Application Note

PCR-RFLP Directly from Blood Using Thermo Scientific Phusion Blood Direct PCR Kit

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Key Words

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

Introduction

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 (for example, 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 (Cat #F-547)
- HhaI (Cat #ER1851)
- Thermo Scientific™ Piko™ 24-well Thermal Cycler (Cat #TCP0024)
- Thermo Scientific Piko UTW PCR Plates (Cat #SPL0241)
- Primers: (Forward and Reverse)

  429 bp fragment of human eukaryotic translation initiation factor 2-alpha kinase 3 gene (EIF2AK3)
  F: AGCTCCTATAGTAACCTCTTCTTGAACTCACTTG 34 nt Tm = 68.6 °C
  R: GCTTTCACGGTCTCGGTCCCACTG 34 nt Tm = 68.6 °C
  24 nt Tm = 75.5 °C

Table 1. Reaction conditions for PCR.

Component	50 µL Reaction	Final Conc.
H_2O	Add to 50 µL	
2x Phusion Blood PCR Buffer	25 µL	1x
Primer F (Forward)	x µL	0.5 µM
Primer R (Reverse)	x µL	0.5 µM
Phusion Blood DNA Polymerase	1 µL	
Whole Blood*	2.5 µL	
Optional Components for Reaction Optimization*		
50 mM $MgCl_2$	1.5 µL	
50 mM EDTA	1.25-2.5 µL	
DMSO	2.5 µL	5%

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

Table 2. Cycling protocols.

	2-step Protocol		
Cycle Step	**Temperature**	**Time**	**Cycles**
Lysis of Cells	98 °C	5 minutes	1
Denaturation	98 °C	1 seconds	
Annealing*	-	-	40
Extension*	72 °C	15-30 s/kb	-
Final Extension	72 °C 4 °C	1 minute hold	1

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

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Restriction Enzyme Digestion

After PCR, the reactions were centrifuged at 1000 × *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/µL). The 10 µL 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_2O 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 the 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.

Results

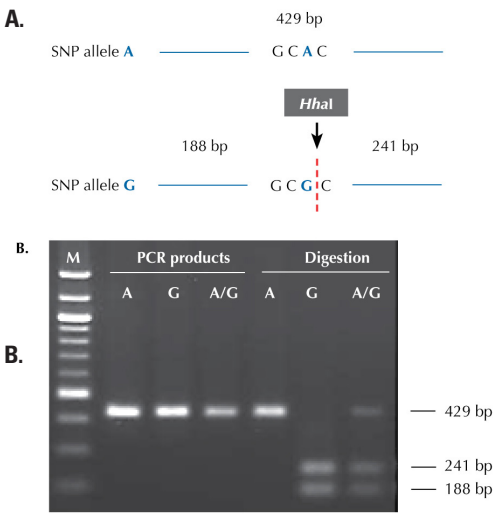

Figure 1. Assay design and results of the SNP genotyping example. **A.** A 429 bp fragment of human EIF2AK3 gene covering the SNP site of interest was first amplified directly from whole blood of different individuals (5% blood in 50 µL reactions). The unpurified PCR products were subsequently digested 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.

Conclusion

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.

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