

FINNZYMES

Phusion® Blood

Direct PCR Kit

Product codes:

F-547S, 100 reactions 20 µl each

F-547L, 500 reactions 20 µl each

Stable for one year from the packaging date. Store at –20°C.

1. Introduction

Finnzymes' Phusion® Blood Direct PCR Kit is designed to perform PCR directly from whole blood with no prior DNA extraction or sample preparation. Blood stored at +4°C or frozen, and preserved with EDTA, citrate or heparin are all suitable for this kit, as is blood dried onto commercially available cards such as Whatman 903® and FTA® cards. Both genomic and exogenous target DNA can be amplified.

The Phusion Blood Direct PCR Kit employs a modified Phusion® Hot Start High-Fidelity DNA Polymerase that exhibits extremely high resistance to inhibitors found in blood. The kit has been optimized to give excellent results from mammalian blood with amplicons up to 7.5 kb. The recommended blood concentration is 1–20 %, although robust amplification with up to 40 % blood can often be achieved with some optimization. This kit is recommended for end-point PCR protocols.

The Phusion Blood Direct PCR Kit includes a pair of universal control primers that are compatible with a number of mammalian and other vertebrate species (see section 7).

Important notes

- The recommended starting amount is 5 % blood added directly to the reaction without further modification.
- Use 98°C for denaturation.
- Anneal at $T_m+3^\circ\text{C}$ (> 20nt) or use 2-step protocol (see 5.2).
- For extension, use 15 s for amplicons ≤ 1 kb or 30 s/kb for amplicons >1 kb.
- After PCR, spin the reactions at 1 000 x g (about 4 000 rpm) for 1–3 minutes to pellet debris from blood.
- For blood concentrations greater than 10 %, optimization of MgCl₂ concentration may be required (see 4.3).
- Note: Phusion DNA Polymerases produce blunt end PCR products.

2. Package information

| Component | F-547S | F-547L |
|--|--------|----------|
| Phusion® Blood DNA Polymerase | 40 µl | 200 µl |
| 2x Phusion® Blood PCR Buffer (includes dNTPs and MgCl ₂) | 1 ml | 5 x 1 ml |
| Universal control primer mix | 40 µl | 40 µl |
| 50 mM EDTA (pH 8.0) | 0.5 ml | 0.5 ml |
| 50 mM MgCl ₂ solution | 1.5 ml | 1.5 ml |
| 100 % DMSO | 0.5 ml | 0.5 ml |

Material safety data sheet (MSDS) is available at www.finnzymes.com.

3. Guidelines for PCR

Carefully mix and spin down all tubes before opening to ensure homogeneity and improve recovery. The PCR setup can be performed at room temperature.

Table 1. Pipetting instructions.

| Components | 20 µl reaction | 50 µl reaction | Final conc. |
|---|----------------|----------------|---------------|
| H ₂ O | add to 20 µl | add to 50 µl | |
| 2x Phusion® Blood PCR Buffer | 10 µl | 25 µl | 1x |
| primer A | x µl | x µl | 0.5 µM |
| primer B | x µl | x µl | 0.5 µM |
| Phusion® Blood DNA Polymerase | 0.4 µl | 1 µl | |
| Whole blood | 1 µl | 2.5 µl | (see 4.1) |
| Optional components for reaction optimization | | | |
| 50 mM MgCl ₂ | 0.6 µl | 1.5 µl | (See 4.3) |
| 50 mM EDTA | 0.5–1.0 µl | 1.25–2.5 µl | (See 4.3) |
| DMSO | 1.0 µl | 2.5 µl | 5 % (See 4.4) |

Table 2. Recommended cycling protocol.

| Cycle step | 2-step protocol | | 3-step protocol | | Cycles |
|--------------------------|-----------------|---------------|-----------------|---------------|--------|
| | Temp. | Time | Temp. | Time | |
| Lysis of cells (see 5.1) | 98°C | 5 min | 98°C | 5 min | 1 |
| Denaturation | 98°C | 1 s | 98°C | 1 s | 35–40 |
| Annealing (see 5.2) | - | - | X°C | 5 s | |
| Extension (see 5.3) | 72°C | 15–30 s/kb | 72°C | 15–30 s/kb | |
| Final extension | 72°C 4°C | 1 min hold | 72°C 4°C | 1 min hold | 1 |

4. Notes about reaction components

4.1 Blood sample

With the Phusion Blood Direct PCR Kit, it is possible to use a wide range of blood concentrations in the reaction (from 1 % to 20 %, or in some cases even up to 40 %). The recommended starting point is 5 %.

In general, if higher blood percentage (>10 %) is used, higher reaction volume (up to 50 µl) is recommended, and some optimization may be required (in particular the MgCl₂ concentration may need to be increased up to 4.5 mM final

concentration). For small amplicons, whole blood up to 40 % can be used (please note that with mouse blood 20 % should not be exceeded).

If blood dried onto a Whatman card is used, a 1 mm punch can be added directly to the reaction without pretreatment. The optimal reaction volume varies depending on the card type used:

- Whatman 903 Card: 10–50 μ l
- Whatman FTA Elute Card: 25–50 μ l
- Whatman FTA Gene Card: 50 μ l

If larger punches or smaller volumes are desired for the FTA cards, the punches may need to be pretreated by washing with 20 μ l H₂O at 50°C for 3 minutes. After washing, the water can simply be removed and the PCR mix added directly to the rinsed punch.

Non-mammalian blood

The Phusion Blood Direct PCR Kit has been successfully tested with a variety of mammalian species. In addition, good results have been obtained with several different bird species. For birds and other species with nucleated blood cells, it may be necessary to reduce the amount of blood in the PCR reaction.

Blood storage

For long term storage, it is recommended to store blood at –20°C or dried on Whatman FTA/903 cards. For short term storage (less than 3 months), blood can be stored at +4°C. EDTA, sodium citrate and heparin all work well with the Phusion Blood Direct PCR Kit.

4.2 Enzyme

Phusion Blood DNA Polymerase is a proofreading polymerase that contains a unique processivity-enhancing domain, making this polymerase accurate and rapid. Phusion Blood DNA Polymerase is a hot start polymerase utilizing a reversibly binding Affibody® protein.^{1,2}

Phusion Blood DNA Polymerase possesses 5'→3' DNA polymerase activity and 3'→5' exonuclease activity. The error rate of Phusion Blood DNA Polymerase is approximately 25-fold lower than that of *Thermus aquaticus* DNA polymerase when determined with a modified *lacI*-based method.³

The recommended amount of Phusion Blood DNA Polymerase is optimized to work with most amplicons. If high blood volume is used, increasing the enzyme amount may improve the results. The volume per reaction can be increased up to 0.6 μ l per 20 μ l reaction, or up to 1.5 μ l per 50 μ l reaction.

When cloning fragments amplified with Phusion Blood DNA Polymerase, blunt end cloning is recommended. If TA cloning is required, it can be performed by adding A overhangs to the blunt PCR product with DyNAzyme™ II DNA Polymerase (F-501), for example. However, before adding the overhangs it is very important to remove all Phusion Blood DNA Polymerase by purifying the PCR product carefully. Any remaining Phusion Blood DNA Polymerase will degrade the A overhangs, creating blunt ends again. A detailed protocol for TA cloning of PCR fragments amplified with any of the Phusion DNA Polymerases can be found on Finnzymes' website (www.finnzymes.com).

4.3 MgCl₂ concentration

The 2x Phusion Blood PCR buffer included in the kit provides 3.0 mM MgCl₂ in the final reaction. Extensive testing has shown 3.0 mM MgCl₂ to be optimal for most PCR reactions using whole blood. There is a separate tube of 50 mM MgCl₂ (F-510MG) included in the kit for adjusting the MgCl₂ concentration (up to 4.5 mM if necessary) for reactions containing very high amounts of blood. It should be noted, though, that excess MgCl₂ may result in spurious PCR products.

If unspecific products are created with the default reaction buffer (providing 3.0 mM MgCl₂), the effective MgCl₂ concentration can be decreased by adding the chelating agent EDTA, which is included in the kit. Typically, adding 0.5 to 1.0 μ l of 50 mM EDTA to a 20 μ l reaction is sufficient to eliminate non-specific products. Note that the optimal conditions will depend on the primers, the percentage of blood in the reaction and/or the type of card used, since anticoagulants and other chemicals impregnating the cards can alter the available Mg²⁺ concentration.

4.4 DMSO

DMSO has been found to improve PCR results for some amplicons (particularly GC-rich templates). With these amplicons it is recommended to add 1–5 % DMSO to the reaction. Note that if high DMSO concentration is used, the annealing temperature must be decreased, as DMSO alters the melting point of the primers. It has been reported that 10 % DMSO decreases the annealing temperature by 5.5–6.0°C.⁴

Other PCR additives such as formamide, glycerol, and betaine are also compatible with Phusion Blood DNA Polymerase.

5. Notes about cycling conditions

5.1 Lysis of cells and DNA denaturation

The initial 5-minute incubation step at 98°C allows the lysis of leukocytes, making genomic DNA available for PCR.

The DNA denaturation step can be very short. It is sufficient that the reaction mixture reaches the required 98°C.

5.2 Primer annealing

Note that the optimal annealing temperature for Phusion Blood DNA Polymerase may differ significantly from that of *Taq*-based polymerases. Always use the T_m calculator and instructions on Finnzymes' website (www.finnzymes.com) to determine the T_m values of primers and optimal annealing temperature. As a basic rule, for primers >20 nt, anneal for 5 seconds at a T_m +3°C of the lower T_m primer. For primers ≤ 20 nt, use an annealing temperature equal to the T_m of the lower T_m primer. If necessary, use a temperature gradient to find the optimal annealing temperature for each template-primer pair combination. The annealing gradient should extend up to the extension temperature (two-step PCR). Two-step cycling without an annealing step is recommended for high-T_m primer pairs (T_m at least 69–72°C). **Note:** Do not perform annealing below 50°C.

5.3 Extension

The extension is performed at 72°C. The optimal extension time varies depending on the amplicon length and the blood concentration in the reaction. The recommended extension time is 15 seconds for amplicons ≤ 1 kb, and 30 s/kb for amplicons >1 kb. When higher blood concentrations are used, longer extension times may improve the results.

6. Post-amplification analysis

After amplification, centrifuge the PCR reaction at 1 000 x g for 1–3 minutes to collect the supernatant for analysis. This step separates the various components of blood that might interfere with subsequent assays, e.g. gel electrophoresis. This is especially important when high blood concentrations are used, as there can be a substantial amount of cell debris, etc. in the tube after the PCR reaction.

If post-PCR enzyme treatment is performed (e.g. PCR-RFLP), it may be necessary to dilute the PCR product 2–4-fold in order to dilute the salts and other inhibitors from the PCR reaction.

7. Control reactions

7.1 Universal control primer mix

This component is a mix of degenerate primers in H₂O that amplify a 237 bp fragment of mammalian genomic DNA. The amplified region is a highly conserved non-coding region upstream of the SOX21 gene,⁵ and the primers are designed to amplify this region from a wide range of vertebrate species. Each primer concentration is 25 µM.

Primer #1 (24-mer)

5'- AGCCCTTGGGGASTTGAATTGCTG -3'

Melting point: 73.5°C

Primer #2 (27-mer)

5'- GCACTCCAGAGGACAGCRGTGTCAATA -3'

Melting point: 72.2°C (R=A), 75.3°C (R=G)

Table 3. Pipetting instructions for control reactions.

| Component | 20 µl reaction | 50 µl reaction |
|-------------------------------|----------------|----------------|
| H ₂ O | add to 20 µl | add to 50 µl |
| 2x Phusion® Blood PCR Buffer | 10 µl | 25 µl |
| Universal control primer mix | 0.4 µl | 1 µl |
| Phusion® Blood DNA Polymerase | 0.4 µl | 1 µl |
| Whole blood (see 4.1) | Up to 1 µl | Up to 10 µl |

Table 4. Cycling instructions for control reactions.

| Cycle step | Temp. | Time | Cycles |
|---------------------|-------------|---------------|--------|
| Lysis of cells | 98°C | 5 min | 1 |
| Denaturation | 98°C | 1 s | 35 |
| Annealing/Extension | 72°C | 15 s | |
| Final extension | 72°C 4°C | 1 min hold | 1 |

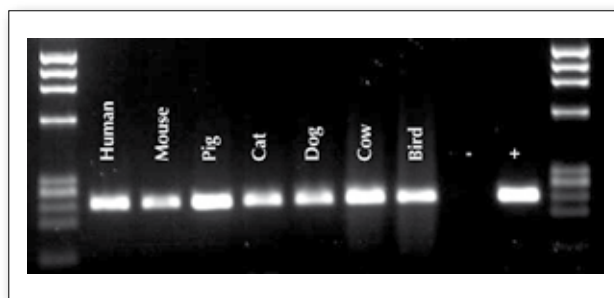


Figure 1. Amplification of the 237 bp control DNA fragment directly from whole blood (5 % in the reaction) of different species using universal control primers included in the Phusion Blood Direct PCR Kit. The blood samples used were stored at -20°C. The + and - denote control reactions with and without purified DNA.

8. Troubleshooting

No product at all or low yield

- Repeat and make sure that there are no pipetting errors.
- Make sure that the cycling protocol was performed as recommended.
- Optimize annealing temperature.
- Titrate the amount of blood in the reaction.
- Increase the number of cycles.
- Check the purity and concentration of the primers.
- Check primer design.
- Increase extension time.
- Increase denaturation time up to 5 seconds.
- Add 1–5 % DMSO (especially for high GC amplicons).
- Add MgCl₂ (1–2 µl/50 µl reaction) if high blood concentration is used.
- Add EDTA (1.25–2.5 µl/50 µl reaction).

Non-specific products - High molecular weight smears

- Make sure that the extension time used was not too long. (Recommended extension time is 15–30 s/kb).
- Increase annealing temperature or perform a temperature gradient PCR.
- Titrate the amount of blood in the reaction.
- Reduce the total number of cycles.
- Decrease primer concentration.
- Add EDTA (1.25–2.5 µl/50 µl reaction).

Non-specific products - Low molecular weight discrete bands

- Increase annealing temperature.
- Titrate the amount of blood in the reaction.
- Shorten extension time.
- Perform a temperature gradient PCR.
- Decrease primer concentration.
- Add EDTA (1.25–2.5 µl/50 µl reaction).
- Design new primers.

9. References

1. Nord K. *et al.* (1997) *Nature Biotechnol.* 15: 772–777.
2. Wikman M. *et al.* (2004) *Protein Eng. Des. Sel.* 17: 455–562.
3. Frey B.F. & Suppmann B. (1995) *Biochemica* 2: 34–35.
4. Chester N. & Marshak D.R. (1993) *Analytical Biochemistry* 209: 284–290.
5. Woolfe A. *et al.* (2005) *PLoS Biology* 3: 116–130.

Shipping and storage

Phusion Blood PCR Kit is shipped on gel ice. Upon arrival, store the components at –20°C. The kit is stable for one year from the packaging date when stored and handled properly.

Warranty

Finnzymes Oy warrants that its products will meet the specifications stated on the technical data section of the data sheets, and Finnzymes Oy agrees to replace the products free of charge if the products do not conform to the specifications. Notice for replacement must be given within 60 days of receipt. In consideration of the above commitments by Finnzymes Oy, the buyer agrees to and accepts the following conditions:

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Version 1.2, June 2009