Preliminary validation of two novel immunoassays for detecting VEGF in human and mouse plasma using single molecule counting technology

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Abstract

Background: Growth in biomarkers as therapeutic targets and as surrogate markers for efficacy presents a need for increasingly sensitive immunoassays to expand biomarker applicability. Improved immunoassays will provide: (1) better evaluation and validation of new drug candidates, (2) better matching of patients to new therapies, (3) accelerated drug approval (4) earlier diagnosis of at-risk patients, and (5) a deeper understanding of cancer biology. Towards this end, Singulex® has developed two ultra-sensitive VEGF Immunoassays for human and mouse vascular endothelial growth factor (VEGF). Here we report the preliminary validation of these two novel assays.

Methods: Two novel assays were developed with the Erenna™ Immunoassay System for detecting VEGF: human (hVEGF) and mouse (mVEGF). Analytical sensitivity, cross-reactivity and precision were determined and compared to an ELISA based VEGF assay. Both the Singulex assay and ELISA assay were used to test a range of specimen types (plasma, cell lysates, conditioned media, and tissue specimens) from humans and mice. Preliminary assays with human plasma and tissue specimens were conducted to compare hVEGF levels between normal and breast cancer samples.

Results: The Singulex hVEGF assay had an LOD of 0.1 pg/mL, an LLOQ of 0.3 pg/mL, and 84–107% spike recovery; 90X more sensitive than the ELISA assay. Human VEGF concentrations were quantified in all specimens tested compared to the ELISA, which quantified VEGF in only 8% of plasma samples, but all of the cell lysate samples. The Singulex mVEGF assay had an LOD of 3.5 pg/mL, LLOQ of 5 pg/mL, and 68–111% spike recovery; 3X more sensitive than the ELISA assay. Cross-reactivity for the two assays was minimal for all specimen types tested, except for human plasma samples where the mVEGF assay demonstrated 80–100% CR.

Conclusions: We show that the Singulex hVEGF and mVEGF Immunoassays can detect VEGF at or below pg/mL levels, and can effectively quantify VEGF levels in plasma, cell lysates, conditioned media, and tissue samples from mice and humans. These novel assays are an important tool when used to assess tumor and normal breast cancer tissue and plasma.

Introduction

VEGF-A, commonly known as VEGF, is one member of a family of secreted glycoproteins that promote endothelial cell growth, survival, migration, and vascular permeability, all of which contribute to angiogenesis. The binding of VEGF to its receptor triggers the activation of a cell signaling pathway that is critical for the growth of blood vessels from pre-existing vasculature. VEGF is implicated in a variety of diseases, including several cancers, age-related macular degeneration, diabetic retinopathy and rheumatoid arthritis. As such, it is an attractive candidate for the development of therapies for these diseases, particularly cancer.

Figure 1: The VEGF Signaling Pathway.

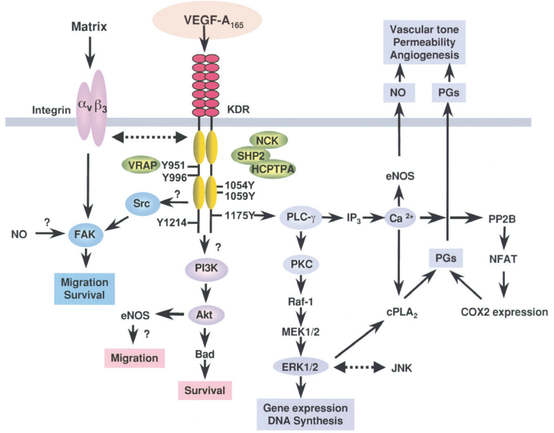


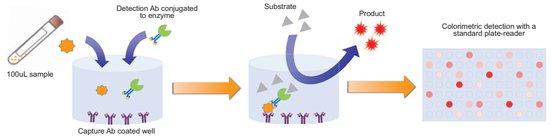
Image from Biochem. Soc. Trans. (2003) 31: 1171-1177.

Methods

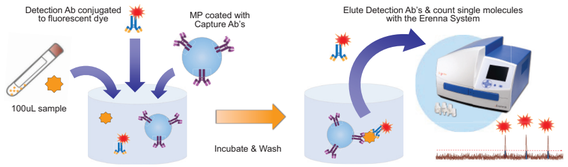
Two novel assays were developed with the Erenna™ Immunoassay System (Figure 1) for detecting VEGF in human (hVEGF) and mouse (mVEGF) samples. Analytical sensitivity, cross-reactivity and precision were determined and compared to an ELISA based VEGF assay (R&D Systems). Both the Singulex assay and ELISA assay were used to test a range of specimen types (plasma, cell lysates, conditioned media, and tissue specimens) from humans and mice. Preliminary assays with human plasma and tissue specimens were conducted to compare hVEGF levels between normal and breast cancer samples.

Figure 2: Comparison of Traditional ELISA Immunoassays with the Novel Erenna Immunoassay System (EIAS).

(A) Traditional ELISA immunoassays consist of 3 major steps: a sandwich immunoassay, enzymatic amplification, and colorimetric detection.



(B) The Erenna Immunoassay System consists of 2 major steps: a modified microparticle (MP) based sandwich immunoassay followed by single-molecule counting (SMC) technology.



Results

Figure 3: Linearity and Accuracy of the Singulex Human and Mouse VEGF Assays. Recombinant human or mouse VEGF analyte was serially diluted and used to develop a standard curve for the hVEGF assay (A, B) or the mVEGF assay (C, D), respectively. The full linear range (A, C) and the low-end range (B, D) is shown for each assay. Error bars represent +/- Std Dev for the average determination resulting from triplicate measurements.

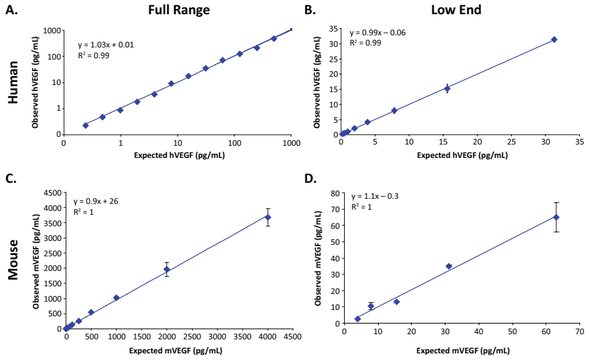


Table 1: Analytical Performance of the Singulex VEGF Assay Compared to an ELISA Assay. Recombinant human and mouse VEGF analyte was used to test the analytical performance of the Singulex hVEGF and mVEGF assays, respectively. Recovery of each assay was tested using corresponding spiked serum samples from humans and mice. Resulting analytical sensitivity, range, recovery and sample volume was compared to an ELISA assay for hVEGF and mVEGF from R&D Systems.

	Human VEGF		Mouse VEGF	
Manufacturer	**Singulex**	**R&D Systems**	**Singulex**	**R&D Systems**
LoD (pg/mL)	0.1	15.6	3.5	39
LLoQ (pg/mL)	0.3	31.2	5	39
ULoQ (pg/mL)	1000	2000	4000	2500
Spike Recovery	84–107%	—	68–111%	—
Sample Volume	100 µL	100 µL	100 µL	100 µL

Table 2: Precision of the Singulex Human and Mouse VEGF Assays. The Singulex hVEGF and mVEGF assays were independently tested for intra- and inter-assay variability using normal plasma samples collected from apparently healthy humans and mice, respectively.

	Human VEGF		Mouse VEGF	
Precision	**Intra-assay**	**Inter-assay**	**Intra-assay**	**Inter-assay**
Samples (n)	3	4	3	4
Replicates	18–21	3	18–21	3
Runs	---	7	---	6
Tested Range of [VEGF] pg/mL	1.9–12.6	29–87	300–635	826–2583
Range of %CV	7–12%	6–17%	14–16%	15–25%

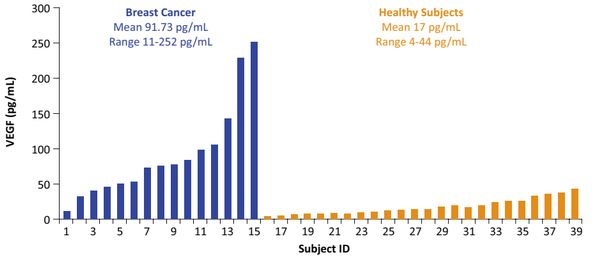
Table 3: Performance Comparison of the Singulex hVEGF Assay in Human Plasma Samples. Plasma samples from apparently healthy human subjects were tested in triplicate using the Singulex hVEGF assay (n=24) and an ELISA assay for hVEGF from R&D Systems (n=12).

	Singulex hVEGF	R&D Systems hVEGF
Samples Tested (n)	24	12
Mean [VEGF] in pg/mL (Range)	17 (4–44)	12.2 (5–44)
Mean %CV (Range)	6.9 (1–16)	8.1 (4–17)
% Detectable (>LoD)	100%	75% (9/12)
% Quantifiable (>LLoQ, <ULoQ)	100%	8% (1/12)

Table 4: Performance Comparison of the Singulex mVEGF Assay in Mouse Plasma Samples. Plasma samples from healthy CD-1 and Balb-C mice (n=20) were tested using the Singulex mVEGF assay (triplicate) and an ELISA assay for mVEGF from R&D Systems (duplicate).

	Singulex mVEGF		R&D Systems mVEGF	
Mouse Strain	CD-1	Balb-C	CD-1	Balb-C
Samples Tested (n)	20	20	20	20
Mean (Range) of [VEGF] in pg/mL	1112 (36–4000)	196 (92–1243)	696 (49–2500)	91 (49–582)
Mean %CV (Range)	15.5 (2–59)	8.4 (3–19)	---	---
% Detectable (>LoD)	100%	100%	100%	100%
% Quantifiable (>LLoQ, <ULoQ)	90%	100%	65% (12/20)	100%

Figure 4: Detection of Elevated hVEGF in Breast Cancer Subjects with the Singulex Assay. Plasma samples from breast cancer patients and apparently healthy control subjects were tested in triplicate with the Singulex hVEGF assay. The average [hVEGF] for each subject was determined.



Conclusions

- We show that the Singulex human and mouse VEGF Immunoassays detect VEGF at or below pg/mL levels, providing: ultra-sensitive quantification from:
  - 0.3 – 1000 pg/mL in humans
  - 5 – 4000 pg/mL in mice
- Robust detection in a broad range of specimen types, including plasma, tissue and cell lysates from mice and humans.
- Sensitivity and dynamic range to quantify VEGF across the full spectrum of disease states, from healthy to diseased subjects.
- These novel VEGF assays will be an important tool for investigating the diagnostic and prognostic value of VEGF as a tumor marker.