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ORIGINAL PAPER

Influence of design probe and sequence mismatches on the efficiency of fluorescent RPA

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Abstract

Recombinase polymerase amplification (RPA) is an isothermal amplification technique. Because of its short detection cycle and high specificity, it has been applied in various fields. However, the design of probe on the efficiency of RPA is not well understood and the effect of sequence mismatches of oligonucleotides on the performance of RPA is rarely discussed. In this study, we found that different primers with the same probe have a slight effect on the efficiency of fluorescent RPA, and different probes with the same amplified region have a great influence on the efficiency of fluorescent RPA. We summarized the design rules of probes suitable for fluorescent RPA by analyzing the experimental data. The rule is that the best distance between fluorescent groups in the probe is 1–2 bases, and the G content should be reduced as far as possible. In addition, we verified this rule by designing a series of probes. Furthermore, we found the base mismatches of the probe had a significant effect on RPA, which can lead to false positives and can change the amplification efficiency. However, 1–3 mismatches covering the center of the primer sequence only affect the amplification efficiency of RPA, not its specificity. And with an increase in the number of primer mismatches, the efficiency of RPA will decrease accordingly. This study suggests that the efficiency of fluorescent RPA is closely related to the probe. We recommend that when designing a fluorescent probe, one must consider the presence of closely related non-targets and specific bases.

Keywords RPA · Probe · Mismatch · Design rule · Efficiency

Introduction

Since it was introduced in 2006, the isothermal recombinase polymerase amplification (RPA) technology for nucleic acid detection has been applied in various fields (Clarke et al. 2016; Daher et al. 2016; Lillis et al. 2016). RPA products can be detected by gel electrophoresis (AGE) or real-time fluorescent probes (Clancy et al. 2015; Euler et al. 2012; Hansen et al. 2016; Santiago-Felipe et al. 2015). Alternatively, they can be visualized by a lateral flow dipstick (LFD) assay (Jaroenram and Owens 2014; Nair et al. 2015; Yang et al. 2017). Since the amplification progress of RPA with a fluorescent probe can be observed in real time, the production of signals indicates the beginning of amplification. The fluorescence RPA is widely used and its results are easier to judge (Cabada et al. 2017).

With PCR, the specificity of amplification resides mainly in primer design and the annealing temperature (Bej et al. 1991; Ishii and Fukui 2001; Jung et al. 2017; Wadle et al. 2016). A failure to discriminate between closely related target molecules results in false positives (Boyle et al. 2009). However, for RPA, the specific mechanism was described in detail in Piepenburg et al. as the first introduction to RPA technology (Piepenburg et al. 2006). The specificity is dependent on the recombinase enzymes' function during the homology search, the target sequence and the reaction temperature (Armes and Stemple 2014; Olaf et al. 2011; Patil et al. 2011). Previous studies have examined the effects that the reaction components and temperature have on RPA (Bianco et al. 1998; Crannell et al. 2014; Faye et al. 2015). And some researches showed the wide application of RPA

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technology (Allerberger and Wagner 2010; Kersting et al. 2014). However, the effects of different oligonucleotides sequences and mismatches on RPA were rarely introduced (Abd et al. 2013; Boyle et al. 2013). Previous study has also shown that with the design of the probes according to the manufacturer's recommendations, the results of RPA will have different problems, such as differences in efficiency or low specificity (Krãµlov et al. 2014). In addition, Daher et al. noticed that the amplification efficiency of the same primer-probe for different positive templates is different (Daher et al. 2015). There is no extensive study on the causes of these problems in RPA reactions. The sequence of the same gene also had base differences in the same species, and these differences can lead to errors in molecular detection (Klungthong and Chinnawirotpisan 2010; Ledeker and De Long 2013). Complementarity between primers and template is crucial for RPA applications, as mismatches can severely reduce the priming efficiency. Further, the effect of base mismatches in RPA efficiency has not been studied. Therefore, our study has a powerful impetus for examining the application of RPA.

To analyze the effect of different oligonucleotides on fluorescent RPA, we designed several approaches. These approaches include analyzing the effects of different primers with same probe on RPA, the effects of different probes with the same of amplified regions on RPA, the effects of the probe sequence and the distance of fluorescent groups on RPA, and the effects of oligonucleotide sequence mismatches on RPA. In addition, we designed a series of probes to verify the probe design rule. All primers and probes were designed according to the specific genes, and each approach was generally developed as an independent method. Based on the results of this study, the wide application of RPA can be promoted.

Materials and methods

Standard strain

Standard strains were purchased from different culture collection centers (Table 1). Genomic DNA was extracted from overnight bacterial cultures by using TakaRa MiniREST Bacterial Genomic DNA Extraction Kit Ver. 3.0 in accordance with the manufacturer's recommendations. The extracted DNA was stored at − 20 °C until ready for use.

Table 1 List of bacterial species used in this study

Species	Strain ID
Listeria monocytogenes	ATCC 19117
Staphylococcus aureus	ATCC 6538
Escherichia coli	ATCC 43895
Streptococcus agaricus	ATCC 43895
Salmonella	ATCC 14028
Streptococcus pyogenes	CICC 10373
Staphylococcus argenteus	DSM 28299

Table 2 The oligonucleotide sequences used to study the effects of different primers on RPA

Target	ID	Sequence (5′–3′)
L. monocytogenes actA CP013288.1	P1	CGGCCAATTGATATGCCGAGCCTACCAG-(FAM-dT)-A-(dSpacer)-(BHQ1-dT)-CCAAAAAGAAGTTAC-C3spacer
	F11	CACCATTTATAGAAAAACAAGCAGAGACAA
	F12	TAAAGCACCATTTATAGAAAAACAAGCAGAG
	F13	CTGAGCTTGGGGAAAATAAAGCACCATTTATAG
	F14	CGCAAGAAACTGAGCTTGGGGAAAATAAAGC
	F15	CACTTCCTATCACGAAAGCGCAAGAAACTG
	F16	CCCCTGTGAATACCGCGCCAAAGCTAGCAG
	F17	GAAAATAAAGCACCATTTATAGAAAAACAAG
	R11	CCTCGGTTTGCGGTTTCATTTCCTCTTTAT
	R12	CCACTTTTTCCTCGGTTTGCGGTTTCATTTCC
	R13	CCTACCACTTTTTCCTCGGTTTGCGGTTTC
	R14	CTTTCTCCTACCACTTTTTCCTCGGTTTGCG
	R15	CACATTATTAGCTGGTTCGCTTTCTCCTAC
	R16	CCGTTCACATTATTAGCTGGTTCGCTTTCTC
	R17	CTTTTTCCTCGGTTTGCGGTTTCATTTCCTC

P probe, *F* forward primers, *R* reverse primers

Table 3 The oligonucleotide sequences used to study the effects of different probes for same amplified region on RPA

Target	ID*	Sequence (5′–3′)
L. monocytogenes CP013288.1 701167–701334	actAP1	CGGCCAATTGATATGCCGAGCCTACCAG-(FAM-dT)-A-(dSpacer)-(BHQ1-dT)-CCAAAAAGAAGTTAC-C3spacer
	actAP1^{I}	AAACAAGCAGAGACAAACAATCGGCCAA(FAM-dT)T(dSpacer)T(BHQ1-dT)ATGCCGAGCCTAC-C3spacer
	actAP1^{II}	CAAGCAGAGACAAACAATCGGCCAATTGA(FAM-dT)(dSpacer)(BHQ1-dT)GCCGAGCCTACCAG-C3spacer
	actAF17	GAAAATAAAGCACCATTTATAGAAAAACAAG
	actAR15	CACATTATTAGCTGGTTCGCTTTCTCCTAC
S.aureus CP020020.1 819916–820022	ClfAP1	CCAGTTACCGGCGTTTCTTCCGTAGTTGCA(FAM-dT)(dSpacer)(BHQ1-dT)GTTGATGATGATTGC3spacer
	ClfAP1^{I}	GCGTTTCTTCCGTAGTTGCATTTGTTGA(FAM-dT)G(dSpacer)(BHQ1-dT)GATTGTGTCGTTTC-C3spacer
	ClfAP1^{II}	CTTCCGTAGTTGCATTTGTTGATGATGAT(FAM-dT)(dSpacer)(BHQ1-dT)GTCGTTTCCTGTTG-C3spacer
	ClfAP1^{III}	GTTACCGGCGTTTCTTCCGTAGTTGCATT(FAM-dT)(dSpacer)(BHQ1-dT)TGATGATGATTGTG-C3spacer
	ClfAP1^{IV}	TTCCGTAGTTGCATTTGTTGATGATGA(FAM-dT)TG(dSpacer)G(BHQ1-dT)CGTTTCCTGTTGT-C3spacer
	ClfAF1	CTTCACCAGTTACCGGTGTTTCTTCTGTAG
	ClfAR1	CACTAATAGTGACGAAACGAATGTAGCGCA
E. coli CP017251.1 1862905–1863111	WzP1	ATTTGCTCCCATGTCTCCAAATACTTGT(FAM-dT)T(dSpacer)(BHQ1-dT)GTAACCGTTCTCCA-C3spacer
	WzP1^{I}	TTGCTCCCATGTCTCCAAATACTTGTTTA(FAM-dT)(dSpacer)(BHQ1-dT)AACCGTTCTCCATA-C3spacer
	WzP1^{II}	CTCCATACGTAGTAACTGATAAAAAATT(FAM-dT)G(dSpacer)(BHQ1-dT)CCCATGTCTCCAAA-C3spacer
	WzF1	ACTAACAGTTCTGCTCCATACGTAGTAACT
	WzR1	AAGTGTTCCATATGTTGTTTCTGAATCTAT

**P* probe, *F* forward primers, *R* reverse primers

Table 4 The oligonucleotide sequences used to study the effects of different probes on RPA

Species	ID	Sequence (5′–3′)	G Content		Fluorescent group	Sequence accession number
			Bases	Percent		
L. monocytogenes	actAF11	GAAAATAAAGCACCATTTATAGAAAAACAAG	–	–	–	CP013288.1 701167–701334
	actAR11	CACATTATTAGCTGGTTCGCTTTCTCCTAC	–	–	–	
	P1	CGGCCAATTGATATGCCGAGCCTACCAG-(FAM-dT)-A-(dSpacer)-(BHQ1-dT)-CCAAAAAGAAGTTAC-C3spacer	9	19.1	2	
L. monocytogenes	actAF21	CATCCAGGGTTGCCATCGGATAGCGCCGCG	–	–	–	CP013288.1 699994–700332
	actAR21	CCCTGCACTTTTATCAACAATTGCTTTCTTC	–	–	–	
	P2	CTTAGATTCTAGCATGCAGTCAGCGGA-(FAM-dT)-G-(dSpacer)-G-(BHQ1-dT)-CTACACCACAACC-C3spacer	9	20	3	
L. monocytogenes	inlAF31	GTCATAAGCGTTCATTGTACTTGTTGTGCTAGC	–	–	–	CP013288.1 986203–986383
	inlAR31	GCTGTACGCTCAATTCACGAAAAATCCTGTGG	–	–	–	
	P3	AGGTGGTGTAGTGTTCCCTCCGTTATTTG-(FAM-dT)-(dSpacer)-G-(BHQ1-dT)-CGGCGGAGTGTTC-C3spacer	12	25.5	1	
L. monocytogenes	actAF41	CCAACAGAAGAAGAGTTGAACGGGAGAGG	–	–	–	CP013288.1 700780–701167
	actAR41	CCCCAAGCTCAGTTTCTTGCGCTTTCGTGA	–	–	–	
	P4	ACAGAAGAAGAAATTGATCGCCTAGCTGA-(FAM-dT)-(dSpacer)-(BHQ1-dT)-AAGAGATAGAGGAAC-C3spacer	16	34.8	2	
L. monocytogenes	actAF51	CCAACAGAAGAAGAGTTGAACGGGAGAGG	–	–	–	CP013288.1 700744–700916
	actAR51	CCCGCATTTCTTGAGTGTTTTCCTGTTCCT	–	–	–	
	P5	CCAACATCTGAAGAATTTAGTTCGCTGAA-(FAM-dT)-(dSpacer)-G-(BHQ1-dT)-GGCGATTTTACAG-C3spacer	10	21.7	2	

P probe, *F* forward primers, *R* reverse primers

Table 5 The oligonucleotide sequences used to verify the probe design rule of RPA

Probe number	Species	ID*	Sequence (5′–3′)	G Content		Fluorescent group distance	Sequence accession number
				Bases	Percent		
1	*E. coli*	WzP1	ATTTGCTCCCATGTCTCCAAATAC TTGT(FAM-dT)T(dSpacer)(BHQ1-dT) GTAACCGTTCTCCA-C3spacer	5	10.87	2	CP017251.1 1862905–1863111
		WzF1	ACTAACAGTTCTGCTCCATACGTA GTAACT	–	–	–	
		WzR1	AAGTGTTCCATATGTTGTTTCTGA ATCTAT	–	–	–	
2	*S. aureus*	ClfAP1	CCAGTTACCGGCGTTTCTTCCGTA GTTGCA(FAM-dT) (dSpacer) (BHQ1-dT) GTTGATGATGATTG-C3spacer	10	22.22	1	CP020020.1 819916–820022
		ClfAF1	CTTCACCAGTTACCGGCGTTTCTT CCGTAG	–	–	–	
		ClfAR1	CACTAATAATGGCGAAACGAGTGT GGCGCA	–	–	–	
3	*E. coli*	RfbEP	ATGTTTTTCACACTTATTGGATGG TCTCAA(FAM-dT)(dSpacer)C(BHQ1-dT)AACTAGGACCGCAGAGG-C3spacer	10	22.22	2	CP017251.1 1866141–1866404
		RfbEF	TGGCATGACGTTATAGGCTACAAT TATAGG	–	–	–	
		RfbER	TGTTTCGATGAGTTTATCTGCAAG GTGATT	–	–	–	
4	*E. coli*	WzP2	GTGGCTGGGAATGCATCGGCCTTC TTTTT(FAM-dT)G(dSpacer)(BHQ1-dT)CCTTTTCTCTCCGT-C3spacer	12	25.53	2	CP017251.1 1863193–1863377
		WzF2	TATAACTGATATTTTCATTTCGTG ATAATCTC	–	–	–	
		WzR2	CCATAGCTCGATAAATTGCGCATT CTATTC	–	–	–	
5	*S. agalactiae*	SipP	CAATCAAGTTTCTGTTGCAGACCA AAAAG(CY3-dT)T(dSpacer)C(BHQ1-dT)CTCAATACAATTTC-C3spacer	5	10.42	3	CP007631.2 47761–47915
		SipF	CTGCCACTTCAATGAAAATAGAAA CACCAGC	–	–	–	
		SipR	CGTTGTTGCTGCTTCTGGTGTCAT ACCTTCC	–	–	–	
6	*S. aureus*	aClfAP	CTTCACCAGTTACCGGCGTTTCTT CCG(FAM-dT)A(dSpacer)T(BHQ1-dT)-GCATTTGTTGATG-C3spacer	12	25.53	3	CP020020.1 819950–820046
		aClfAF	CTTGATTCGTTGTCGTAGTAGTAG CTTCAC	–	–	–	
		aClfAR	CCAGCACAACAGGAAACGACACAA TCATC	–	–	–	
7	*S. aureus*	CoaP1	TCAGTACCTTGTGGTTTTATTTCA AGTT(FAM-dT)A(dSpacer)A(BHQ1-dT)GAGCTACCTTCAA-C3spacer	8	17.39	3	LS483319.1 235347–235459
		CoaF1	TGATTCAGTACCTTGTGGTTTTAT TTCAAG	–	–	–	
		CoaR1	GCGGCCCATCATTAAGCAATAATT ATACAA	–	–	–	

Table 5 (continued)

Probe number	Species	ID*	Sequence (5′–3′)	G Content		Fluorescent group distance	Sequence accession number
				Bases	Percent		
8	*Salmonella*	IrpP	CATCACTACCGGAATGTCATACTTAGCGGCAA(FAM-dT)C(dSpacer)G(BHQ1-dT)GGTCCAACGAC-C3spacer	10	20.83	3	CP025554.1 1117763–1118011
		IrpF	GTCAGAAAGTGATTTCGTCACGCCTTTGAT	–	–	–	
		IrpR	CAGGAGGCATTACGAAAAGAAAATAACATTGG	–	–	–	
9	*S. aureus*	CoaP2	GTGGTTTTATTTCAAGTTTAGATGAGC(FAM-dT)AC(dSpacer)T(BHQ1-dT)CAAGACCTTCTAA-C3spacer	8	17.39	4	LS483319.1 235321–235473
		CoaF2	CCTTGTGGTTTTATTTCAAGTTTAGATGAG	–	–	–	
		CoaR2	CCCATCATTAAGCAATAATTATACAAACCC	–	–	–	
10	*S. pyogenes*	SpeBP	CCCCAACCCCAGTTAACATGGTAGAAG(FAM-dT)T(dSpacer)CG(BHQ1-dT)CCGTCAGCACCATCG	9	18.75	4	LS483335.1 1767596–1767765
		SpeBF	CCGTTGAAGCCGCCTGCGCCGCCACCAGTAC	–	–	–	
		SpeBR	CAAGGTGTCGGTAAAGTAGGCGGACATGCCTTTG	–	–	–	
11	*S. pyogenes*	SdaBP	CAACTCGTCTGCGTTATAGATTGGAGCAGC(FAM-dT)T(dSpacer)A(BHQ1-dT)AATAAAGATAGCC-C3spacer	10	20.83	3	LS483332.1 1,695,349–1,695,509
		SdaBF	CTGTGTTGTAAACTAATACTTTCTCGTTGATG	–	–	–	
		SdaBR	ACAAAGAGCTCAAGAATGGTTAGAAGCAAATC	–	–	–	
12	*S. agalactiae*	CfbP	CTGAACATTATCTTTGATATTTC(CY5-dT)C(dSpacer)AC(BHQ1-dT)GAATGCTATCTTGATC-C3spacer	5	11.11	4	LS483387.1 1894114–1894270
		CfbF	CAGGGTTGGCACGCAATGAAGTCTTTAATTTTTC	–	–	–	
		CfbR	AATAATCAAGCCCAGCAAATGGCTCAAAAGC	–	–	–	
13	*S. argenteus*	NrpsP	CTGAAGGAGACGACAACCAAGGCTGTG-(CY5-dT)-AG-(dSpacer)-A-(BHQ1-dT)-GGGTCAATTCATA-C3spacer	13	28.26	4	CP015758.1 148866–148950
		NrpsF	CTGAAGGAGACGACAACCAAGGCTGTGTAG	–	–	–	
		NrpsR	CTATATTGATTATGAAGTGCTTTGTCATAGG	–	–	–	

**P* probe, *F* forward primers, *R* reverse primers

Primers and probes design

The oligonucleotides that studied the effect of different primers on RPA are shown in Table 2. F17R15 were chosen as reference to compare because all the other primers were based on this pair of primers. The oligonucleotide sequences used to study the effects of different probes for same amplified region on RPA are shown in Table 3. The oligonucleotide sequences used to study and verify the rules of probe design are shown in Tables 4 and 5. To thoroughly

Table 6 Perfect match and mismatch primers and probes used in this study

Target	Oligonucleotide ID*	Sequence (5′–3′)	GC%	Mismatch bases
S.aureus CP020020.1 819916–820022	ClfAP1	CCAGTTACCGGCGTTTCTTCCGTAGTTGCATTTGTTGATGATGATTG	44.7	No mismatch
	ClfAP2	CCAGTTACCGGCGTTTCTTCTGTAGTTGCATTTGTTGATGCTGATTG	44.7	C–T, A–C
	ClfAP3	CCAGTTACCGGTGTTTCTTCTGTAGTTGCATTTGTTGATGCTGATTG	42.6	C–T, C–T, A–C
	ClfAF1	CTTCACCAGTTACCGGTGTTTCTTCTGTAG	46.7	No mismatch
	ClfAF2	CTTCACCAGTTACCGGCGTTTCTTCTGTAG	50.0	T–C
	ClfAF3	CTTCACCAGTTACCGGTGTTTCTTCCGTAG	50.0	T–C
	ClfAF4	CTTCACCAGTTACCGGCGTTTCTTCCGTAG	53.3	T–C, T–C
	ClfAR1	CACTAATAGTGACGAAACGAATGTAGCGCA	43.3	No mismatch
	ClfAR2	CACTAATAATGACGAAACGAATGTAGCGCA	40.0	G–A
	ClfAR3	CACTAATAGTGGCGAAACGAATGTAGCGCA	46.7	A–G
	ClfAR4	CACTAATAGTGACGAAACGAGTGTAGCGCA	46.7	A–G
	ClfAR5	CACTAATAGTGGCGAAACGAGTGTAGCGCA	50.0	A–G, A–G
	ClfAR6	CACTAATAATGGCGAAACGAATGTAGCGCA	43.3	G–A, A–G
	ClfAR7	CACTAATAATGACGAAACGAGTGTAGCGCA	43.3	G–A, A–G
	ClfAR8	CACTAATAATGGCGAAACGAGTGTAGCGCA	46.7	G–A, A–G, A–G

**P* probe, *F* forward primers, *R* reverse primers

underlined mismatch base

investigate the effects that oligonucleotide-template mismatches have on RPA detection, a large number of RPA primers and probes were designed containing 1–3 mismatches (Table 6). All oligonucleotides were synthesized by Sangon Biotech, Shanghai, China.

RPA assay conditions

The RPA reaction was performed in a 50 µL volume using a TwistAmp™ exo kit (TwistDX, Cambridge, UK). Briefly, each reaction contained 29.5 µL of rehydration solution, 11.2 µL of nuclease-free water, 2.1 µL of each primer (10 mM), 0.6 µL of the RPA probe (10 mM), 2 µL of template, and 2.5 µL of magnesium acetate (280 mM). All reagents were configured as pre-mixed solutions, except for template and magnesium acetate, and were added to each reaction tube containing a dried enzyme pellet. Subsequently, targets were added to each reaction. Next, the supplied magnesium acetate was added to the lid of each tube containing a reaction. The lids were closed, and the magnesium acetate was centrifuged into the tubes using a minispin centrifuge, then the tubes were immediately placed into Tube Scanner (Twista, TwistDx Ltd, Cambridge, UK) and the reaction was carried out at 39 °C for 15 min.

RPA reaction of different primer-probes

The RPA experiments were carried out by using the different primer-probes listed in Tables 2, 3 and 4. We compared the effects of different primer-pairs with the same probe and different probes for same amplified region on RPA. And we examined the impact of different probes with the corresponding primers on RPA. The G content of the probes designed according to *L. monocytogenes* is shown in the Table 4.

Evaluation of probe design rules

Based on the experience with probe design described in Table 4, a series of probes with corresponding primers were designed for RPA specifically detecting pathogenic bacteria (Table 5). The fluorescence groups of probes numbered 1–4 are separated by 1–2 bp, but the fluorescent groups of probes numbered 5–13 are separated by 3–4 bases. And the G content of all probes is randomly distributed. According to the amplification efficiency of the RPA assay, we can evaluate the probe design rules.

Influence of sequence mismatches on amplification

The effects of the RPA assay in the presence of oligonucleotide mismatches were evaluated using a large number of primers and probes containing mismatch bases (Table 5). In addition, based on the specific sequence of *S. aureus*, the perfect match primers and probe were designed. The perfect match primers

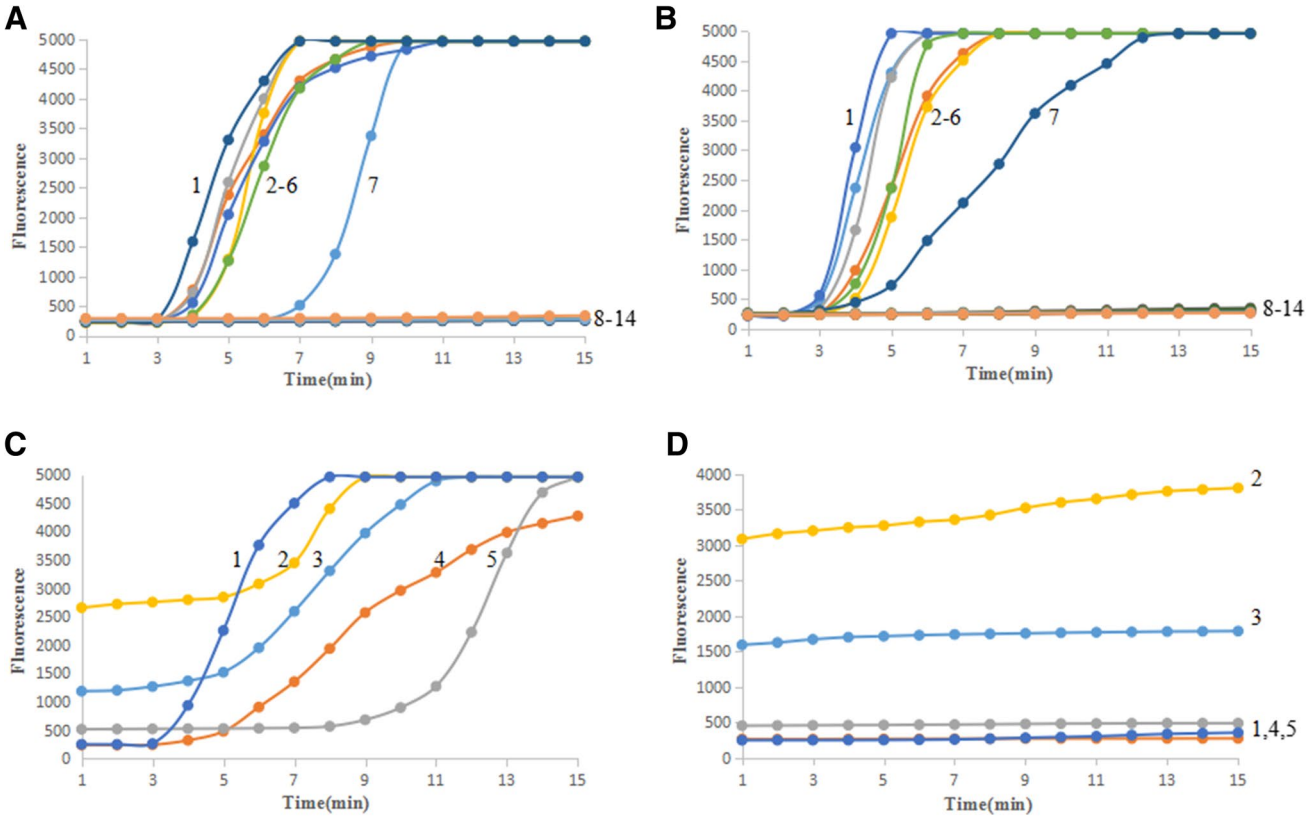

Fig. 1 Influence of different primer-probes on fluorescent RPA. **a** The effect of combinations of R15, P1 and different forward primers on fluorescent RPA. Curve 1–7 are the amplification results of the combination of F17R15P1, F16R15P1, F15R15P1, F14R15P1, F13R15P1, F12R15P1 and F11R15P1, respectively. Curve 8–14 are the amplification results of the blank control of F17R15P1, F16R15P1, F15R15P1, F14R15P1, F13R15P1, F12R15P1 and F11R15P1, respectively. **b** The effect of combinations of F17, P1 and different reverse primers on fluorescent RPA. Curve 1–7 are the amplification results of the combination of F17R11P1, F17R12P1, F17R13P1, F17R14P1, F17R15P1, F17R16P1 and F17R17P1, respectively. Curve 8–14 are the amplification results of the blank control of F17R11P1, F17R12P1, F17R13P1, F17R14P1, F17R15P1, F17R16P1 and F17R17P1, respectively. **c**, **d** The effect of different probes on fluorescent RPA. C represents the amplification curve of the positive control; **d** represents the amplification curve of the blank control. 1, 2, 3, 4 and 5 represent the amplification results of P1, P2, P5, P3 and P4 in Table 3, respectively

and probe were suitable for RPA according to their verification and analysis, then add 2 μL template to the reaction system, and were used as a positive control. At the same time, using equivalent volume of water to replace the required template as a blank control, other conditions remain unchanged. The effects of primers and probes with sequence mismatches on RPA were analyzed.

Results

RPA efficiency of different primer-probes

The results obtained for different primers of the same probe showed that the primers had slightly different effects on RPA efficiency and had no effect on RPA specificity (Fig. 1a, b). The influence of different probes for same amplified region on RPA were shown in the Fig. 2. The results showed that the amplification efficiency of positive control with different probes in the same amplied region were significantly different. The amplification results of the blank control of actAP1^{I} and clfAP1^{IV} were false positive, which could not achieve the purpose of detection of the target bacteria. In addition, all five different primer-probes of *L. monocytogenes* were tested for specific amplification, and the results of different primer-probes had an obvious discrepancy. As shown in Fig. 1c, P1 is the best of these combinations in the positive amplification test based on either the time of start of amplification or the amount of fluorescence increase. In addition, in Fig. 1d, the fluorescence of P2 increased significantly in the blank control, while the other four probes showed no significant change in fluorescence.

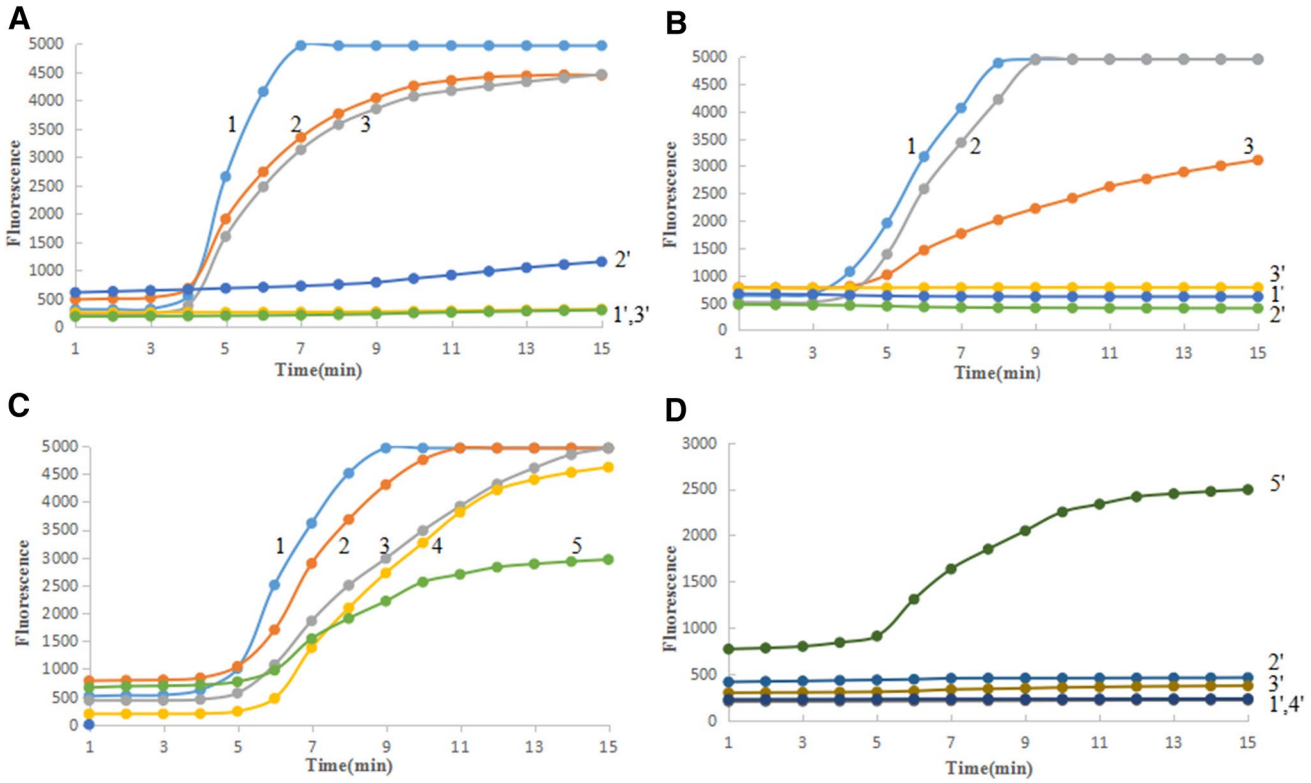

Fig. 2 Influence of different probes for same amplified region on fluorescent RPA. **a** The RPA amplification results of *L.monocytogenes* probes for same amplified region. Curve 1–3 are the amplification results of positive control of actA P1, actA $P1^{I}$ and actA $P1^{II}$, respectively; Curve 1′–3′ are the amplification results of blank control of actA P1, actA $P1^{I}$ and actA $P1^{II}$, respectively. **b** The RPA amplification results of *E. coli* probes for same amplified region. Curve 1–3 are the amplification results of positive control of wzP1, $wzP1^{I}$ and $wzP1^{II}$, respectively; Curve 1′–3′ are the amplification results of blank control of wzP1, $wzP1^{I}$ and $wzP1^{II}$, respectively. **c, d** The RPA amplification results of *S. aureus* probes for same amplified region. C represents the amplification curve of the positive control. Curve 1–5 are the amplification results of positive control of clfAP1, $clfAP1^{I}$, $clfAP1^{II}$, $clfAP1^{III}$ and $clfAP1^{IV}$, respectively. **d** The amplification curve of the blank control. Curve 1′–5′ are the amplification results of positive control of clfAP1, $clfAP1^{I}$, $clfAP1^{II}$, $clfAP1^{III}$ and $clfAP1^{IV}$, respectively

Analysis of different probes

In studying the effects of different primer-probes on fluorescent RPA, we found that the change of the primers did not have a significant impact. In contrast, probes played an important role in RPA. Therefore, we assumed that the influence of the probes on fluorescence RPA is caused by their own sequences and structures. The detailed information of the 5 probes used in method 2.4 is shown as follows (Table 4). We found the contents of G in different probes were different. The content of G varied from 19.1 to 34.8%. We also noticed that there were some differences in the distance between the fluorescent groups. We found that the amplification efficiency was related to G content of probe. With the decrease of G content, RPA amplification efficiency is higher (As shown in Fig. 1c, P1 > P2 > P5 > P3 > P4). At the same time, the distance between the fluorescent groups in the probe is different, and the amplification curve of the blank control also has obvious difference. When the fluorescence groups were separated by 3 bases, the amplification curve of the blank control presented false positive (As shown in Fig. 1d, P2).

Evaluation of probe design rules

To verify the rules of probe design, we designed a series of specific probes for detecting pathogenic bacteria. The sequence information of the 13 probes used in method 2.5 is shown as follows (Table 4). From Table 4, we can see that the G content of probe number 1–4 increases gradually, and the distance between fluorescent groups is 1–2 bases. The fluorescent groups of probes numbered 5–13 differ from 3 to 4 bases. Figure 3b showed that the amplification efficiency of the positive control of probe number 1–4 will increase gradually with the decrease of the content of G. And there were no false positive results in the blank control with probe number 1–4 (Fig. 3a). However, we also found

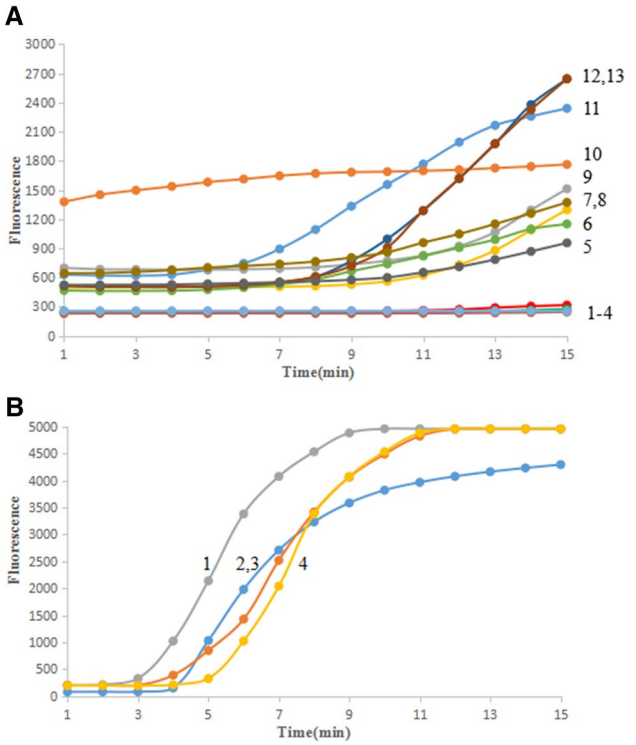

Fig. 3 Effect of different probes on fluorescent RPA of the detection of different pathogenic bacteria. **a** The RPA amplification results of blank control with different probes. Curves 1–13 correspond to probe No. 1–13 in Table 4. **b** The RPA amplification results of positive control with different probes. Curves 1–4 were the positive control result of probe No. 1–4 in Table 4

that the fluorescence intensity of the blank control with P7 increased significantly (Fig. 3a).

Effect of mismatched primers and probes on RPA

Based on the perfect matching primers and probe of *S. aureus*, the mismatched oligonucleotides were synthesized. The number of mismatches varied from 1 to 3 (Table 6). When the base mismatch number was small, the mismatch primers had a slight effect on RPA amplification (Fig. 4a, b). However, the results of the probe mismatch greatly affected RPA. Compared with the non-mismatch probe, the efficiency of mismatch probes for RPA was significantly improved when observing the positive amplification curves (Fig. 4c). In addition, we noticed that the fluorescence of the blank control curve of the mismatch probe increased significantly, thereby affecting the determination of the results (Fig. 4d).

Discussion

In previous reports, the use of the real-time fluorescent RPA method has been widely described and applied in various fields (Crannell et al. 2014; Lai et al. 2017). In addition, the primers and probes applicable to RPA are designed according to the manufacturer's recommendations. However, the oligonucleotides designed in accordance with the manufacturer's recommendations also cause many problems in the amplification process, such as false positives and low efficiency (Lutz et al. 2010). In the present study, we demonstrated that the efficiency of RPA was related to the design of probes and primers. Using the same probe, we compared the effect of different primers on RPA and found that there was a slight difference in the effects that different primers had on RPA. However, different probes had different amplification curves in positive control experiments; moreover, the amplification curves of the blank control were significantly different (Figs. 1c, d, 2). The probes had different G contents (ranging from 13.7 to 34.8%). We noticed that the amplification curve of fluorescent RPA was more ideal with a decreased G content. Therefore, compared with the primers, we inferred the probe had a great influence on fluorescent RPA. In addition, we also found that the amplification efficiency of RPA was related to the G content. Therefore, we need to select the region to design probe with relatively low G content on target sequences. And it is not necessary to select regions with low G content as target sequences.

As is known, the probes applicable to RPA contain an abasic nucleotide analogue, which replaces a nucleotide in the target sequence flanked by a dT-fluorophore and a corresponding dT-quencher group (Murinda et al. 2014). In addition, probes are blocked from any potential polymerase extension by a suitable 3′-modification group. The fluorescent reporter group is 1–4 bases away from the quencher (Crannell et al. 2015). In this study, when the fluorescence reporter group and the fluorescence quencher group were separated by 3 or 4 bases, the fluorescence quantity of the blank control was obviously increased (Fig. 1d). Further, when they were separated by 1–2 bases, we noticed that the fluorescence quantity of the blank control was basically unchanged. Thus, there is a strong relationship between the fluorescence variation of the blank control and the distance between the fluorophores. In addition, the results indicate that the shorter the distance is between fluorophores, the more favorable the blank control is. And the best distance of the fluorophores is 1 or 2 bases. This may be due to the fact that the distance between the fluorophores is inversely proportional to their binding strength. In addition, it should be pointed out that the design of probes is not only related to G content and the distance between fluorescent groups, but also related to other factors, such as secondary structure

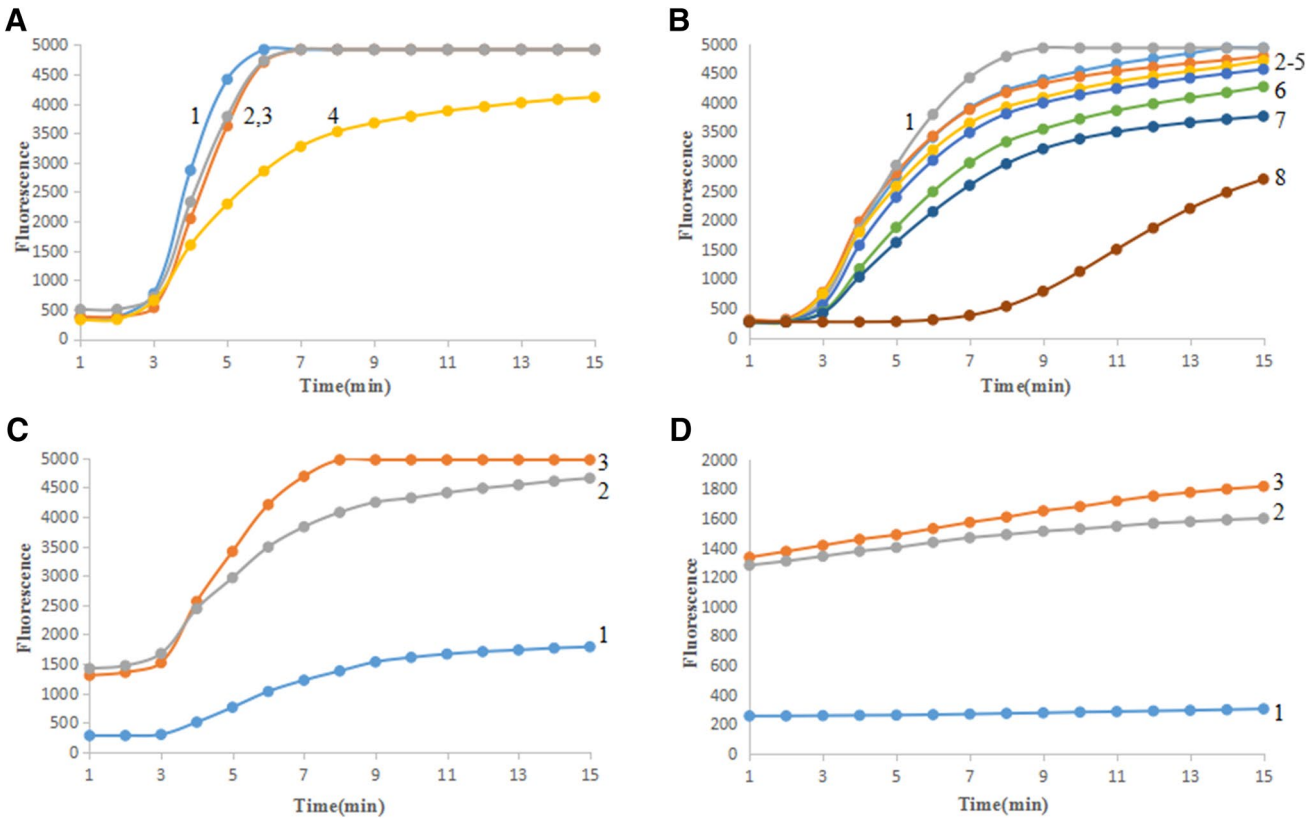

Fig. 4 Effect of mismatched primers and probes on fluorescent RPA of the detection of *S. aureus*. **a** The effect of mismatched forward primers on fluorescent RPA. Curves 1–4 are the positive control amplification results of ClfAF1R1P1, F2R1P1, F3R1P1 and F4R1P1, respectively. **b** The effect of mismatched reverse primers on fluorescent RPA. Curves 1–8 are the positive control amplification results of ClfAF1R1P1, F1R2P1, F1R3P1, F1R4P1, F1R5P1, F1R6P1, F1R7P1 and F1R8P1, respectively. **c** and **d** The effect of mismatched probes on fluorescent RPA. C represents the amplification curve of the positive control; **d** The amplification curve of the blank control. Curve 1, 2 and 3 represent the amplification results of ClfAP1, P2 and P3 in Table 4, respectively

content and the distribution of bases. However, the influence of these factors on probes is still unclear at present.

Similar to PCR, the mismatches of the primers and probe had an impact on RPA efficiency. Based on the study of Daher et al., we analyzed the effect of the random mismatch of 1–3 bases covering the center of the primer sequences on RPA. We found that as the number of primer base mismatches increases, the amplification efficiency of RPA also decreases. As observed from Fig. 4, the effect of the mismatches of primer on RPA was less than that of the probe. For probes, the mismatches of probes had a great impact on the efficiency of RPA, especially the blank control. We noticed that the fluorescence variation of the blank control of the mismatches of probes was obviously increased, which was in sharp contrast to the perfectly matched probe. The mismatch of probes mainly affected the efficiency of RPA and the amplification results of the blank control. The main reason for the difference might be that the mismatch bases change the structure and GC content of the probe. Therefore, research on the mismatch of oligonucleotide sequences is helpful for the application of RPA.

Conclusion

In conclusion, we were able to provide insight into the efficiency of RPA technology and analyze the effect of oligonucleotide mismatches on fluorescent RPA. We summarized the design rules of probes. The rule is that the best distance between fluorescent groups in the probe is 1–2 bases, and the G content should be reduced as far as possible. Compared to the existing design methods, the rules of our summary are more efficient and simple.

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Compliance with ethical standards

Conflict of interest No potential conflicts of interest are disclosed.

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