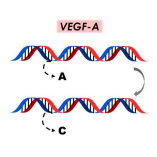

Feb 02, 2021

PCR-RFLP protocols for genotyping *VEGF-A* rs28357093

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ABSTRACT

The VEGF-A gene encodes a homonymous protein, responsible for regulating angiogenesis and vascular permeability. In addition, in the central nervous system, it acts as a neurotrophic factor, stimulating neurogenesis and cell survival. *VEGF-A* rs28357093 is located in the promoter region of the gene. There are few studies with this polymorphism, with no reports on the consequences. However, it is in a regulatory region, important for the control of gene transcription, and may influence gene and protein expression. In this document, we share our protocol for amplification and enzymatic digestion for this SNP, using the PCR-RFLP technique and silver staining for genotyping analysis.

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KEYWORDS

PCR-RFLP, Polymorphisms, VEGF-A, Molecular biology

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GUIDELINES

For electrophoresis in 15% PAGE, consider the following configuration for a good separation of DNA bands:
80 V
50 mA
3 W

06:30:00

MATERIALS TEXT

Reagents for PCR master mix:

10X PCR Buffer	Sinapse INC ® (São Paulo - Brazil)
MgCl2 50mM	Sinapse INC ® (São Paulo - Brazil)
dNTPs	Sinapse INC ® (São Paulo - Brazil)
Taq Polymerase	Sinapse INC ® (São Paulo - Brazil)
Primers (Reverse and Forward Mix)	Integrated DNA Technologies® (IDT) (Coralville, Iowa, US).

Reagents for electrophoresis run:

Loading dye	Sinapse INC ® (São Paulo - Brazil)
100 bp DNA ladder	Sinapse INC ® (São Paulo - Brazil)
20 bp DNA ladder	BIO-RAD ® (Hercules, Califórnia, US)

(If you choose for check success of DNA amplification in 3% agarose, use **2 µl** of ethidium bromide. Prefer to run the restriction product in 15% polyacrylamide, to avoid losing very small fragments)

Reagents for RFLP mix:

Enzyme buffer	(Thermo Fisher Scientific™, Waltham, Massachusetts, US)
HhaI restriction enzyme	(Thermo Fisher Scientific™, Waltham, Massachusetts, US)

SAFETY WARNINGS

Most of the reagents used are toxic (e.g. acrylamide, ethidium bromide, formaldehyde). Be careful with the handling of these products, respecting the biosafety rules stipulated in your laboratory.

BEFORE STARTING

Make sure to be following your laboratory's biosafety rules for DNA manipulation

PCR amplification of the *VEGF-A* rs28357093 locus

1

Be sure to use coat, mask and gloves.
Be careful when manipulate all components of the master mix, preventing contamination.

Amplication by conventional PCR using the primer sequences (Please, check *note* box in the end of this section for primer paper reference):

Forward: 5' - CCC CTG CCC CCT TCA ATA -3'

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Reverse: 5'- AGC CTC AGC CCC TCC ACA -3'

Master mix for **25 µl** reactions are sufficient for PCR amplicons and RFLP genotyping analysis

It is a good recommendation include a negative control (master mix without the DNA template) for controlling contamination of the reaction.

Example of one 25 µl PCR reaction:

A	B	C
Components	Final concentration	Volume
10X PCR Buffer	1x	2.5 µL
MgCl2 50mM	1,0 mM	1.5 µL
dNTPs 10 mM Mix	2.5 mM	1.0 µL
Primers (Reverse and Forward Mix) 2.5 µM	0.1 mM	4.0 µL
Ultrapure Water (Milli-Q®)	-	13.8 µL
Taq polymerase (5U/µL)	1.2U/µL	0.2 µL
DNA template	-	2.0 µL
Total volume	-	25 µL

Were used 10X PCR Buffer, MgCl2, Taq Polymerase and dNTPs from Sinapse INC ® (São Paulo - Brazil). Primers are from Integrated DNA Technologies® (IDT) (Coralville, Iowa, US).

Thermal Cycler Setup

A	B	C
Steps	Temperature	Time
Initial denaturation	94°C	5 min
Denaturation	94°C	1 min
Annealing	60°C	1 min
Elongation	72°C	1.30 min
GO TO STEP 3 35X	-----	-----
Final elongation	72°C	7 min

The amplification process according to this thermal cycler setup takes approximately 3 hours.

Thermal cycler

T100 PCR thermal cycler

BIO-RAD https://www.bio-rad.com/pt-br/product/t100-thermal

***VEGF-A* rs28357093 primers were previously described in:**

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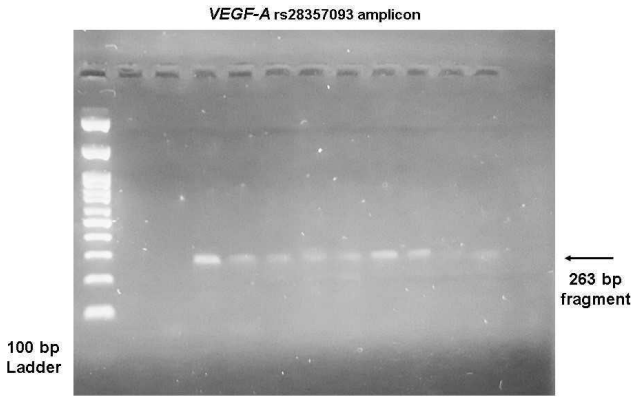
Holt RCL, Ralph SA, Webb NJA, Watson CJ, Clark AGB, Mathieson PW *et al.* Steroid-sensitive nephrotic syndrome and vascular endothelial growth factor gene polymorphisms. *European Journal of Immunogenetics.* 2003; 30(1): 1-3.

Electrophoresis for check amplification

2

To check amplification success, mix **5 µl** of the PCR product with **3 µl** of DNA loading dye. Use **3 µl** of 100 bp DNA ladder (Sinapse INC® 100 bp ladder). Run on a 3% agarose (or in Polyacrylamide gel electrophoresis - PAGE) with TBE buffer.
The amplified fragment must have 263 bp.



Electrophoresis in 3% agarose gel. Were used a 100 bp ladder. The fragment of interest (with 263 bp) it is located between 200 and 300 bp.

If you chose for an electrophoresis in agarose and use ethidium bromide as DNA intercalant follow the biosafety measures (wear gloves/avoid direct contact to the skin). Also, take care to not expose the eyes or skin in the UV-light when detecting the DNA bands.

Enzimatic digestion and RFLP analysis for *VEGF-A* rs28357093

3 For RFLP, a new mix must be prepared, using the restriction enzyme *HhaI* (GCG^C) from Thermo Fisher Scientific®

Enzimatic digestion mix for one reaction

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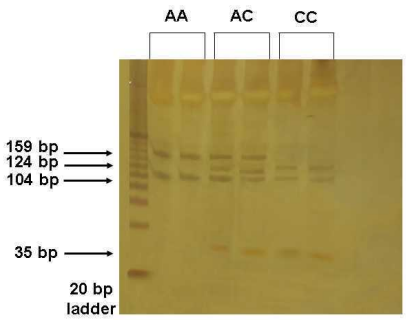
A	B
Components	Volume
Enzyme buffer	2.0 µL
HhaI restriction enzyme 10U/µL	0.5 µL
Ultrapure Water (Milli-Q®)	17.5 µL
Amplicon	10.0 µL
Total volume	30 µL

Incubate the reactions for 6 h at 37°C, with 20 min in 80ºC for enzimatic inactivation (total reaction time: 06 h and 20 min). After DNA digestion, run an electrophoresis to view the genotypes for the *VEGF-A* rs28357093.

Prepare a mix with **10 µl** of the digests with **3 µl** of DNA loading dye. Use **3 µl** of 20 bp DNA ladder (BIO-RAD 20 bp ladder). Run it in a 15% PAGE with TBE buffer
For a good separation, the electrophoretic run must be cover the whole length of the gel.
To reveal the genotypes, use silver staining.



Expected results:
Electrophoresis in 15% PAGE staining with silver, which enables the genotypes analysis.
AA - 159, 104 bp
AC - 159, 124, 104, 35 bp
CC - 124, 104, 35 bp

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