Real-Time Reverse Transcription-PCR Quantification of Vascular Endothelial Growth Factor Splice Variants,

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Vascular endothelial growth factor (VEGF) is an endothelial cell–specific mitogen and a key regulator of angiogenesis in a variety of physiologic and pathologic processes *(1)*. The human gene for VEGF resides on chromosome 6p21.3 and is organized into 8 exons *(2, 3)*. Alternative exon splicing of the *VEGF* gene produces various splice variants (subscripts denote the number of amino acids after signal sequence cleavage): $VEGF_{206}$, $VEGF_{189}$, $VEGF_{183}$, $VEGF_{165}$, $VEGF_{148}$, $VEGF_{145}$, and $VEGF_{121}$ *(4–8)*. Exons 1–5 and 8 are preserved in all variants, whereas the presence or absence of exons 6a, 6b, and 7 distinguish variants. $VEGF_{121}$ lacks these exons *(4)*, $VEGF_{165}$ contains the exon 7-encoded sequence *(4)*, and $VEGF_{148}$ has the same amino acid sequence as $VEGF_{165}$ apart from a 35-bp–long deletion at the end of exon 7 *(5)*. $VEGF_{189}$ contains exons 6a and 7 *(4)*, whereas in $VEGF_{183}$ the final 18 bp of exon 6a are missing *(6)*. $VEGF_{145}$ contains exon 6a but lacks exon 7 *(7)*. $VEGF_{206}$ is the full-length form containing, in addition to exons 6a and 7, a 51-bp part from intron 3, exon 6b, neighboring exon 6a *(8)*.

VEGF isoforms differ in their heparin and heparan sulfate binding capacities as well as in their receptor affinities *(9)*, but their exact biological roles remain unclear. Most VEGF-producing cells appear to preferentially express $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ *(4)*. $VEGF_{183}$ has a wide tissue distribution and may have not been detected earlier because of confusion with $VEGF_{189}$ *(6)*. $VEGF_{145}$ is one of the main variants produced by several cell lines derived from carcinomas of the female reproductive system *(7)*, and it has also been detected in ovarian and breast cancer tissue samples *(10)*. $VEGF_{148}$ was identified in human glomeruli *(5)*, but there are no data concerning its expression in other tissues. $VEGF_{206}$ is a very rare isoform found in a human fetal liver library *(8)*.

We developed a real-time reverse transcription-PCR (RT-PCR) method for the quantification of 6 VEGF splice variants ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{148}$, $VEGF_{165}$, $VEGF_{183}$ and $VEGF_{189}$) and applied it to 2 human breast cancer cell lines, MCF-7 and MDA-MB-231, and a human colorectal cancer cell line, COLO-205.

The sequences of the primers and probes used are presented in Table 1, and the principle of the proposed assay is shown in Fig. 1A. A common forward primer (VEGFex3F) and a common pair of hybridization probes (VEGF-FL and VEGF-LC) were used (TIB MOLBIOL). The VEGFex3F primer and the VEGF-FL probe are located on exon 3, whereas the VEGF-LC probe spans the exon 3/4 boundaries to avoid genomic DNA detection. For the quantification of total VEGF mRNA, we used the VEGFex8R reverse primer located on exon 8 (common for all variants), which generated amplicons of various sizes (233, 305, 330, 365, 419, and 437 bp for $VEGF_{121}$, $VEGF_{145}$, $VEGF_{148}$, $VEGF_{165}$, $VEGF_{183}$, and $VEGF_{189}$, respectively). Amplification of each variant was performed with reverse primers spanning variant-specific exon boundaries: 5/8 for $VEGF_{121}$ (VEGFR121; 223 bp), 6a/8 for $VEGF_{145}$ (VEGFR145; 295 bp), 7a/8 for $VEGF_{148}$ (VEGFR148; 320 bp), 5/7 for $VEGF_{165}$ (VEGFR165; 224 bp), 6a/7 for $VEGF_{183}$ (VEGFR183; 274 bp), and 6a/7 for $VEGF_{189}$ (VEGFR189; 289 bp). The VEGFR165 reverse primer is not specific for $VEGF_{165}$ because $VEGF_{148}$ can also be amplified because the region exon 5/exon 7 is common in both variants.

Real-time PCR was performed in the LightCycler Instrument (Roche Applied Science) in a total volume of 10 μL per glass capillary. For each reaction, 1 μL of cDNA was placed in a 9-μL reaction mixture containing 0.1 μL of a temperature-released *Taq* DNA polymerase (5 U/μL; Platinum DNA Polymerase; Invitrogen), 1 μL of the supplied 10× PCR buffer, 0.7 μL of the supplied $MgCl_2$ (50 mM), 0.2 μL of deoxynucleotide triphosphates (10 mM; Invitrogen), 0.15 μL of bovine serum albumin [10 μg/μL; Sigma *(11)*], 0.5 μL of the primers and probes (3 μM), and diethylpyrocarbonate-treated H_2O. The cycling protocol was identical for all splice variants and total VEGF and consisted of an initial 5-min denaturation step at 95 °C for activation of the DNA polymerase, followed by 50 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 15 s, and extension at 72 °C for 20 s.

For the development and analytical evaluation of the assay, we generated and used as calibrators PCR amplicons corresponding to the 6 VEGF splice variants studied. For this purpose, total RNA was extracted from MCF-7 cells; cDNA was synthesized and served as a template for the amplification of the targets of interest by real-time PCR. PCR products were separated on a 2% agarose gel.

Table 1. Sequences of primers and hybridization probes used in this study.

	Name	Oligonucleotide sequence, 5′–3′
Forward primer	VEGFex3F	ATCTTCAAGCCATCCTGTGTGC
Reverse primers	VEGFR121	TGCGCTTGTCACATTTTTCTTG
	VEGFR145	TCGGCTTGTCACATACGCTCC
	VEGFR148	TCGGCTTGTCACATCTTGCAAC
	VEGFR165	CAAGGCCCACAGGGATTTTC
	VEGFR183	GCCCACAGGGACGGGATTT
	VEGFR189	CACAGGGAACGCTCCAGGAC
	VEGFex8R	GCTCACCGCCTCGGCTTGT
Hybridization probes	VEGF-FL	AGTGTGTGCCCACTGAGGAGTCC-FL[a]
	VEGF-LC	LC Red 640-ACATCACCATGVAGATTATGCG-Ph[b]

[a] 3′-End-labeled with fluorescein (FL).

[b] 5′-End-labeled with LC Red 640; phosphorylated (Ph) on the 3′ end to avoid extension of the probe.

The bands were excised and purified by use of the QIAQuick Gel Extraction Kit (Qiagen), and the amplicons were quantified with the PicoGreen DNA Quantification Kit (Molecular Probes). Concentrations were converted to copies/μL by use of the Avogadro constant and the molecular weight of each amplicon [number of bases of the PCR product multiplied by the mean molecular weight of a pair of nucleic acids which is 660 *(12)*].

To establish a specific, sensitive, and reproducible real-time RT-PCR assay, we performed extensive optimization of primers, probes, and $MgCl_2$ concentrations as well as reaction temperatures and times. The analytical evaluation of the assay was performed with the prepared VEGF

Fig. 1. Realtime RT-PCR analysis of VEGF and VEGF splice variants.

(*A*), positions of primers and probes for the quantification of total VEGF and VEGF splice variants. *FRET*, fluorescence resonance energy transfer; *Eex*, excitation wavelength; *Eem* emission wavelength. (*B*), agarose gel electrophoresis of real-time PCR products for the amplification of VEGF splice variants, showing specificity of the assay. *Lane 1*, 100-bp DNA marker; *lane 2*, VEGF$_{121}$ (223 bp); *lane 3*, VEGF$_{145}$ (295 bp); *lane 4*, VEGF$_{148}$ (320 bp); *lane 5*, VEGF$_{165}$ (224 bp); *lane 6*, VEGF$_{183}$ (274 bp); *lane 7*, VEGF$_{189}$ (289 bp); *lane 8*, 100-bp DNA marker. (*C*), schematic representation of the percentages of VEGF splice variants in 3 cancer cell lines.

calibrators. For each splice variant, a calibration curve was generated from serial dilutions ranging from 10^6 to 10 copies/μL of the target of interest. All calibration curves showed linearity over the entire quantification range with correlation coefficients >0.99. The between-day imprecision of the calibration curves ranged from 0.5% to 3.9% (Table 1 of the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol51/issue8/). The analytical detection limit of the method, defined as 3.3 times the SD of the crossing point for the less concentrated calibrator divided by the mean slope of the calibration curve, corresponded to 1 copy/μL for each target, whereas the limit of quantification, defined as 3 times the detection limit, was 3 copies/μL.

Because the amplicons generated by PCR have different sizes, the reaction efficiency could vary. However, the mean (SD) slopes of the 6 calibration curves ranged from −3.63 (0.29) to −3.95 (0.08), indicating comparable PCR efficiencies (1.79–1.89), and the mean (SD) intercepts of the calibration curves ranged from 37.3 (0.9) to 38.3 (0.6) copies/μL from day to day. When we used all of the data generated from the 6 different calibration curves to plot a single calibration curve, the correlation coefficient was 0.99, indicating that this grand mean calibration curve could be used for the quantification of all 6 splice variants as well as total VEGF. The assay was highly specific for each splice variant. When cDNA from 10^5 MCF-7 cells was used as a template for real-time PCR, agarose gel electrophoresis of the PCR products after 50 cycles revealed only one specific product of the desired length for each splice variant (Fig. 1B).

We investigated the expression of VEGF splice variants in 3 cancer cell lines (MCF-7, MDA-MB-231, and COLO-205). VEGF$_{121}$ and VEGF$_{165}$ were the major variants expressed, followed by VEGF$_{189}$, whereas VEGF$_{145}$, VEGF$_{148}$, and VEGF$_{183}$ were present only in small amounts (Fig. 1C). VEGF$_{148}$ was detected for the first time in cancer cell lines.

In conclusion, we have developed a sensitive, specific real-time RT-PCR assay for the quantification of VEGF splice variants and total VEGF. A similar approach has already been described, but only the major VEGF variants VEGF$_{121}$, VEGF$_{165}$, and VEGF$_{189}$ were investigated *(11)*. In addition to these splice variants, we also included in our work the less-studied variants VEGF$_{145}$, VEGF$_{148}$, and VEGF$_{183}$. The analytical performance of the assay was validated through a series of experiments based on VEGF calibrators, which were specifically designed, synthesized, and quantified in a novel way in our laboratory *(13)*. The use of PCR amplicon–based calibrators to generate calibration curves has also been described by others *(14)*. This approach is an alternative to the use of plasmid vectors and is useful for laboratories that do not have the facilities for transformation of bacterial cultures and subsequent plasmid DNA isolation.

The biological significance of the alternative splicing of the VEGF gene is still uncertain. We believe that the developed real-time RT-PCR assay, which provides rapid,
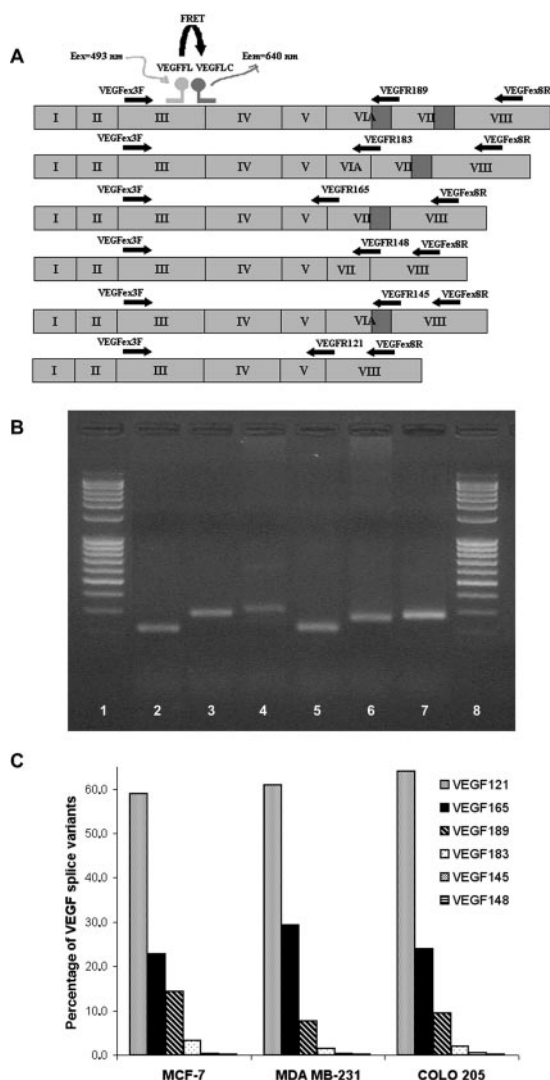
accurate quantification of the expression of 6 VEGF variants, can be a promising tool for the elucidation of their role in promoting physiologic and pathologic angiogenesis. Our method can be applied to various samples for the separate quantification of a single VEGF splice variant of interest or of all 6 variants together and could serve as a model for the development of real-time RT-PCR assays for the quantification of other alternatively spliced genes.

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Comparing Whole-Genome Amplification Methods and Sources of Biological Samples for Single-Nucleotide Polymorphism Genotyping, *Ji Wan Park,*[1,2] *Terri H. Beaty,*[1] *Paul Boyce,*[2] *Alan F. Scott,*[2] *and Iain McIntosh*[2*] ([1] Department of Epidemiology, Bloomberg School of Public Health, and [2] McKusick-Nathans Institute of Genetic Medicine, School of Medicine, Johns Hopkins University, Baltimore, MD; * address correspondence to this author at: Johns Hopkins University, McKusick-Nathans Institute of Genetic Medicine, 733 N. Broadway/BRB 407, Baltimore, MD 21205; fax 410-502-5677, e-mail mcintosh@jhmi.edu)

High-throughput genotyping systems promise to be an efficient means of identifying susceptibility genes involved in the etiology of non-Mendelian disorders. Adequate amounts of high-quality DNA are essential, however, for large-scale genotyping studies *(1)*. The supply of genomic DNA is frequently limited, and the quality of DNA obtained from oral buccal swabs or Guthrie cards has not been thoroughly evaluated for high-throughput single-nucleotide polymorphism (SNP) genotyping *(2, 3)*. Whole-genome amplification (WGA) technologies offer the opportunity to expand DNA from depleted biological samples. The first generation of WGA strategies (i.e., PCR-based methods) *(4, 5)*, however, was limited by substantial amplification bias and incomplete coverage of genetic markers *(6, 7)*. Recently, new strategies for WGA, such as multiple displacement amplification (MDA) or OmniPlex® WGA technology (Rubicon Genomics) have been developed. MDA is an isothermal amplification with the bacteriophage ϕ29 DNA polymerase *(6, 8)*, whereas OmniPlex uses in vitro libraries with fragmented DNA (~1.5 kb) to amplify the entire genome by PCR *(9)*.

To apply WGA technology to BeadArray™ genotyping (Illumina), the utility of MDA and/or OmniPlex on DNA samples derived from lymphoblast cells has been evaluated *(9, 10)*. In this study, we determined the genotyping success rate and reliability of 2 MDA variants *(8, 11)* and OmniPlex with and without 7-deaza-dGTP, using buccal swabs, whole blood, dried blood spots, and sheared genomic DNA on 1260- and 1228-SNP BeadArray panels. The 7-deaza-dGTP nucleotide analog was included in an attempt to ensure amplification of GC-rich DNA.

After Institutional Review Board approval and informed consent were obtained, DNA samples were collected from participants in a study of oral clefts *(12)*. Genomic DNA samples were prepared from peripheral blood by protein precipitation *(13)*. Buccal cells were obtained by rubbing the inside of cheeks with a brush (Medical Packaging), and DNA was extracted with 600 μL of 50 mmol/L NaOH *(2)*. For blood spot samples dried on Guthrie cards, two to three 2-mm circles punched from the blood spots with a micro-punch (Fitzco) were placed in 5 g/100 mL Chelex® 100 chelating resin (Bio-Rad Laboratories) *(14)* and treated as described (blood spot A) *(15)* or stored at −20 °C for ~3 years (blood spot C). As an alternative method, 2 to 3 circles punched from the blood spots were placed in a cell lysis solution (10 μL of 0.2