

# PCR-RFLP directly from blood using Thermo Scientific Phusion Blood Direct PCR Kit

Pia Kuusisto, Thermo Fisher Scientific

## Abstract

This application protocol describes how PCR-RFLP (restriction fragment length polymorphism) assays can be performed from blood samples without DNA purification. In the first step, genomic DNA is amplified directly from whole blood using Thermo Scientific Phusion Blood Direct PCR Kit. The PCR product is subsequently digested with a restriction enzyme that recognizes a specific sequence of interest (e.g. a SNP site) in this DNA fragment. Based on the sample genotype, the digestion produces DNA fragments which are then analyzed by agarose gel electrophoresis. Presented here is a specific example of a PCR-RFLP based SNP (single-nucleotide polymorphism) genotyping assay performed directly from blood without DNA purification before PCR or digestion (see Figure 1A for assay design).

## Materials and methods

- Human whole blood with EDTA as anticoagulant
- Phusion® Blood Direct PCR Kit
- *HhaI*
- Thermo Scientific Piko 24-well Thermal Cycler
- Thermo Scientific Piko UTW (ultra-thin walled) Piko® PCR Plates
- Primers: (Forward and Reverse)

429 bp fragment of human eukaryotic translation initiation factor 2-alpha kinase 3 gene (EIF2AK3)

F: AGCTCCTATAGTAACCTCTTCTTGA  
ACTCACTTG 34 nt T<sub>m</sub>=68.6°C

R: GCTTTCACGGTCTCGGTCCCACTG  
24 nt T<sub>m</sub> = 75.5°C

**Table 1.** Reaction conditions for PCR

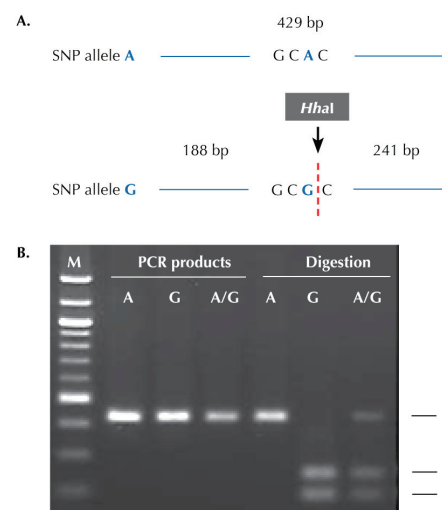
Component	20 µL reaction	50 µL reaction	Final conc.
H <sub>2</sub> O	Add to 20 µL	Add to 50 µL	
2x Phusion Blood PCR Buffer	10 µL	25 µL	1x
Primer F (Forward)	x µL	x µL	0.5 µM
Primer R (Reverse)	x µL	x µL	0.5 µM
Phusion Blood DNA Polymerase	0.4 µL	1 µL	
Whole blood*	1 µL	2.5 µL	
Optional components for reaction optimization*			
50 mM MgCl <sub>2</sub>	0.6 µL	1.5 µL	
50 mM EDTA	0.5 - 1.0 µL	1.25 - 2.5 µL	
DMSO	1.0 µL	2.5 µL	5%

\* See Phusion Blood Direct PCR Kit manual for more instructions related to optional components.

**Table 2.** Cycling protocols

Cycle step	2-step protocol		3-step protocol		Cycles
	Temp.	Time	Temp.	Time	
Lysis of cells	98°C	5 min	98°C	5 min	1
Denaturation	98°C	1 s	98°C	1 s	35-40
Annealing*	-	-	x°C	5 s	
Extension*	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

\* See Phusion Blood Direct PCR Kit manual for more instructions.



**Figure 1. Assay design and results of the SNP genotyping example.** **A.** A 429 bp fragment of human EIF2AK3 gene covering the SNP site of interest was first amplified directly from whole blood of different individuals (5% blood in 50 µL reactions, 2-step cycling protocol, 40 cycles). The unpurified PCR products were subsequently digested as described with *HhaI* restriction enzyme that recognizes only the G allele in the SNP site of interest. **B.** The resulting fragments revealing the genotype of each individual were analyzed on agarose gel in parallel with the untreated PCR products. M, size marker; A, G and A/G correspond to the SNP alleles of each sample.

## Key words

- RFLP
- Direct PCR
- Genotyping
- Human Blood
- SNP Alleles
- Restriction Digest

### Restriction enzyme digestion

After PCR, the reactions were centrifuged at  $1000 \times g$  for 2 minutes and the supernatants were collected for restriction digestion. The digestions were prepared directly in the supernatants with *HhaI* (0.4 U/ $\mu$ L). The 10  $\mu$ L reactions were incubated for one hour at 37°C. The resulting fragments were analyzed on agarose gel and compared to untreated PCR products.

#### Note 1: Restriction digestion in Phusion Blood PCR Buffer

When amplifying DNA directly from whole blood, the PCR product contains blood and PCR derived components that may interfere with the subsequent digestion. The inhibitor tolerance varies from enzyme to enzyme and therefore further optimization of the reaction conditions may be needed. Several restriction enzymes tolerate inhibitors such as salt and blood constituents quite well and the digestion can be performed directly in a PCR reaction after removing most of the blood components by centrifugation. If the restriction enzyme used is not fully active in Phusion Blood PCR buffer, please follow these instructions below:

1. The most convenient option is to dilute the PCR reaction before digestion. Usually diluting the PCR product 1:2 in H<sub>2</sub>O helps to dilute the buffer components and/or inhibitors in the reaction, allowing the restriction enzyme to perform optimally. If the PCR yield is high, it is possible to dilute even more.
2. For some restriction enzymes dilution of the PCR reaction is not enough to provide optimal conditions for efficient digestion. In this case, the PCR product needs to be purified before digestion.
3. Additionally, further optimization

of the reaction conditions, such as adjustment of the reaction time and restriction enzyme amount, as well as adding restriction enzyme's unique reaction buffer to the digestion, may be needed.

#### Note 2: Restriction digestion producing cohesive-ends for cloning

The presence of Phusion Blood DNA Polymerase in digestion reaction may alter the cohesive ends of the DNA fragments that have been cleaved, affecting subsequent ligation. Therefore, for cloning applications, the digestions producing cohesive ends should be performed on purified PCR products.

#### Technical Support

In North America (US, Canada) and Central/South America  
Techservice.genomics@thermofisher.com  
+1 (800) 235-9880  
+1 (303) 604-9499

In Europe, Middle East, and Africa  
Techservice.emea.genomics@thermofisher.com  
(+44) 1372 840410

In Other Countries  
[www.thermoscientific.com/pcrordering](http://www.thermoscientific.com/pcrordering)

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The Thermo Scientific Direct PCR approach allows for amplification of DNA directly from various starting materials such as blood, mouse ear and tail tissues, plants, and FFPE tissue samples. For more information about the Direct PCR products and protocols, please visit

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