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*Genome Res.* 1995 4: 376-379

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# Single-tube Nested PCR with Room-temperature-stable Reagents

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The application of nested PCR in medical diagnostics implies that the same type of reaction is performed repetitively in the same laboratory over long periods of time, such as in the longitudinal monitoring of immunosuppressed patients for virus infections,<sup>(1)</sup> or in the monitoring of donated blood for transmissible infectious agents.<sup>(2-5)</sup> To ensure inter-assay conformity, it is desirable to use preformulated PCR reagent mixes that may be prepared in advance in batches and may be kept on stock, instead of preparing a fresh reagent mix for every PCR assay.

False-positive reactions in PCR may arise from selfcontamination with amplified DNA ("carryover"). This problem is aggravated in nested PCR, because amplified DNA (first-round PCR product) has to be transferred manually into the tubes for the second-round PCR. To avoid this additional risk of carryover, it is desirable to perform first-round and second-round PCR in one reaction tube without opening it during the entire process.<sup>(6)</sup>

We modified nested PCR so that it is performed in a single tube, using preformulated PCR reaction mixes embedded in a trehalose matrix.<sup>(7-10)</sup> These preformulated mixes may be stored for  $\geq 6$  months at ambient temperature. We compared the performance of this single-tube nested PCR with conventional nested PCR. Blood samples from different patients with virus infections were assayed, including infections with human immunodeficiency virus type 1 (HIV 1), hepatitis C virus (HCV), and cytomegalovirus (CMV).

## MATERIALS AND METHODS

### Thermocycler, PCR Tubes, and Enzymes

The thermocycler was a Varius V 80 (five blocks, each holding 16 0.2-ml PCR tubes) (Landgraf, Hannover, Germany), with heating/cooling rates of 2.5°C/sec.

The PCR tubes were 0.2 ml (Perkin Elmer, Vaterstetten, Germany).

Enzymes: DNA polymerases GoldStar (Eurogentech, Seraing, Belgium), AmpliTaq (Perkin-Elmer, Vaterstetten, Germany), and Moloney murine leukemia virus reverse transcriptase (Mo-MLV RT, Pharmacia, Freiburg, Germany).

### DNA Molecular Weight Marker

The marker was a *Hpa*II digest of pUCBM21, containing fragments of 1114, 900, 692, 501/489, 404, 320, 242, 190, 147, 124, 110, 62, 37, 34, 26, and 19 bp (Standard VIII, Boehringer Mannheim, Germany).

### Control DNA for Determination of Lower Detection Limit

For CMV, plasmid clone pRR47 containing a 6.7-kb viral DNA fragment of the CMV major immediate early gene region was used.<sup>(16)</sup> For HCV and HIV 1, first-round PCR products were purified by preparative agarose gel electrophoresis.

### Positive Control DNA and RNA

Nucleic acid extracts from peripheral blood of AIDS patients (HIV 1 proviral DNA), heart transplant recipients with CMV reactivation (CMV DNA), and patients with hepatitis C (HCV RNA) were obtained from routine diagnostic samples.

### Other Reagents

Deoxynucleoside triphosphates (Pharmacia, Freiburg, Germany), salts and buffers (Merck, Darmstadt, Germany), and mineral oil (Perkin-Elmer, Vaterstetten, Germany) were used.

### Blood Samples

For HIV 1 and CMV detection, anticoagulated venous blood (1.6 mg K-EDTA/ml) was collected with 2.7 ml standard systems for hematological purposes (Sarstedt, Nümbrecht, Germany). Thirty-eight samples from anti-HIV positive patients were assayed with HIV 1 DNA-specific PCR, and 56 samples from immunosuppressed heart transplant recipients under continuous cyclosporin A therapy were used for CMV-specific PCR. One hundred twenty serum samples from anti-HCV-positive patients with hepatopathies were assayed with HCV-specific RT-PCR.

### Nucleic Acid Extraction

Nucleic acids were extracted with QIAamp spin columns (Diagen GmbH, Hilden, Germany) according to the manufacturer's instructions: Two hundred

**TABLE 1** Oligonucleotide Primers in Preformulated PCR Mixes

Virus	Primer	Sequence (5' → 3')	Literature
First-round PCR product 434 bp, nested PCR product 338 bp			
HIV	outer 5'	AA TGT CAG CAC AGT ACA ATG TAC	13
	outer 3'	CA GTA GAA AAA TTC CCC TCC ACA ATT	
	nested 5'	CT GCT GTT AAA TGG CAG TCT AGC	
	nested 3'	T TTC TGG GTC CCC TCC TGA GGA	
First-round PCR product 340 bp, nested PCR product 286 bp			
HCV	NCR 1 (outer 5')	GG CGA CAC TCC ACC ATA GAT	14
	NCR 2 (outer 3')	GT GCA CGG TCT ACG AGA CCT	
	NCR 3 (nested 5')	C CAC CAT AGA TCT CTC CCC TGT	
	NCR 4 (nested 3')	CA CTC TCG AGC ACC CTA TCA GGC AGT	
First-round PCR product 434 bp, nested PCR product 110 bp			
CMV	MIEA 4 (outer 5')	C CAA GCG GCC TCT GAT AAC CAA GCC	15,16
	MIEA 5 (outer 3')	C AGC ACC ATC CTC CTC TTC CTC TGG	
	MIEA 6 (nested 5')	A GTG TGG ATG ACC TAC GGG CCA TCG	
	MIEA 7 (nested 3')	G GTG ACA CCA GAG AAT CAG AGG AGC	

microliters of guanidinium salt solution and 25  $\mu$ l of proteinase K stock solution (both supplied by the manufacturer) were mixed with 200  $\mu$ l of anticoagulated whole blood and incubated at 70°C for 10 min. Isopropanol (210  $\mu$ l) was added, and the mixture was vortexed briefly, applied to a QIAamp spin column, and centrifuged at 6000g for 1 min. Twice, 500  $\mu$ l of wash buffer was added and centrifuged at 6000g for 1 min. The nucleic acids were eluted with 100  $\mu$ l of hot water (70°C, 1 min centrifugation at 6000g). For RNA, diethyl pyrocarbonate-treated water was used. Nucleic acid eluate (5  $\mu$ l) was used for PCR or reverse transcription and brought to 20  $\mu$ l with water and appropriate buffer.

### Reverse Transcription of HCV RNA

Five microliters of RNA extract was reverse transcribed with Mo-MLV RT in a 20- $\mu$ l reaction,<sup>(12)</sup> according to the manufacturer's instructions. Five microliters of the cDNA solution was used for PCR.

### Single-tube Nested PCR

Preformulated PCR mix (without buffer) contained trehalose, the four dNTPs, DNA polymerase (either GoldStar or AmpliTaq), and primers. The components are listed in Tables 1 and 2. For a 20- $\mu$ l reaction, 3.3  $\mu$ l of preformulated first-round mix and 3.3  $\mu$ l of second-round mix were required (a 50- $\mu$ l reaction requires 8.3  $\mu$ l of each mix).

### Preparation of PCR tubes

Preformulated PCR mix for the first round was pipetted into the bottom of the tube and second-round preformulated PCR mix into the inside of the cap. The open tubes were then kept in an incubator at 35–40°C for 2–3 hr until the reagents had dried. Once dried, the tubes could be stored at room temperature in a dry place (desiccator or sealed plastic bag) for several months.

### PCR

The aqueous solution of template DNA (cDNA in case of HCV-specific PCR) was adjusted to the salt and buffer concentrations recommended by the manufacturers of the DNA polymerases by addi-

tion of 10 $\times$  PCR buffer. The PCR buffer (1 $\times$ ) for GoldStar polymerase was 75 mM Tris-Cl (pH 9.0), 20 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.5 mM MgCl<sub>2</sub>, and 0.01% Tween 20; the PCR buffer (1 $\times$ ) for AmpliTaq Polymerase was 20 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, and 0.01% gelatin. Upon addition of the template DNA into the PCR tube, the reagents embedded in the trehalose matrix dissolved without agitation. The aqueous layer was overlaid with 40  $\mu$ l of liquid paraffin. Cycle conditions are listed in Table 3. After first-round PCR, the closed tube was inverted to dissolve the second-round reagents inside the cap, briefly centrifuged, and returned into the thermocycler for second-round PCR. Trace amounts of xylene cyanol or bromophenol in the second reagent mix helped to visualize its complete dissolution.

The optimal concentration of first-round primers was determined experimentally. These primers had to be employed at reduced concentrations (Table 2) to ensure their complete consumption during first-round PCR and to suppress formation of unwanted PCR products arising from combinations of first- and second-round primers.

### Conventional Nested PCR

Conventional nested PCR was performed in 20- $\mu$ l first-round reactions. The reaction mixes had the same composition as described for single-tube nested PCR with the exception that no trehalose was present and the first-round primers were 1  $\mu$ M in all reactions. First-round product (2  $\mu$ l) was transferred into

**TABLE 2** Components of PCR Mix and Preformulated Reagent for 20- $\mu$ l Reactions

Component	Quantity	Concentration in preformulated mix <sup>a</sup>	Concentration in the PCR
Outer primers			
(HCV)	0.8 pmoles	0.24 $\mu$ M	0.04 $\mu$ M
(CMV)	2.0 pmoles	0.61 $\mu$ M	0.1 $\mu$ M
(HIV)	2.9 pmoles	0.88 $\mu$ M	0.14 $\mu$ M
Nested primers (all)	20 pmoles	6 $\mu$ M	1 $\mu$ M
dATP, dCTP, dGTP, dTTP	5 nmoles	1.5 mM	250 $\mu$ M
DNA polymerase (Gold Star)	0.35 units	0.11 U/ $\mu$ l	0.0175 U/ $\mu$ l
Trehalose	0.44 mg	13.3% (wt/vol)	2.2% (wt/vol)
MgCl <sub>2</sub>		N.I.	2.5 mM
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>		N.I.	20 mM
Tris-chloride (pH 9.0)		N.I.	75 mM
Tween 20		N.I.	0.01%

<sup>a</sup>(N.I.) Not included.

**TABLE 3** PCR Parameters

Virus	Cycles (no. per round)	Cycle profile (first and second rounds)
HIV1	first: 40 second: 40	30 sec at 94°C, 45 sec at 55°C, 50 sec at 72°C
HCV	first: 28 second: 28	30 sec at 94°C, 40 sec at 50°C, 30 sec at 72°C
CMV	first: 30 second: 30	25 sec at 94°C, 35 sec at 57°C, 35 sec at 72°C

The same number of cycles and the same temperature profile were used both for single-tube nested PCR and conventional nested PCR.

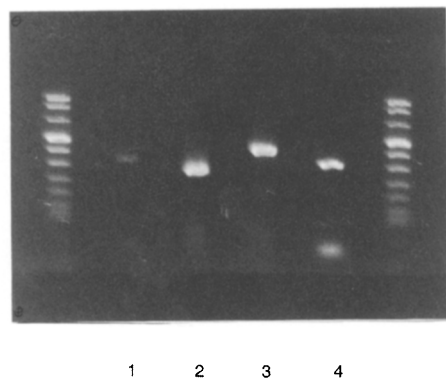
18  $\mu$ l of second-round PCR mix. The compositions of the PCR mixes are given in (Table 2).

### Analysis of Amplified DNA

PCR products were separated on 3% agarose gels and visualized by ethidium bromide staining. Chain lengths were determined by coelectrophoresis of a DNA molecular weight marker.

### RESULTS AND DISCUSSION

PCR has become an important tool of medical diagnostics because of its high



**FIGURE 1** Agarose gel electrophoresis of PCR products from single tube nested PCR. *Left* and *right*: DNA chain-length marker. Fragment lengths are 1194, 900, 692, double band 501/489, 404, 320, 242, 147, 124, and 110 bp (faintly visible). (Lane 1) HCV-specific conventional nested PCR, first-round product at 340 bp (6  $\mu$ l applied). (lane 2) HCV-specific single-tube nested PCR. Only second-round product is visible at 286 bp; no first round product is visible at 340 bp (6  $\mu$ l applied). (lane 3) HIV 1-specific conventional nested PCR. Reamplified first-round product, at 434 bp. (Lane 4) HIV 1-specific single-tube nested PCR. Only second-round product is visible at 338 bp; no first-round product is visible at 434 bp (6  $\mu$ l applied). Primer dimer in the front.

sensitivity; however, this method is not yet standardized. Many modifications of standard protocols exist, and interassay consistency is not yet acceptable.<sup>(17)</sup> Quantitative PCR, in particular, requires uniform, stable reagents. To achieve the standards of clinical chemistry for PCR (precisely defined reaction conditions, reproducibility of the method in different laboratories, and within one laboratory over time) it is desirable to use stable, preformulated reagents. We compared the detection of HIV 1 proviral DNA, CMV DNA, and HCV cDNA by single-tube nested PCR and by conventional nested PCR.

### Primer Concentrations

The optimal ratio of first- to second-round primer concentrations in a single-tube nested PCR depends on the type of template DNA. This ratio was determined experimentally. Using a positive control DNA, the primer ratio (first-

round primer/second-round primer) was lowered stepwise until PCR products derived from combinations of first- and second-round primers disappeared in the agarose gel (Fig. 1). Optimal primer ratios varied between 1:7 (first-round/second-round) for the amplification of HIV 1 provirus DNA, 1:10 for CMV DNA, and 1:25 for HCV cDNA (Table 1).

### Sensitivity of Single-tube Nested PCR

Serial dilutions of control DNA (purified first-round PCR products obtained from HCV cDNA and HIV 1 proviral DNA, and *Eco*RI-linearized pRR47 plasmid DNA containing CMV DNA) were amplified from concentrations in the range of 0.1–100 copies. The detection limits were between 1 and 10 copies (Table 4). Single-tube nested PCR detected viral nucleic acid in all samples that were positive in conventional nested PCR and in some samples that were negative in conventional nested PCR. (Table 5).

In conclusion, nested PCR in a single tube with preformulated, room temperature stable reaction mixes offers several advantages over the conventional procedure: (1) reagents can be prepared in advance in large batches, providing reproducible performance, and thus interassay (and interlaboratory) conformity; (2) after initial amplification, there is no tube-to-tube transfer. One major source of selfcontamination with amplified DNA is eliminated, and (3) nucleic acid standards can be added to the reagents to permit quantitative PCR.

**TABLE 4** Lower Detection Limits of CMV-, HCV-, and HIV 1-specific single-tube Nested PCR

Control DNA	Copy number of template DNA	Positive reactions in 10 PCR assays (no.)
CMV (plasmid pRR47)	100	10
	10	8
	1	1
	0.1 <sup>a</sup>	0
HCV (purified first-round PCR product)	100	10
	10	10
	1	4
	0.1 <sup>a</sup>	0
HIV 1 (purified first-round PCR product)	100	10
	10	10
	1	6
	0.1 <sup>a</sup>	0

<sup>a</sup>Nominally 0.1.

**TABLE 5** Comparison of Single-tube Nested PCR Technique and Conventional Nested PCR

Virus	Samples	Single-tube PCR (positive)	Conventional PCR (positive)
HIV <sup>a</sup>	38	34	34
HCV <sup>b</sup>	120	59	55
CMV <sup>c</sup>	56	23	21

<sup>a</sup>Anti-HIV positive patients. Nucleic acids were extracted from anticoagulated whole blood (EDTA blood).

<sup>b</sup>Anti-HCV positive patients with hepatopathies. Nucleic acids were extracted from serum.

<sup>c</sup>Immunosuppressed heart transplant patients. Nucleic acids were extracted from anticoagulated whole blood (EDTA blood).

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Received September 13, 1994; accepted in revised form February 27, 1995.