ACG TTT TCA TTA ATC AAG AAC GA-3′) labeled with fluorescein (FL2) at 3′ end and probe 2 (5′-TTA GGG GAT CGA AGA TGA TCA GAT ACC-3′) (Fricke et al. unpublished data) labeled with LightCyler 640 Red (LC2). Store at –20°C.
7. Molecular grade water (free of DNAse and RNAse). Store at 4°C or room temperature.
8. LightCycler® 480 Multiwell Plate 96 and plate foil covers (LightCycler® 480 Sealing Foil, Roche Diagnostics, Roche Applied Science, Mannheim, Germany). Store at room temperature.

2.3. Gel Electrophoresis

1. Tris–Acetate–EDTA (TAE) buffer (50×) as running buffer for gel electrophoresis. The buffer should be diluted 1:50 for a 1× concentrated working solution. Store the TAE (50×) stock solution at 4°C.
2. High grade agarose for the separation of PCR fragments. Store at room temperature.
3. Blue/Orange 6× Loading Dye to determine the DNA concentration and to observe the progress of gel electrophoresis. Use 3 µl of the Loading Dye for 10 µl of the PCR sample. Store at 4°C.
4. 100 base pair (bp) DNA size marker (see Note 2). Store at 4°C.

5. Ethidium bromide solution 10 mg/ml for DNA staining of PCR fragments (see Note 3). Store at 4°C.
6. Comb for providing slots in the agarose gel.
7. Gel tray for casting the agarose gel.
8. Standard electrophoresis chamber for separating the amplified gene fragments according to their length.
9. Power pack as a power source for gel electrophoresis.
10. Image acquisition system for detection of PCR fragments under ultra violet (UV) light.
11. Microwave for preparing the agarose gel solution.

3. Methods

3.1. Finding the Target Gene Sequence for Fungal 18SrRNA

1. Open the online gene database NCBI (http://www.ncbi.nlm.nih.gov/guide/), which is the most used database for finding the target sequence. Put the name of the species in the search field, e.g., *Candida albicans* and the target gene 18SrRNA. A new window will be opened (see Note 4).
2. Click on "Nucleotide" and a new window will be opened with all matches of the *database*.
3. *Search* for the target gene "*Candida albicans* 18SrRNA gene" to see the information about the gene sequence.
4. Retrieve the DNA sequence and copy the sequence to a new work sheet or to available alignment software, e.g., VectorNTI (http://www.invitrogen.com), because the sequence will be important for the design of primers and hybridization probes.

3.2. Design of Primers

3.2.1. Selection of Primers

It is important that for real-time PCR the length of the amplified fragment should not exceed 200 bp (see Note 5).

1. There are some free online primer design tools that can be used, e.g., Primer3 (http://www.frodo.wi.mit.edu/primer3/)(8).
2. For Primer3 put your sequence in the search field (numbers in front of gene sequence can be ignored) and define the parameters. Most online tools for primer design have selected automatically the best primer standard conditions, e.g., G/C concentration of 40–60% or a length of 18–20 bp.
3. Select "Pick primers."
4. A window will appear with a collection of different primer pairs with information about their sequence, length of the amplified fragment, melting temperature (T_m), and position in the sequence (see Notes 6 and 7).

3.2.2. Verification of Primers

1. For quality control and verification of PCR primers, free online tools, e.g., Netprimer under http://www.premierbiosoft.com/netprimer/index.html (registration needed) or Beacon Designer™ Free Edition can be used. The generated primers should not form dimers or crossdimers (see Note 8).
2. Add the sequence of the sense and anti-sense primer in the respective field (Sequence) in 5′-3′ direction.
3. Select the button "Analyze" and information about melting temperature, molecular weight, hairpins, dimers, crossdimers, and palindromes will be generated.

3.3. Design of Hybridization Probes

Hybridization probes are short fluorescent labeled oligonucleotide sequences (about 20 bp), which specifically bind within the target sequence. Hybridization probes are more sensitive than common intercalating DNA dyes (e.g., SYBR green) because of a higher specificity (see Note 9). For the detection of different *Candida* spp., hybridization probes were designed because of their advantage in comparison to TaqMan probes (see Note 10). Thus, some manufacturers like Roche Applied Science developed an online database Roche UniversalProbeLibary (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp) where primers and compatible hybridization probes can be found and bought for common species, e.g., human, mouse, drosophila, or arabidopsis thaliana. For other species, especially fungi, hybridization probes have to be designed manually by the same procedure described in Subheading 3.2 but some specific criteria are necessary as follows:

1. The sequence which is amplified by the designed primers is necessary (see Subheading 3.2, step 4) because hybridization probes have to bind within this sequence.
2. Both hybridization probes bind on the same strand. The distance between the two hybridization probes should be 1–5 bases (see Note 11).
3. The melting temperature (T_m) of the hybridization probes should be 5–10°C over the T_m of the collected primers but <80°C (see Notes 12 and 13).
4. The hybridization probes should not form dimers with the primers and should be phosphorylated at the 3′ so they cannot serve as primers.
5. The hybridization probe in the 5′-3′ direction should be labeled at the 3′ end with fluorescein and the opposite hybridization probe should be labeled at the 5′ end with LightCycler Red 640/705 or other compatible dyes.
6. The position of the hybridization probes should bind closer to the 3′ end than the 5′ end of the target sequence (see Note 14).

Table 1
Conditions for detection of different *Candida* spp. and *Aspergillus* spp. by using real-time PCR on LightCycler® 480

Step	Target (°C)	Hold (s)	Ramp-rate (°C/s)	Acquisition (per °C)	Acquisition mode
Denaturation (1 cycle)					
	95	600	4.40		None
Amplification (35 cycles), quantification					
	95	5	4.40		None
	62	15	2.20		Single
	72	10	4.40		None
Melting curve (1 cycle)					
	95	30	4.40		None
	40	30	2.20		None
	85		0.19	3	Continuous
Cooling					
	40		20		None

3.4. Real-Time PCR on the LightCycler® 480 System Using Hybridization Probes

1. Hybridization probes: Dilute each probe (1 nmol) to a final concentration of 3 µM (=3 pmol/µl). The fluorescent labeled hybridization probes should not be exposed to light for a long period of time. Hybridization probes should be stored at −20°C.
2. Primer solution: Dilute the sense primer to a final concentration of 5 µM and the anti-sense primer to a final concentration of 20 µM (see Note 15). For this, prepare a fresh primer solution containing both primers before real-time PCR and add 2.5 µl of the sense primer (100 pmol/µl) and 10 µl of the anti-sense primer (100 pmol/µl) to 37.5 µl water.
3. Add 10 µl LightCycler 480 Probes Master (2×), 5 µl water, 1 µl primer solution (see step 2), 1.5 µl of FL1 probe or FL2 probe (3 µM), 1.5 µl LC1 probe or LC2 probe (3 µM), and 1 µl DNA template in a 96-well plate (see Note 16). It is recommended to prepare a mastermix (see Note 17).
4. Seal plates with a foil plate seal and centrifuge at 1,500 × *g* for 2 min. Perform real-time PCR on LightCycler® 480 system for the amplification of fungal DNA under the following conditions (Table 1). Ensure that the channels Red640 and Red705 are selected prior to starting the run.

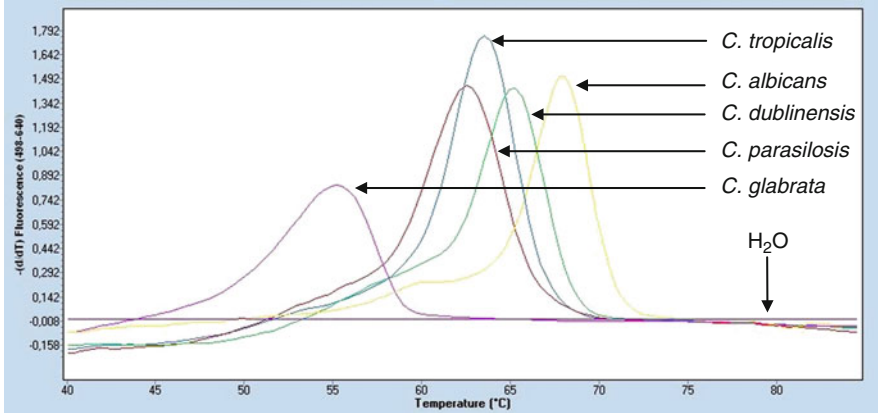

Fig. 2. Analysis of melting peaks of different *Candida* spp. after real-time PCR by using probes FL1 and LC1. Water control shows no melting peak.

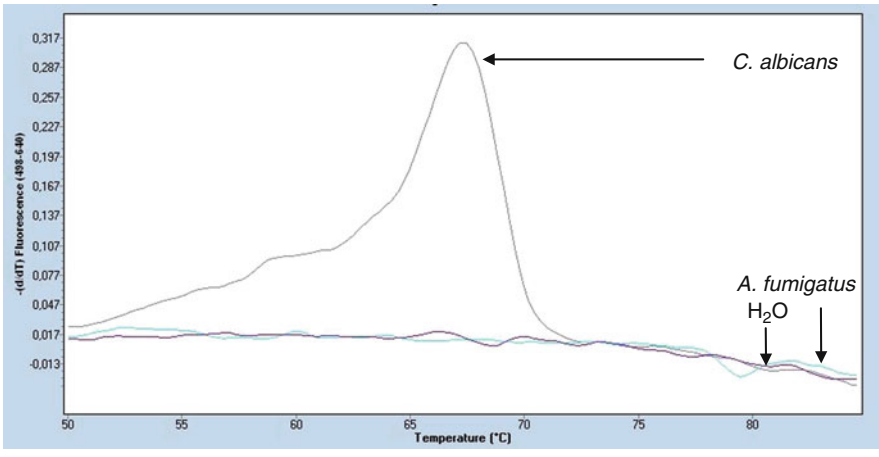

Fig. 3. Analysis of melting peak of *Candida albicans* after real-time PCR by using probes FL1 and LC1. *Aspergillus fumigatus* could not be detected by using these probes.

5. After real-time PCR using the hybridization probes FL1 and LC1, different *Candida* spp. can be detected by melting curve analysis (Fig. 2) (see Note 18). By using the hybridization probes FL2 and LC2 the discrimination between *Candida* and *Aspergillus* within the same sample is possible (Figs. 3 and 4).

3.5. Gel Electrophoresis

Gel electrophoresis of the PCR products is recommended to prove that the correct size of the DNA fragment has been amplified after real-time PCR.

1. For a 1× concentrated working solution of Tris–Acetate–EDTA buffer (TAE) dissolve a 50× concentrated stock solution of TAE buffer in a dilution of 1:50 with water. Store at room temperature.

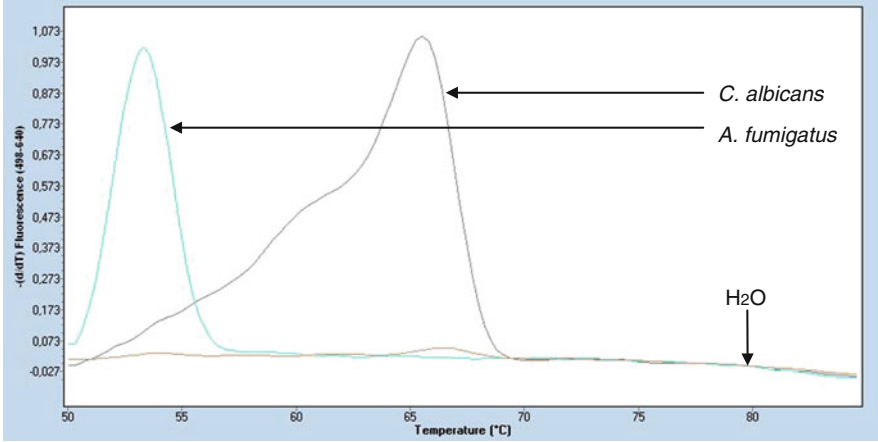

Fig. 4. Analysis of melting peaks for detection of *Candida albicans* and *Aspergillus fumigatus* within one sample after real-time PCR by using probes FL2 and LC2. *C. albicans* (approx. 66°C) and *A. fumigates* (approx. 53°C) showed different melting points. Water control shows no melting peak.

2. Prepare a 2% agarose gel (see Note 19) by mixing 2 g agarose with 100 ml 1× TAE buffer. Dissolve the agarose by boiling the mixture in a microwave at 800 W for 3 min until the solution is clear.
3. Let the gel solution cool down to 60°C.
4. Put the comb into the gel chamber. Fill in the liquid agarose solution and gently remove air bubbles. Be sure that the gel chamber is sealed to avoid spilling the liquid agarose.
5. After polymerization for 15 min, transfer the gel to an electrophoresis chamber and fill the chamber with 1× TAE buffer until the gel is completely covered. Remove the comb carefully.
6. Add 10 µl of the DNA size marker in one slot.
7. Mix 3 µl loading buffer with 10 µl cDNA per sample amplified by the PCR and transfer the samples carefully into the slots.
8. Connect the electrophoresis chamber with the power station and set the voltage and runtime. For a 2% agarose gel (100 ml) the settings are approximately 110 V for 60 min (see Note 20).

3.5.1. Staining of DNA Fragments in the Agarose Gel

1. To stain the DNA in the agarose gel, transfer the gel to an ethidium bromide bath after completion of the electrophoresis and incubate it for about 30 min (see Notes 3 and 21). For this, dissolve 300 µl of the ethidium bromide solution (10 mg/ml) in 500 ml water. Alternatively ethidium bromide or other dye can be added to the gel at the set-up stage
2. After incubation the gel should be washed for 10–20 min in water to remove excess ethidium bromide.

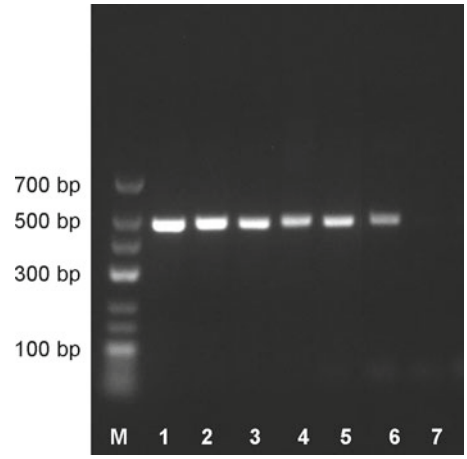

Fig. 5. Analysis of the length of the amplified 18sRNA fragments of *Candida* spp. and *Aspergillus* spp. after real-time PCR by gel electrophoresis: (1) *C. tropicalis*, (2) *C. parapsilosis*, (3) *C. dublinensis*, (4) *C. glabrata*, (5) *C. ablicans*, (6) *A. fumigatus*, (7) negative control (water). The base pair marker (GeneRuler™ Low Range DNA Ladder, Fermentas) is shown in the first lane.

3. Transfer the gel to the image acquisition system, which can detect dyes activated by UV rays. Take a picture according to the manufacturer's recommendations (Fig. 5).
4. Compare the length of the PCR fragments with the size marker and check the correct length of the amplified fragment has been generated.

4. Notes

1. For the isolation of DNA, kits and reagents from different companies are available. DNA isolation procedure often depends on the kind of fungi and sample material. Special modifications in the DNA isolation protocol for *Aspergillus* spp. should be made (7). After isolation, the DNA concentration should be measured before the PCR run (e.g., by photometry).
2. The use of the marker depends on the length of the amplified PCR fragment. Commonly used markers are 100 bp markers but should be adapted to the length of the amplified PCR product.
3. Ethidium bromide is a mutagenic dye, which intercalates between double-stranded DNA. It is strongly recommended to wear specialized gloves (e.g., nitrile gloves) for protection if you are working with ethidium bromide.

4. The target sequence should be unique for the species investigated by real-time PCR.
5. Longer products may decrease the exponential phase during the amplification process and the efficiency of PCR assays. The efficiency depends also on the nucleotide concentration. Longer fragments decrease the concentration of the nucleotides during the process and therefore decrease the PCR efficiency. However, amplification of longer fragments can work and is target dependent.
6. The length of the fragment has to be detected by gel electrophoresis after real-time PCR to prove that the correct size product was amplified.
7. The melting temperature (T_m) is calculated from the length of the primer and depends on the base pairs A, T, C, and G. There are other methods to calculate the melting temperature (e.g., Wallace rule). The melting temperature describes the temperature where 50% of the primers are bound to the DNA and is critical in the determination of the annealing temperature. The annealing temperature of the primers should be 3–5°C under the T_m and is defined as the maximal temperature where the primers bind to the complementary DNA. No samples will be amplified if the annealing temperature is too high. In contrast, low annealing temperatures lead to an unspecific binding of primers.
8. Primers often form dimers or crossdimers because of their short length and complementarity. Primer dimers are mostly seen in water controls. They align with themselves (dimers) or sense and anti-sense primer align with each other (crossdimers). They give false-positive fluorescence signals during the amplification process. After analysis of the melting curves, they can be seen as additional peaks. Primer dimers mostly show a lower melting temperature than the target gene fragments.
9. Common fluorescence dyes like SYBR green are intercalating dyes and are incorporated in double-stranded DNA. Therefore, fluorescence signals of primer dimers or other unspecific amplified products can be measured and can decrease the specificity of the PCR assay.
10. TaqMan probes consist of only one probe with bound dyes (quencher and reporter). The quencher suppresses the fluorescence signal of the reporter as long as the probe binds to the target sequence. The Taq polymerase cleaves the probe during the elongation process and quencher and reporter dye are removed from each other leading to an increased fluorescence signal. TaqMan probes are highly specific and one disadvantage is the requirement of different probes for differ-

ent sequences. In comparison, hybridization probes are able to distinguish between different species dependent on changes in the base pair composition of the target sequence.

11. There will only be a fluorescence signal, if the hybridization probes bind simultaneously on the target gene fragment and are near enough to each other.
12. After denaturation of DNA, primers and hybridization probes bind to their complementary target sequence on the DNA. A higher annealing temperature leads to a stronger hybridization to the target sequence. Primers and hybridization probes bind to the same target sequence and can compete with each other. Thus, the melting temperature of the hybridization probes should be higher than the melting temperature of the primers, which results in a stronger binding of the hybridization probes leading to higher fluorescence intensity.
13. Both hybridization probes bind to the same single strand, whereas one of the two primers binds to the sense strand ($5' \rightarrow 3'$ direction, sense primer). The second primer binds to the anti-sense strand ($3' \rightarrow 5'$ direction, anti-sense primer). After binding of primers and hybridization probes, the Taq polymerase extends the strand in $5' \rightarrow 3'$ direction (Amplification). The optimum temperature for the Taq polymerase is 72°C. Therefore, melting temperatures should be chosen lower than 80°C. Too high melting temperatures could lead to an interference of the amplification process with the annealing process of the hybridization probes.
14. Because of the existing competition between primers and hybridization probes, the hybridization probes should lay closer to the 3' end of the target sequence and thus far away from the sense primer. During the amplification process, the hybridization probes dissociate faster from the single strand when they are closer to the 5'-end of the sense primer because of interference with the Taq polymerase. This could lead to incorrect measurements of fluorescence signals. It is recommended to order and produce the designed probes by specialized firms (e.g., TIB MOLBIOL, Berlin, Germany).
15. This is known as asymmetric PCR and it results in the increased amplification of the strand to which the probes bind, ultimately increasing the signal. The optimum concentration must be tested for each primer.
16. Mostly in real-time PCR, the final concentration of cDNA should not exceed 100 ng.
17. Prepare two mastermix for each probe pair (do not put them together in one mix) and prepare each mastermix for two samples more.

18. The melting points are dependent on the sequence homology between the amplified PCR product and the hyb-probes.
19. The percentage of the agarose gel depends on the length of the amplified target fragment. The smaller the fragment, the higher should be the agarose concentration.
20. The running time and voltage for gel electrophoresis depends on the size and concentration of the agarose gel.
21. Ethidium bromide can also be added directly in agarose gel before electrophoresis. Ethidium bromide is positively charged thus it runs in the opposite direction to the negatively charged DNA.

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