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Amplification Technical Note

Step-by-Step Protocol for Multiplex PCR with the FastStart High Fidelity PCR System and the PCR Optimization Kit

Michaela Schubert*, Brigitte Hloch** and Barbara Rüger*
* Application Lab Core Biochemicals Penzberg, Germany
** Global Marketing, Penzberg, Germany

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1. Purpose of this Note

The Roche Applied Science FastStart High Fidelity PCR System[1] is the product of choice for multiplex PCR. As this Note demonstrates, the system amplifies up to 18 PCR fragments simultaneously after the reaction is optimized with the Roche Applied Science PCR Optimization Kit[2].

2. Applications

The FastStart High Fidelity PCR System can be used for:

- simultaneous amplification of many targets in a single reaction (if more than one pair of primers is used).
- genotyping applications that require simultaneous analysis of multiple markers, such as typing of normal and genetically modified animals and plants, detection of pathogens or genetically modified organisms (GMOs), or microsatellite analyses.
- typing and detection of bacteria and viruses.
- amplification of multiple DNA regions for SNP analysis.
- semi–quantitative analysis of differentially expressed mRNA.

  Note: For multiplexing that starts from RNA, we recommend using a two-step RT-PCR. For the first step (producing the cDNA for multiplex PCR), use the Roche Applied Science Transcriptor First Strand cDNA Synthesis Kit[2].

3. Advantages of the FastStart High Fidelity PCR System

The Roche Applied Science FastStart High Fidelity Enzyme Blend is suitable for a wide range of multiplex PCR applications because it:

- has been tested for simultaneous amplification with up to 18 primer sets.
- can successfully amplify DNA or cDNA targets of the following sizes:

  50 bp – 600 bp
  450 bp – 1100 bp
  1100 bp – 1800 bp

  Note: Our experience indicates that these ranges give optimal results (i.e., allow equal amplification of all primer sets). However, our collaborators have shown that broader size ranges can also be successfully amplified.
- can amplify as little as 0.375 ng of target (depending on the primer sets).
- can successfully amplify targets with GC contents between 40% and 60% (as shown in the examples in this Technical Note).
- provides higher fidelity than regular Taq DNA polymerase.
- allows reactions to be set up at room temperature (RT), because the FastStart High Fidelity Enzyme Blend is inactive prior to the necessary activation step (94°C) and will not elongate the nonspecific primer template hybrids that may form at lower temperatures.

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3. Advantages of the FastStart High Fidelity PCR System continued

- the PCR Optimization Kit provides quick and simple optimization of multiplex PCR. This kit allows you to increase the yield and specificity of the reaction by optimizing the $MgCl_2$ concentration and the pH of the reaction, as well as test several performance-enhancing additives.
- carryover contamination can be prevented by the incorporation of dUTP and subsequent incubation with Uracil-DNA Glycosylase.
- the entire multiplex PCR workflow uses products from Roche Applied Science. No additional products are needed except primer and template!

4. Materials

Standard Solutions and Reagents

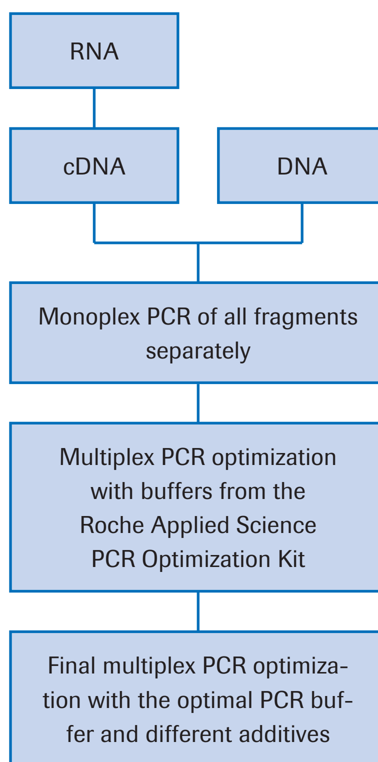
- Template (DNA, RNA)
- Appropriate primers
- Ethidum bromide
- DNA sample buffer

Products Supplied by Roche Applied Science for the Multiplex PCR Workflow

	Product	Pack Size	Cat. No.
Nucleic Acid Purification	TriPure Isolation Reagent	50 ml 200 ml	11 667 157 001 11 667 165 001
	High Pure PCR Template Preparation Kit	100 purifications	11 796 828 001
	High Pure RNA Tissue Kit	50 isolations	12 033 674 001
	High Pure RNA Isolation Kit	50 reactions	11 828 665 001
	High Pure PCR Product Purification Kit	50 purifications 250 purifications	11 732 668 001 11 732 676 001
PCR	FastStart High Fidelity PCR System[1]	125 U for 50 reactions 500 U for 200 reactions 2,500 U for 1,000 reactions	03 553 426 001 03 553 400 001 03 553 361 001
	PCR Optimization Kit[2]	1 kit	11 636 138 001
	PCR Nucleotide Mix	100 reactions (200 µl) 1,000 reactions (10 x 200 µl)	11 581 295 001 11 814 362 001
	Water, PCR Grade	25 vials of 1 ml each 1 vial of 25 ml 4 vials of 25 ml each	03 315 932 001 03 315 959 001 03 315 843 001
RT-PCR	Transcriptor First Strand cDNA Synthesis Kit[2]	50 reactions	04 379 012 001
Primer design	LightCycler® Probe Design Software 2.0[2]	1 software package	04 342 054 001
Gel Electrophoresis	Agarose MP	100 g 500 g	11 388 983 001 11 388 991 001
	DNA Molecular Weight Marker VI	50 µg	11 062 590 001
	Molecular Weight Marker VIII	50 µg	11 336 045 001

5. Methods

5.1 Workflow



5.2 General Hints for Multiplex PCR

- Activate FastStart High Fidelity Enzyme Blend for ≥ 4 min.

  Note: To increase the yield of product, use a longer activation time (up to 10 min) and more PCR cycles.

- Set up all reactions at RT!
- Purchase and use only HPLC-purified primers!
- Optimization of multiplex PCR depends greatly on the sizes of the products. It is difficult to amplify very long and short products at the same time with equivalent yields, because the polymerase will amplify the shorter products more efficiently. Therefore, consider designing primers for amplicons within one of these optimal size ranges: 50 – 600 bp, 450 – 1100 bp, and 1100 – 1800 bp.

5.3 Using LightCycler® Probe Design Software 2.0

The principle use of LightCycler® Probe Design Software 2.0[2] is finding optimal combinations of PCR primers and probes for amplifying a given DNA sequence by real-time PCR on the LightCycler® Instrument. However, the software can also be used to design specific primer sets for multiplex applications on a conventional thermal cycler.

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General Notes

In addition, the software can be used to:

- analyze the primer sets in detail for cross-complementarities.
- perform a BLAST search on the primers chosen by the program.
- print a design report.
- copy the chosen primer sets and paste them directly into an order form.
- use existing oligos in a design (*e.g.*, to extend or shorten amplicons by redesigning only one primer).

Simplified, Step-by-Step Procedure for Designing Primers

1. Launch LightCycler® Probe Design Software 2.0.
2. Import (from Toolbox Menu, choose *Sequence* and *Import*) or enter (from Toolbox Menu, choose *Insert*) your DNA sequence.
3. On the right side of the screen, choose the experiment type. *Primers only*.
4. If you want to use a fixed oligo (forward or reverse), select *Fixed Oligo* from the Tools Menu and enter the oligo sequence.
5. Edit a sequence name (if required).
6. On the right side of the screen, click on *Details* and select .

   Reaction Conditions:
   – Choose LC FastStart DNA Master HP as buffer.

   Experiment Settings:
   – Choose *primers only*.
   – Amplicon size. Insert the amplicon size range (min – max) for which the primers will be used.
   – If necessary, adjust *Primer Tm* to accommodate primer sets that already exist.
7. Click on the *Analyze* button and select *Single Set Search*.
8. To check primers (chosen by the program) against other genome sequences, use the Tools Menu to perform a BLAST search (select *BLAST-import current selection*). In the “Select From” box, choose the organism to search.
9. Analyze chosen primers against the Primer Probe Sets for cross-complementarities. Double click the primers you want to check. If cross-complementarities are found, choose a different primer set (if possible).
10. Order the chosen primer sets by directly copying and pasting the sequences into an order form.

Notes:

- The following commands function as they do in other Windows programs: *New, Open, Save, Save as*.
- Choose *Print window* to print the contents of the current window.
- For more information, see the LightCycler® Probe Design Software 2.0 manual.

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6. Two-step RT-PCR, Starting from RNA

When you want to perform a multiplex PCR starting from RNA, we recommend using a two-step RT-PCR that starts with the Transcriptor First Strand cDNA Synthesis Kit, as described below.

General Notes

- Numbered vials in the procedure are components of the Transcriptor First Strand cDNA Synthesis Kit.
- Set up reactions on ice!
- Always wear gloves and use RNase-free reagents when working with RNA.
- We recommend scaling up the RT-PCR to obtain enough material to perform the optimization steps described in this Note.

Reverse Transcription Procedure

Set up the RT reaction mix to generate enough cDNA for one or more PCRs:

Component	1 Reaction (µl)	10 Reactions[1] (µl)	Final Conc. 50 µl rxn
Total RNA (1 µg/µl)	variable[2]	variable	10 ng – 5 µg
Anchored-oligo(dT)$_{18}$ Primer (50 pmol/µl) (vial 5)	1	10	2.5 µM
Water, PCR Grade (vial 9)	add to 13	add to 130	
Total Volume	**13**	**130**	

[1] To make enough cDNA for the optimization steps in sections 7–9, scale up the reaction mix tenfold. (The extra reaction volume allows for pipetting losses.)
[2] We recommend starting with 1 µg total RNA/reaction.

- Incubate at 65 °C for 10 min.
  Note: This step ensures denaturation of RNA secondary structures.
- Place on ice immediately.
- Add the following reagents to the tube:

Component	1 Reaction (µl)	10 Reactions (µl)	Final Conc. 50 µl rxn
Transcriptor RT Reaction Buffer (5×) (vial 2)	4	40	1×
Protector RNase Inhibitor (40 U/µl)* (vial 3)	0.5	5	20 U
Deoxynucleotide Mix (10 mM each) (vial 4)	2	20	1 mM each
Transcriptor Reverse Transcriptase (vial 1)	0.5	5	10 U
Total Volume	**20**	**200**	

* Protector RNase Inhibitor is active at temperatures up to 60°C.

- Mix well by pipetting up and down several times.
- Centrifuge the tube briefly in a microfuge.
- Incubate 30 min at 55°C.
- **Note:** If the mRNA is longer than 4 kb, we recommend incubating at 50°C for 1 h.
- Inactivate the Transcriptor Reverse Transcriptase by heating to 85°C for 5 min.
- Place the tube on ice (for short term storage) or freeze at –20°C.
  Use aliquots of the cDNA product as PCR templates in the following procedures.

7. Monoplex PCR of All Fragments Separately

Monoplex PCR Procedure

First, test each primer pair individually using identical PCR and cycle conditions. Therefore, set up each monoplex PCR at room temperature by adding the components below:

Component	Each Reaction (µl)	Final Conc.
10× FastStart High Fidelity Reaction Buffer with $MgCl_2$	5	1× (includes 1.8 mM $MgCl_2$)
dNTP Mix, 10 mM each	1	200 µM each
cDNA Template	5	5 µl
or DNA Template	variable	200 ng
Upstream Primer	variable	0.4 µM
Downstream Primer	variable	0.4 µM
FastStart High Fidelity Enzyme Blend	0.5	2.5 U
Water, PCR Grade	variable	
Total Volume	**50**	

Note: For simplification, prepare a Master Mix of all components except the primers, aliquot into separate tubes and add the appropriate primers to each tube separately.

Typical Thermo-cycling Profile

Cycles	Time (min)	Temperature (°C)	Remark
1×	4[1]	95	Activation
30×	1 1 1 – 2[3]	95 60[2] 72	Denaturation Annealing Elongation
1×	7	72	Elongation

[1] Increase activation time of enzyme for difficult amplifications.
[2] Annealing temperature depends on the melting temperature of the primers.
[3] We recommend using 1 min for 1.0 kb targets, 2 min for targets up to 1.8 kb.

Gel Analysis of PCR Products

Separate the products (3–5 µl monoplex reaction/lane) for 1 hour at 100 V on a 1 – 4% Agarose Gel (see Note below) containing 1µg/ml ethidium bromide.
Note: Use 1% agarose gel to separate fragments >1000 bp. Use 3 – 4% agarose gel to separate fragments ranging from 50 bp to 1000 bp.

Interpretation of Results

Each of the monoplex PCRs should generate specific single products. If necessary, adjust the thermocycling conditions until one program works for all the monoplex PCRs.

8. Initial Optimization of Multiplex PCR with Buffers from the PCR Optimization Kit

After you have generated specific monoplex amplification products using the same PCR conditions you can begin developing the multiplex PCR. During this initial optimization, use the PCR Optimization Kit to define the optimal multiplex PCR buffer.

General Hints

- Set up reactions at room temperature!
- Set up 17 multiplex reactions, using buffers A-P (1-16) from the PCR Optimization Kit and the FastStart High Fidelity Reaction Buffer for comparison.
- As positive control load a mix of all individual monoplex PCR's (3 µl each) in one lane.
- The $MgCl_2$ concentration and pH value for each buffer in the kit are shown in the following table:

pH	$MgCl_2$ Concentration (mM)			
	1.0	1.5	2.0	2.5
8.3	A	B	C	D
8.6	E	F	G	H
8.9	I	J	K	L
9.2	M	N	O	P

Multiplex PCR Procedure for 1st Optimization

Prepare the Master Mix at room temperature by adding the components below:

Component	Each Reaction (µl)	Master Mix for 18 Reactions (µl)	Final Conc. (50 µl rxn)
dNTP Mix, 10 mM each	1	18	200 µM each
cDNA Template	5	90	5 µl
or DNA Template	variable	variable	200 ng
Multiplex Primer Mixture	variable	variable	0.4 µM each
FastStart High Fidelity Enzyme Blend	0.5	9	2.5 U
Water, PCR Grade	add to 45	add to 810	
Total Volume	**45**	**810**	

For each reaction, dispense 45 µl of Master Mix to each of 17 tubes and add 5 µl of PCR Optimization Buffer A-P or FastStart High Fidelity Reaction Buffer with $MgCl_2$.

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8. Initial Optimization of Multiplex PCR with Buffers from the PCR Optimization Kit continued

Typical Thermo-cycling Profile

Cycles	Time (min)	Temperature (°C)	Remark
1×	4[1]	95	Activation
30×	1 1 1 – 2[3]	95 60[2] 72	Denaturation Annealing Elongation
1×	7	72	Elongation

[1] Increase activation time of enzyme for difficult amplifications.

[2] Annealing temperature depends on the melting temperature of the primers.

[3] We recommend using 1 min for 1.0 kb targets, 2 min for targets up to 1.8 kb.

Gel Analysis

Follow the guidelines for agarose gel electrophoresis in Section 7., but use 10 µl of each multiplex reaction/lane.

Interpretation of Results

Determine the best performing buffer, which is best suited to amplify most of the desired products and if possible shows the best performance and yield.

9. Final Optimization of Multiplex PCR with the Best Performing PCR Buffer and Different Additives

After identifying the best performing PCR buffer (in Section 8 above), test the different additives from the PCR Optimization Kit to see if they enhance the yield and specificity of the multiplex PCR. This will require 12 multiplex reactions.

Function of the different Additives

- DMSO reportedly (1-3, 5) reduces nonspecific priming and secondary structure, and facilitates strand separation. DMSO changes the Tm of primer-template hybridization. We recommend using DMSO for templates with high GC content.
- Glycerol (4, 5) stabilizes the DNA polymerase during PCR, thereby increasing product yield.
- Gelatin (4) stabilizes the DNA polymerase during PCR, thereby increasing product yield.
- Ammonium sulfate improves PCR yield and facilitates strand separation.

Multiplex PCR Procedure for 2nd Optimization

At room temperature, set up the Master Mix by adding the components below:

We suggest the following starting concentrations of the respective additives

Component	Single Reaction (µl)	Master Mix for All Reactions (µl)	Final Conc. (50 µl rxn)
Best performing Buffer (from Section 8), 10× conc.	5	65	1×
dNTP Mix, 10 mM each	1	13	200 µM each
FastStart High Fidelity Enzyme Blend	0.5	6.5	2.5 U
Total Volume	**6.5**	**84.5**	

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9. Final Optimization of Multiplex PCR with the Best Performing PCR Buffer and Different Additives continued

Dispense 6.5 µl of Master Mix to each of 12 tubes, then add the following components:

Component	Single Reaction (µl)	Final Conc. (50 µl rxn)
cDNA Template[1]	5	5 µl
or DNA Template[1]	variable	200 ng
Multiplex Primer Mixture[1]	variable	0.4 µM each
One of the following Additives:		
Water, PCR Grade	2	Control (No Additive)
DMSO (100%)	1	2% DMSO
DMSO (100%)	2	4% DMSO
Glycerol (50%)	5	5% Glycerol
Glycerol (50%)	10	10% Glycerol
Glycerol (50%)	15	15% Glycerol
Gelatin (1%)	0.5	0.01% Gelatin
Gelatin (1%)	2.5	0.05% Gelatin
$(NH_4)_2SO_4$ (500 mM)	0.5	5 mM $(NH_4)_2SO_4$
$(NH_4)_2SO_4$ (500 mM)	1	10 mM $(NH_4)_2SO_4$
$(NH_4)_2SO_4$ (500 mM)	2	20 mM $(NH_4)_2SO_4$
FastStart High Fidelity Enzyme Blend + $MgCl_2$	0.2 µl Enzyme Blend + x µl 25 mM $MgCl_2$	3.5 U Enzyme (total) + 3.5 mM $MgCl_2$ (total)
Water, PCR Grade	add to 50 µl	
Total Volume	**50**	

[1] To simplify the preparation of these samples, you can prepare a mixture that contains template plus primers (enough for 13 reactions, to allow for pipetting losses) and then aliquot the mixture to each of the 12 reaction tubes.

Typical Thermo-cycling Profile

Cycles	Time (min)	Temperature (°C)	Remark
1×	4[1]	95	Activation
30×	1 1 1 – 2[3]	95 60[2] 72	Denaturation Annealing Elongation
1×	7	72	Elongation

[1] Increase activation time of enzyme for difficult amplifications.
[2] Annealing temperature depends on the melting temperature of the primers.
[3] We recommend 1 min for 1.0 kb targets, 2 min for targets up to 1.8 kb.

Gel Analysis

Follow the guidelines for agarose gel electrophoresis in Section 7., but use 10 µl of each multiplex reaction/lane.

Interpretation of Results

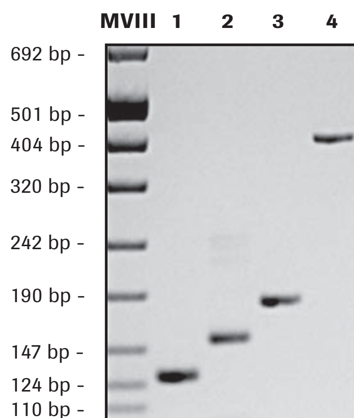
Determine which additive enhances the yield and specificity of the multiplex PCR.

10. Results

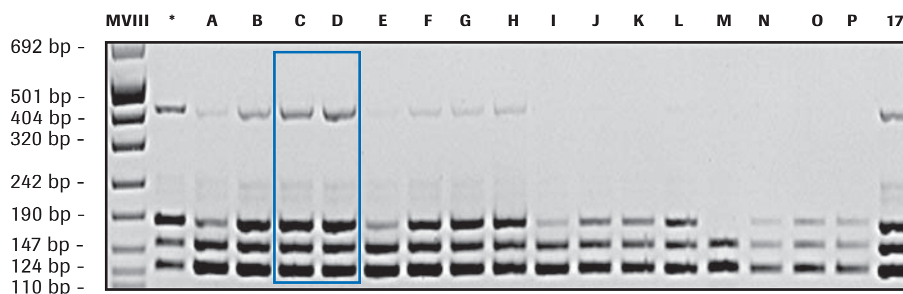
10.1 Primer Set 1: 4-plex (124 bp – 394 bp)

(Template: cDNA from human skeletal muscle total RNA)

Monoplex PCR:



Multiplex PCR with Buffers from the PCR Optimization Kit:



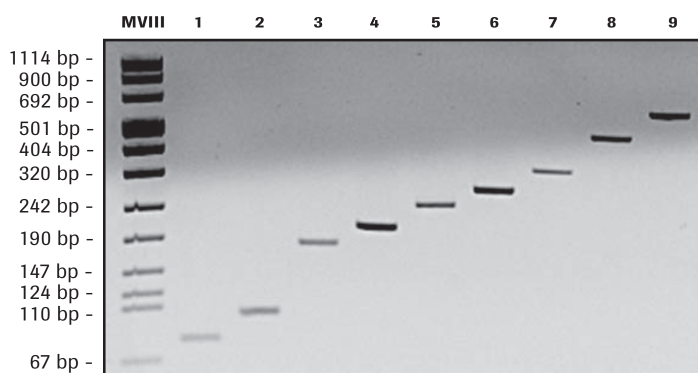
* Control mixture containing 3 µl from each monoplex product (from separate amplifications)
Samples A-P: Buffers from the PCR Optimization Kit
Sample 17: FastStart High Fidelity Reaction Buffer as comparison

Note: Optimization with additives was not necessary for this primer set. All PCR products were amplified successfully in buffers C and D from the PCR Optimization Kit!

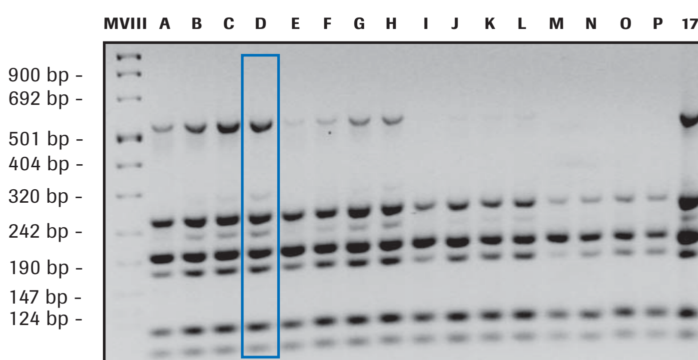
10.2 Primer Set 2: 9-plex (89 bp – 540 bp)

(Template: cDNA from human skeletal muscle total RNA)

Monoplex PCR:

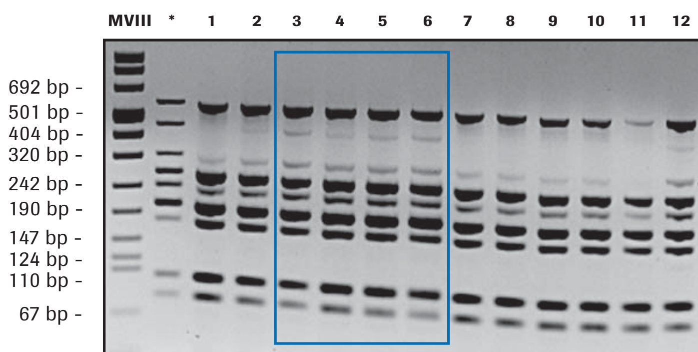


Multiplex PCR with Buffers from the PCR Optimization Kit:



Samples A-P: Buffers from the PCR Optimization Kit
Sample 17: FastStart High Fidelity Reaction Buffer as comparison

Multiplex PCR with the Best Performing PCR Buffer (D) and Different Additives:



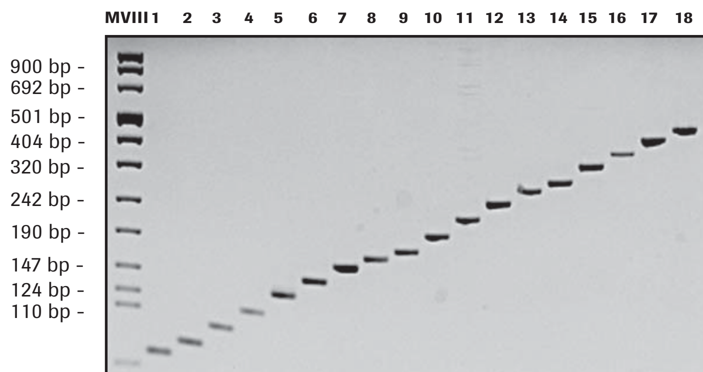
* Control mixture containing 3 µl of each monoplex product (from separate amplifications)

Additives:
1: PCR Opt. Buffer D; no additives
2: PCR Opt. Buffer D + 4% DMSO
3: PCR Opt. Buffer D + 6% DMSO
4: PCR Opt. Buffer D + 5% Glycerol
5: PCR Opt. Buffer D + 10% Glycerol
6: PCR Opt. Buffer D + 15% Glycerol
7: PCR Opt. Buffer D + 0.01% Gelatin
8: PCR Opt. Buffer D + 0.05% Gelatin
9: PCR Opt. Buffer D + 5 mM $(NH_4)_2SO_4$
10: PCR Opt. Buffer D + 10 mM $(NH_4)_2SO_4$
11: PCR Opt. Buffer D + 20 mM $(NH_4)_2SO_4$
12: FastStart HiFi Buffer + 3.5 U FastStart HiFi Enzyme Blend + 3.5 mM $MgCl_2$

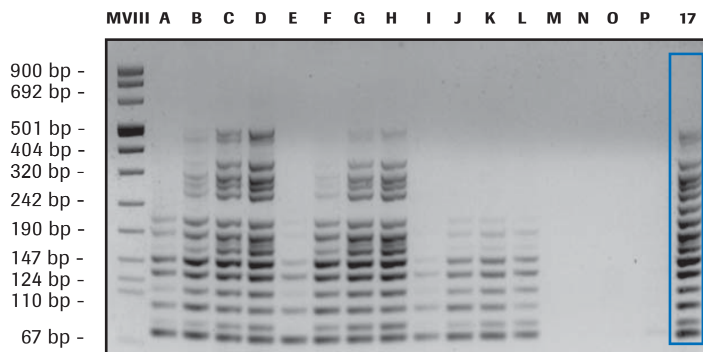
10.3 Primer Set 3: 18-plex (74 bp – 470 bp)

(Template: human genomic DNA)

Monoplex PCR:



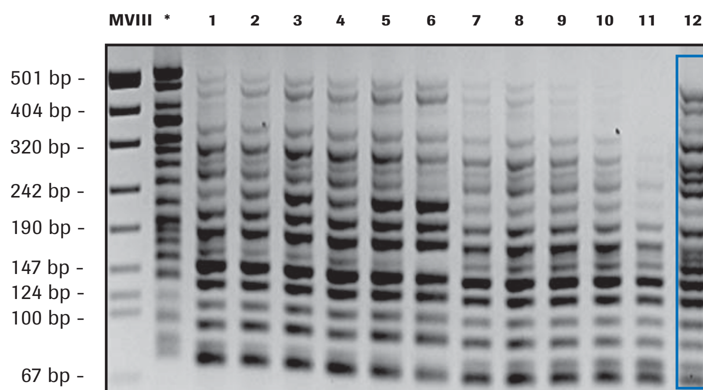
Multiplex PCR with Buffers from the PCR Optimization Kit:



Samples A-P: Buffers from the PCR Optimization Kit

Sample 17: FastStart High Fidelity Reaction Buffer as comparison

Multiplex PCR with the Best Performing PCR Buffer (FastStart High Fidelity Buffer) and Different Additives:



Additives:

1: FastStart HiFi Buffer; no additives
2: FastStart HiFi Buffer + 2% DMSO
3: FastStart HiFi Buffer + 4% DMSO
4: FastStart HiFi Buffer + 5% Glycerol
5: FastStart HiFi Buffer + 10% Glycerol
6: FastStart HiFi Buffer + 15% Glycerol
7: FastStart HiFi Buffer + 0.01% Gelatin
8: FastStart HiFi Buffer; + 0.05% Gelatin
9: FastStart HiFi Buffer + 5 mM $(NH_4)_2SO_4$
10: FastStart HiFi Buffer + 10 mM $(NH_4)_2SO_4$
11: FastStart HiFi Buffer + 20 mM $(NH_4)_2SO_4$
12: FastStart HiFi Buffer + 3.5 U FastStart HiFi Enzyme Blend + 3.5 mM $MgCl_2$

* Control mixture containing 3 µl of each monoplex product (from separate amplifications)

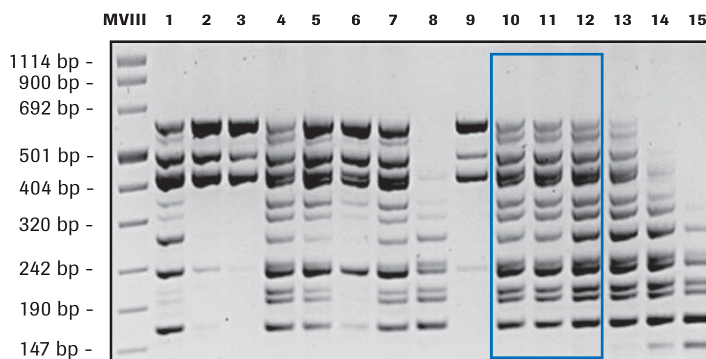
Further typical results

For each of the remaining examples, we show only the final optimization in the presence of different additives.

10.4 Primer Set 4: 13-plex (139 bp – 598 bp)

(Template: human genomic DNA)

Multiplex PCR with the Best Performing PCR buffer (D) and Different Additives:



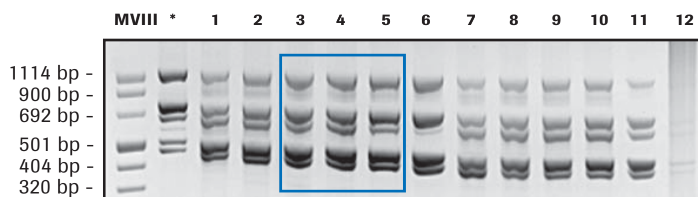
Additives:

1: FastStart HiFi Buffer; no additives
2: FastStart HiFi Buffer + 4% DMSO
3: FastStart HiFi Buffer + 6% DMSO
4: PCR Opt. Buffer D; no additives
5: PCR Opt. Buffer D + 4% DMSO
6: PCR Opt. Buffer D + 6% DMSO
7: PCR Opt. Buffer D + 5% Glycerol
8: PCR Opt. Buffer D + 10% Glycerol
9: PCR Opt. Buffer D + 15% Glycerol
10: PCR Opt. Buffer D + 0.01% Gelatin
11: PCR Opt. Buffer D + 0.05% Gelatin
12: PCR Opt. Buffer D + 5 mM $(NH_4)_2SO_4$
13: PCR Opt. Buffer D + 10 mM $(NH_4)_2SO_4$
14: PCR Opt. Buffer D + 20 mM $(NH_4)_2SO_4$
15: PCR Opt. Buffer D + 30 mM $(NH_4)_2SO_4$

10.5 Primer Set 5: 5-plex (450 bp – 1100 bp)

(Template: human genomic DNA)

Multiplex PCR with the Best Performing PCR Buffer (D) and Different Additives:



* Control mixture containing 3 µl of each monoplex product (from separate amplifications)

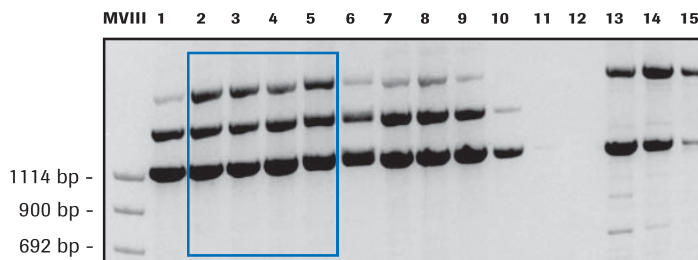
Additives:

1: PCR Opt. Buffer D; no additives
2: PCR Opt. Buffer D + 4% DMSO
3: PCR Opt. Buffer D + 6% DMSO
4: PCR Opt. Buffer D + 5% Glycerol
5: PCR Opt. Buffer D + 10% Glycerol
6: PCR Opt. Buffer D + 15% Glycerol
7: PCR Opt. Buffer D + 0.01% Gelatin
8: PCR Opt. Buffer D + 0.05% Gelatin
9: PCR Opt. Buffer D + 5 mM $(NH_4)_2SO_4$
10: PCR Opt. Buffer D + 10 mM $(NH_4)_2SO_4$
11: PCR Opt. Buffer D + 20 mM $(NH_4)_2SO_4$
12: FastStart HiFi Buffer + 3.5 U FastStart HiFi Enzyme Blend + 3.5 mM $MgCl_2$

10.6 Primer Set 6: 3-plex (1100 bp – 1800 bp)

(Template: human genomic DNA)

Multiplex PCR with the Best Performing PCR Buffer (FastStart HiFi) and Different Additives:



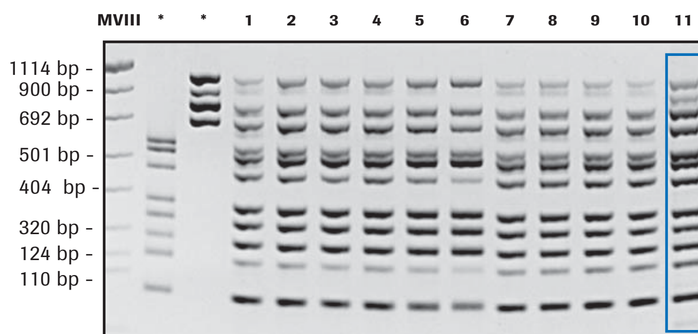
Additives:

1: FastStart HiFi Buffer; no additives
2: FastStart HiFi Buffer + 2% DMSO
3: FastStart HiFi Buffer + 4% DMSO
4: FastStart HiFi Buffer + 5% Glycerol
5: FastStart HiFi Buffer + 10% Glycerol
6: FastStart HiFi Buffer + 15% Glycerol
7: FastStart HiFi Buffer + 0.01% Gelatin
8: FastStart HiFi Buffer + 0.05% Gelatin
9: FastStart HiFi Buffer + 5 mM $(NH_4)_2SO_4$
10: FastStart HiFi Buffer + 10 mM $(NH_4)_2SO_4$
11: FastStart HiFi Buffer + 20 mM $(NH_4)_2SO_4$
12: FastStart HiFi Buffer + 30 mM $(NH_4)_2SO_4$
13: PCR Opt. Buffer D; no additives
14: PCR Opt. Buffer D + 4% DMSO
15: PCR Opt. Buffer D + 6% DMSO

10.7 Primer Set 7: 12-plex (93 bp – 450 bp)

(Template: human genomic DNA)

Multiplex PCR with the Best Performing PCR Buffer (FastStart HiFi) and Different Additives:



* Control mixtures containing 3 µl of each monoplex product (from separate amplifications)

Additives:

1: FastStart HiFi Buffer; no additives
2: FastStart HiFi Buffer + 2% DMSO
3: FastStart HiFi Buffer + 4% DMSO
4: FastStart HiFi Buffer + 5% Glycerol
5: FastStart HiFi Buffer + 10% Glycerol
6: FastStart HiFi Buffer + 15% Glycerol
7: FastStart HiFi Buffer + 0.01 % Gelatin
8: FastStart HiFi Buffer + 0.05 % Gelatin
9: FastStart HiFi Buffer + 5 mM $(NH_4)_2SO_4$
10: FastStart HiFi Buffer + 10 mM $(NH_4)_2SO_4$
11: FastStart HiFi Buffer + 3.5 U FastStart HiFi Enzyme Blend + 3.5 mM $MgCl_2$

11. Results from Outside Laboratories

11.1 5-plex PCR of Transgene Sequences in Plants

This example was kindly provided by Dr. Peterhaensel, Sonja Toepsch and Rashad Kebeish, RWTH Aachen, Germany.

Overview

The objective of this experiment was the amplification of five transgene sequences from *Arabidopsis thaliana*. The system developed in this experiment allows parallel detection of five transgenes from less than 6 ng genomic DNA template.
Product lengths range from 259 bp - 847 bp.

Primers and Products

Different PCR systems were established. Five forward primers and one reverse primer the latter was applied in five fold excess were used. The expected product lengths are:

System 1 (GCL)	259 bp
System 2 (TSR)	384 bp
System 3 (GLCF)	550 bp
System 4 (GLCE)	723 bp
System 5 (GLCD)	847 bp

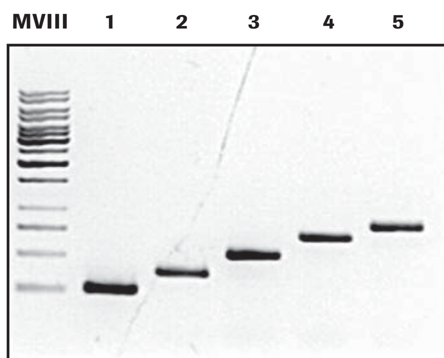
Thermocycling Profile

Cycles	Time	Temperature	Remark
1×	2 min	95°C	Activation
35×	20 sec	95°C	Denaturation
	20 sec	60°C	Annealing
	1 min	72°C	Elongation
1×	5 min	72°C	Elongation

Results

Monoplex PCR:

MVIII 1 2 3 4 5



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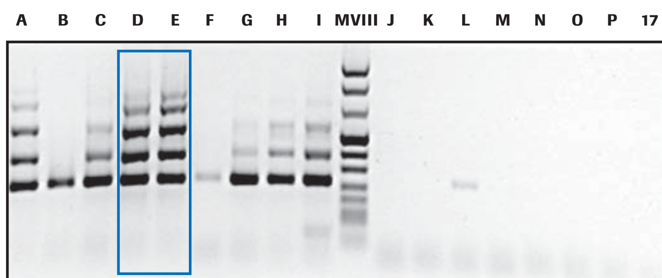
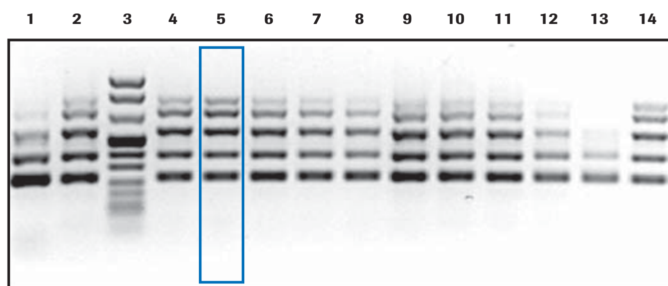
Multiplex PCR with Buffers from the PCR Optimization Kit:



Samples A-P : Buffers from the PCR Optimization Kit
Sample 17: FastStart High Fidelity Reaction Buffer as comparison

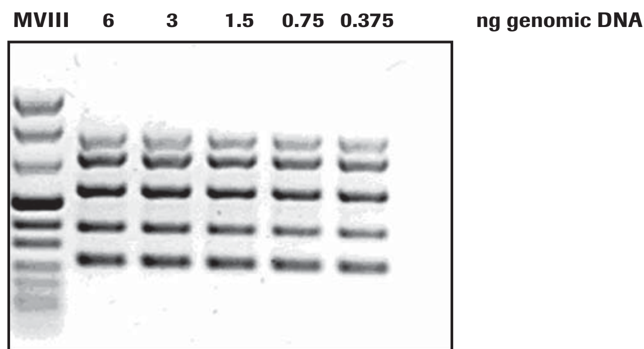
Multiplex PCR with the Best Performing PCR Buffer (D) and Different Additives:



Additives:

1: FastStart HiFi Buffer; no additives
2: PCR Opt. Buffer D; no additives
3: Roche Molecula r Weight Marker VIII
4: PCR Opt. Buffer D + 2% DMSO
5: PCR Opt. Buffer D + 4% DMSO
6: PCR Opt. Buffer D + 5% Glycerol
7: PCR Opt. Buffer D + 10% Glycerol
8: PCR Opt. Buffer D + 15% Glycerol
9: PCR Opt. Buffer D + 0.01 % Gelatin
10: PCR Opt. Buffer D + 0.05 % Gelatin
11: PCR Opt. Buffer D + 5 mM $(NH_4)_2SO_4$
12: PCR Opt. Buffer D + 10 mM $(NH_4)_2SO_4$
13: PCR Opt. Buffer D + 20 mM $(NH_4)_2SO_4$
14: PCR Opt. Buffer D + 3.5 U FastStart HiFi Enzyme Blend + 3.5 mM $MgCl_2$

Sensitivity:



11.2 4-plex PCR of Genomic Plant DNA

This example was kindly provided by Dr. Lahaye and Patrick Roemer, University Halle, Germany.

Overview

The objective of this experiment was the amplification of four different loci from pepper (*Caspasum annum* ECW-30R). The system developed in this experiment allows parallel amplification and detection of these four loci.

Product Lengths

192 bp Marker: B128-T7-02
275 bp Marker: B104-SP6-01
850 bp Marker: Y65-2
1300 bp Marker: 1464

Monoplex PCR with DNA Template

Set up each monoplex PCR by adding the components below:

Component	Each Reaction (µl)	Final Conc.
10× FastStart High Fidelity Reaction Buffer with $MgCl_2$	2	1× (1.8 mM $MgCl_2$)
dNTP Mix	2	200 µM
DNA Template	5	100 ng
Upstream Primer	3	30 ng
Downstream Primer	3	30 ng
FastStart High Fidelity Enzyme Blend	0.5	2.5 U
Water, PCR Grade	4.5	
Total Volume	**20**	

Thermocycling Profile

Cycles	Time	Temperature	Remark
1×	4 min	94°C	Activation
35×	10 sec 20 sec 35 – 90 sec[2]	94°C 60 – 63 °C[1] 72°C	Denaturation Annealing Elongation
1×	5 min	72°C	Elongation

[1] Annealing temperature depends on the melting temperature of the primers.
[2] Elongation time depends on the product size. Use 1 min for 1 kb product.

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11.2 4-plex PCR of Genomic Plant DNA continued

Gel Analysis

PCR products were separated for 1.3 hours at 100 V on a 1% agarose gel that contained 1µg/ml ethidium bromide.

Multiplex Optimization

Multiplex PCR with Buffers from the PCR Optimization Kit

Component	Single Reaction (µl)	Final Conc.
dNTP Mix	5	200 µM
DNA	5	100 ng
Upstream Primers	2.5	0.4 µM
Downstream Primers	2.5	0.4 µM
FastStart High Fidelity Enzyme Blend	0.5	2,5 U
Water, PCR grade	add to 45	
Total Volume	**45**	

Add 5 µl PCR Optimization Buffer A-P or FastStart High Fidelity Reaction Buffer with $MgCl_2$ or normal Taq-buffer as comparison.

Thermocycling Program

Cycles	Time	Temperature	Remark
1×	4 min	94°C	Activation
35×	10 sec 20 sec 35 – 90 sec[2]	94°C 53 °C[1] 72°C	Denaturation Annealing Elongation
1×	5 min	72°C	Elongation

[1] Annealing temperature depends on the melting temperature of the primers.

[2] Elongation time depends on the product size. Use 1 min for 1 kb product.

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Gel Analysis

PCR products were separated for 1.5 hours at 100 V on a 1% agarose gel that contained 1µg/ml ethidium bromide.

Final Multiplex Optimization with Best Performing Buffer

Multiplex PCR with the best performing PCR buffer and different additives

Component	Single Reaction (µl)	Final Conc.
Best performing Buffer	5	
dNTP Mix	5	200 µM
DNA	5	100 ng
Upstream Primers	2.5	0.4 µM
Downstream Primers	2.5	0.4 µM
Additives one of: DMSO (100%) Glycerol (50%) Gelantin (1%) $(NH_4)_2SO_4$ (500mM)	 2 or 3 2.5 or 3.75 0.5 or 2.5 0.5 or 1 or 2 or 3	 4 or 6% 10 or 15% 0.01 or 0.05% 5 or 10 or 20 or 30 mM
FastStart High Fidelity Enzyme Blend	0.5	2.5 U
Water, PCR grade	add to 50	
Total Volume	**50**	

Thermocycling Profile

Cycles	Time	Temperature	Remark
1×	4 min	94°C	Activation
35×	10 sec 20 sec 90 sec[2]	94°C 53°C[1] 72°C	Denaturation Annealing Elongation
1×	5 min	72°C	Elongation

[1] Annealing temperature depends on the melting temperature of the primer
[2] Elongation time depends on the product size (1 min for 1 kb)

Gel Analysis

PCR products were separated for 1.5 hour at 100 V on a 1% agarose gel that contained 1µg/ml ethidium bromide.

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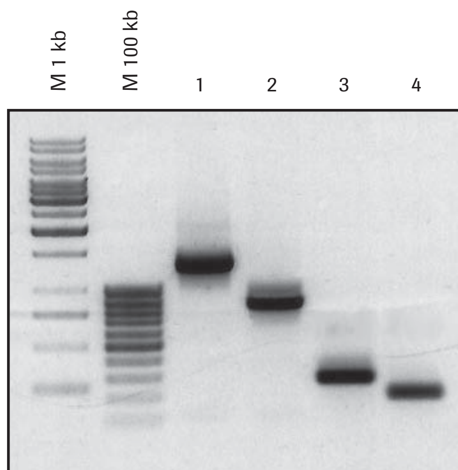
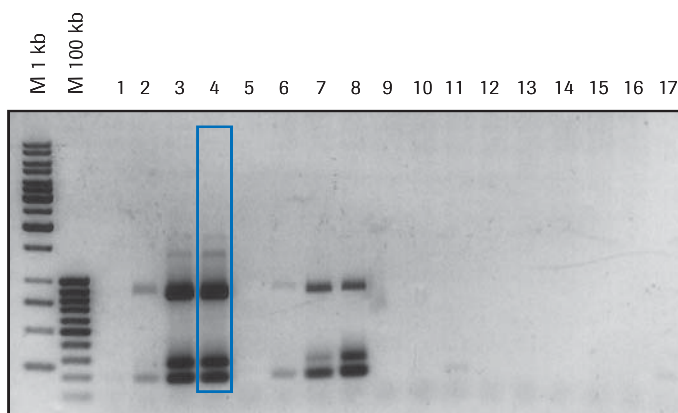
Results

Monoplex PCR:
(Template: genomic DNA *Capsicum annuum* ECW-30R)



1: marker 1464; 2: marker Y65-2; 3: marker B104-Sp6-01; 4: marker B128-T7-02.

Multiplex PCR to Determine the Best Performing Buffer from the PCR Optimization Kit:
(Template: genomic DNA *Capsicum annuum* ECW-30R; Polymerase: FastStart High Fidelity Enzyme Blend)



Samples 1-16: Buffers A-P from the PCR Optimization Kit
Sample 17: Taq-PCR-Buffer as comparison

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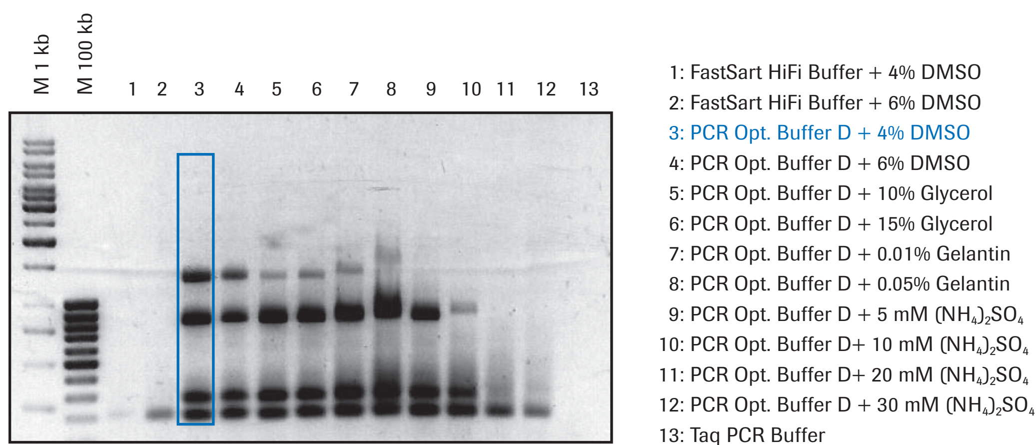
11.2 4-plex PCR of Genomic Plant DNA continued

Multiplex PCR with the Best Performing Buffer (D) and Different Additives:
(Template: genomic DNA *Capsicum annuum* ECW-30R; Polymerase:
FastStart High Fidelity Enzyme Blend

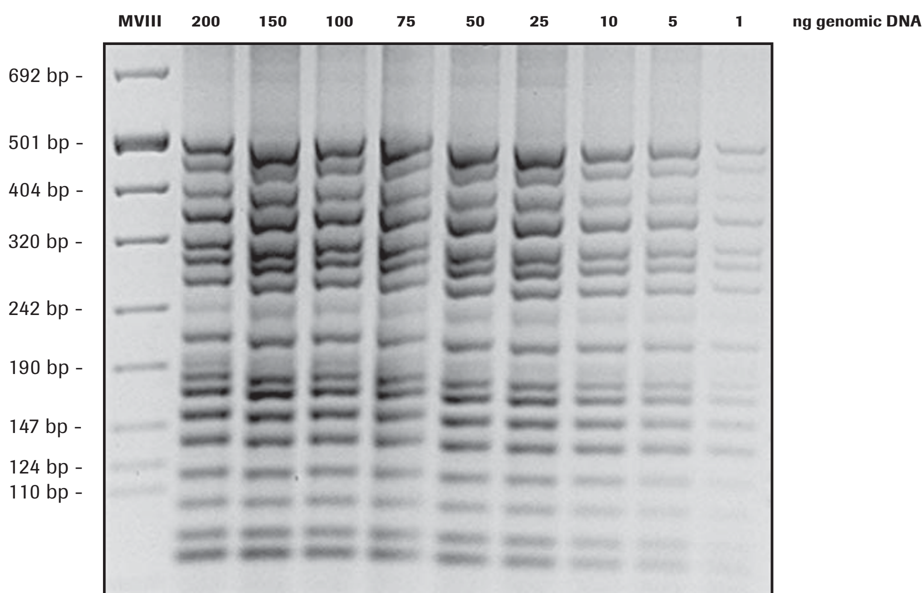


12. Sensitivity

12.1 Primer Set 3: 18-plex (74 bp – 470 bp)

(Template: human genomic DNA)

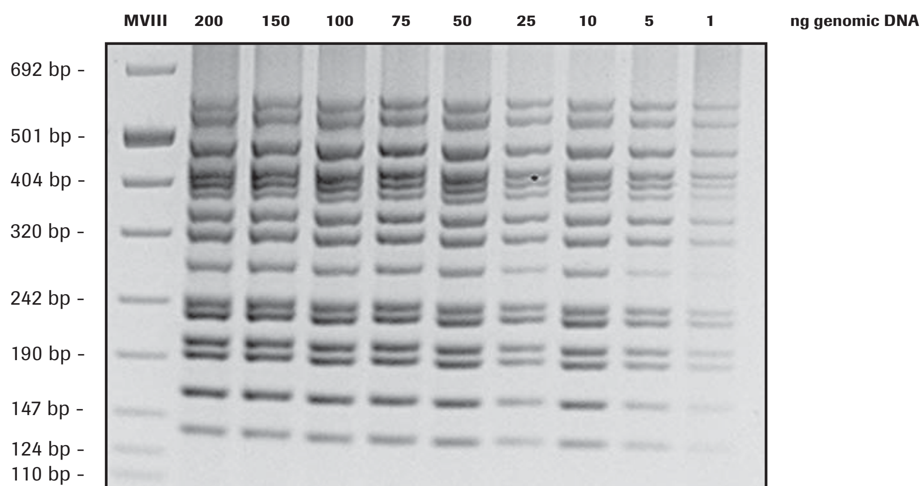
Sensitivity Test using Optimal Conditions (FastStart High Fidelity PCR Buffer + 3.5 U FastStart High Fidelity Enzyme Blend + 3.5 mM $MgCl_2$)



12.2 Primer Set 4: 13-plex (139 bp – 598 bp)

(Template: human genomic DNA)

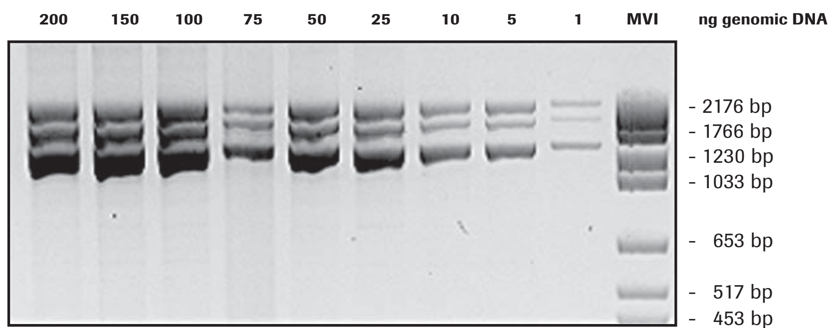
Sensitivity Test using Optimal Conditions (PCR Optimization Buffer D + 0.01% Gelatin; + 5 mM $(NH_4)_2SO_4$ + 3.5 U FastStart High Fidelity Enzyme Blend + 3.5 mM $MgCl_2$)



12.3 Primer Set 6: 3-plex (1100 bp – 1800 bp)

(Template: human genomic DNA)

Sensitivity Test using Optimal Conditions (FastStart High Fidelity Buffer + 4% DMSO)



13. Troubleshooting

Problem	Possible Cause	Recommendation
PCR doesn't work with a cDNA template	Hexamer primers can interfere with subsequent PCR	Use anchored-oligo$(dT)_{18}$ primer instead of hexamer primers.
	RNA is degraded	Check quality of RNA.
Nonspecific product bands	Nonspecific binding of primers	Redesign primers to get more specific binding to target and/or to allow a higher annealing temperature.
	Formation of primer-dimers	1) Increase annealing temperature or redesign primers (*e.g.*, with the LightCycler® Probe Design Software 2.0). 2) Check primer sets for cross complementarities.
	Priming by shorter fragments that are impurities in the primer preparation	Use HPLC-purified primers.
Difficult amplicons are used and not all PCR products are amplified	Activation time of FastStart High Fidelity Enzyme Blend is too short.	Increase the activation time up to 10 min.
	Primer design is not optimal.	Design alternative primers.
	Primer concentration is not optimal.	Titrate primer.
	Too little FastStart High Fidelity Enzyme Blend was added to the reaction.	Increase the amount of enzyme and $MgCl_2$ to get higher specificity and yield (*e.g.*, 3.5 U FastStart High Fidelity Enzyme Blend + 3.5 mM $MgCl_2$).
Product bands can not be individually distinguished	Amplicon sizes are too close together or agarose gel is not the correct concentration.	1) Redesign primer sets. 2) Products with differences from 20-50 bp can be separated on a 3–4% agarose gel. Products >1000 bp can be separated on a 1–2% agarose gel. 3) As positive control load a mixture containing 3 µl of each monoplex product.
Faint bands visible	Too little of the PCR product was loaded on the gel.	Load more PCR product on each gel lane.
In the presence of additives, multiplex PCR does not give a satisfactory result	Too little FastStart High Fidelity Enzyme Blend was added to the reactions.	Increase the amount of enzyme and $MgCl_2$ to get higher specificity and yield (*e.g.*, 3.5 U FastStart High Fidelity Enzyme Blend + 3.5 mM $MgCl_2$).

14. Summary

This Technical Note shows that multiplex PCR can easily be optimized in three simple, straightforward steps, even when the primer set is very complex (up to 18 different PCR products). The three optimization steps[1] are:

1. Monoplex PCR of all fragments individually[2]
2. Initial optimization of multiplex PCR with buffers from the Roche Applied Science PCR Optimization Kit
3. Final optimization of multiplex PCR with the best performing PCR buffer and different additives

(All these steps can use either DNA or cDNA as target.)

[1] These steps use the FastStart High Fidelity Enzyme Blend and components of the PCR Optimization Kit, which are both available from Roche Applied Science.

[2] Starting with amplification of individual primer sets (monoplex) is important, since this step establishes that each single primer pair produces the expected PCR product.

15. References

1. Pomp, D. & Medrano, J. F. (1991) *BioTechniques* **10** (1), 58-59.
2. Filichkin, S. A. & Gelvin, S. B. (1992) *BioTechniques* **12** (6), 828-830.
3. Masoud, S. A. Johnson, L.B. & White, F. F. (1992) *PCR Methods and Applications* **2**, 89-90.
4. Rolfs, A. et al. (1992) PCR: *Clinical Diagnostics and Research Springer Laboratory*, 51-67.
5. Landre, P.A., Gelfand, D.H. and Watson, R. M. (1995) PCR Strategies (Academic Press; ed. Innis, M.A. Gelfand, D.H., Sninsky, J. J.) 3-16.

16. Acknowledgement

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We thank Dr. Suzanne Cheng, Roche Molecular Diagnostics, USA, for kindly providing the 12-plex example with primer set 7 and Dr. Ruediger Rueger, Roche Pharmaceuticals, for kindly providing the 9-plex example with primer set 2.

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Roche Diagnostics GmbH
Roche Applied Science
68298 Mannheim
Germany

www.roche-applied-science.com

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