

DNA *in situ* Hybridizations for VEGFA Gene Locus (6p12) in Human Tumor Tissue

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[Abstract] Over the last decades numerous regulators of angiogenesis have been identified and characterized. Among the others the vascular endothelial growth factor (VEGFA) appears undoubtedly important in several pathophysiological processes. Moreover, VEGFA represents one of the most attractive targets of anticancer therapy, given its major role in the growth and development of different tumor types. Here we describe a method to detect the copy number variation (CNV) status of the VEGFA gene by fluorescence *in situ* hybridization (FISH). FISH analysis is a reliable method for investigating VEGFA amplification or increased gene copy number and may represent an alternative method to immunohistochemical analysis for investigating the deregulation of VEGFA expression levels.

Materials and Reagents

1. BAC clone No. RPCIB753M0921Q (GenomeCUBE)
2. LB medium (Sigma-Aldrich, catalog number: L3522-250G)
3. Large-construct kit (QIAGEN, catalog number: 12462)
4. SP6 (EuroFins MGW Operon LLC A Eurofins Genomics Company, catalog number: SP050-1) and T7 (EuroFins MGW Operon LLC A Eurofins Genomics Company, catalog number: SP401) primers
5. *AluI* restriction enzyme (Life Technologies, Invitrogen™, catalog number: 45200-029)
6. Cy3-dUTP (GE Healthcare Dharmacon, catalog number: 45-000-738)
7. BioPrime array CGH kit (module) (Life Technologies, Invitrogen™, catalog number: 18095-012) (it includes 2.5 Random primers solution, 10x dUTP nucleotide mix, Exo-Klenow fragment)
8. Amicon Ultra-0.5 centrifugal filter unit with Ultracel-30 membrane (Merck KGaA, catalog number: UFC503096)
9. Human Cot-1 DNA (Life Technologies, Invitrogen™, catalog number: 15279-011)
10. DAPI solution (1 µg/ml) (Abbott Laboratories, catalog number: 06J49-001)
11. Spectrum Green-labeled Chr6 centromeric probe (CEP6) and hybridization buffer (Abbott Laboratories, catalog number: 06J37-016 and 07J36-001)
12. Micron-centrifugal filters (Merck KGaA, catalog number: MRCF0R030)
13. TE Buffer, 1X Solution 500ml (USB, catalog number: 75893)
14. 20x SSC (Abbott Laboratories, catalog number: 02J10-032)

15. 0.3 % NP40 (Abbott Laboratories, catalog number: 07J05-001)
16. Pretreatment reagent VP 2000 (Abbott Laboratories, catalog number: 02J06-030)
17. Protease 1 (250 mg) (Abbott Laboratories, catalog number: 02J08-032)
18. Ethanol (Sigma- Aldrich, catalog number: 02860)
19. Rubber cement (Carfa AG, catalog number: 00494)
20. Protease 1 (250 mg in 500 ml protease buffer) (Sauter *et al.*, 1995)
21. Wash buffer (0.4 SSC + 0.3% NP40-pH 7-7.5) (Sauter *et al.*, 1995)

Equipment

1. Incubator for slides (Memmert GmbH + Co.KG, Mode: II IPP 300)
2. Pipette (Eppendorf)
3. Nanodrop spectrophotometer assay (Thermo Fisher Scientific, model: NanoDrop 2000)
4. Slide (Thermo Fisher Scientific, catalog number: No. 10143352)
5. Cover slide (Biosystem 24 x 50 mm, catalog number: 14071135638 and R. Langenbrinck 21 x 26 mm, catalog number: 01-2126)
6. Hotplate for denaturation (Medax GmbH & Co.KG, catalog number: 12895)
7. Zeiss fluorescence microscope using a 63x objective (Carl Zeiss AG, model: Axioskop 40)
8. AxioVision software (Carl Zeiss AG, model: SE64 Rel.4.9)

Procedure

A. How to make the probe

1. Inoculate starter culture with the BAC clone (*e.g.* glycerol stock) in 2-5 ml LB medium, containing the appropriate selective antibiotic (chloramphenicol).
2. Incubate over-night 25/30 °C, shaking at 200 rpm.
3. Dilute 0.5 ml of the starter culture in 500 ml selective LB medium (1/500 to 1/1,000), containing the appropriate antibiotic (chloramphenicol).
4. Incubate over-night with shaking (200 rpm).
5. Isolate the BAC-DNA using the Large-Construct Kit according to the manufacturer's instructions.
6. Verify BAC identity by sequencing using 1 µg of isolated DNA and 20 pmol of SP6, T7 primers, respectively.
7. Digest ~1 µg BAC-DNA with *A*/uI restriction enzyme according to the manufacturer's instructions.
8. Suspend the digested BAC-DNA in sterile water to a final volume of 21 µl (BioPrime array CGH kit).

9. Add 20 µl of 2.5X Random primers solution (BioPrime array CGH kit) to each sample and incubate at 95 °C for 5 min.
10. On ice, add 5 µl of 10xdUTP Nucleotide Mix (BioPrime array CGH kit).
11. Add 3 µl of Cy3-dUTP.
12. Add 1 µl of Exo-Klenow fragment (BioPrime array CGH kit).
13. Mix gently and perform a quick spin down (5 sec) and incubate at 37 °C for 2 h.
14. Add 5 µl of Stop Buffer to each tube and place on ice (BioPrime array CGH kit).

Labeling mix	
Reagent Added per Sample	Volume Added per Sample
2.5X Random primers solution (BioPrime array CGH kit) and incubate at 95 °C for 5 min	20 µl
On ice, 10x dUTP Nucleotide Mix	5 µl
Cy3-dUTP	3 µl
Exo-Klenow fragment	1 µl
Mix gently and perform a quick spin down (5 sec) then incubate at 37 °C for 2 h	
Stop Buffer to each tube and place on ice (long term storage at -20 °C)	5 µl

15. Label the Micron tubes on both the lid and the side with sample identifiers, as the lids tend to break. Also label the side of the filter with an identifier.
16. Load the Micron filter into the 1.5 ml tubes provided with the kit, so that the filter is sifting on the ridges inside the tube.
17. Pipette 450 µl of TE 1x into each filter.
18. Load the samples into the filters.
19. Centrifuge at 8,000 x g for 10 min at room temperature.
20. Check the filter (visual inspection by eyes) to make sure the Cy3-Dye labelled DNA has stuck to it.
21. Add 500 µl of TE to the filter and centrifuge at 8,000 x g for 10 min.
22. Discard the flow through and place in a new 1.5 ml filter tube upside-down to elute.
23. Centrifuge at 8,000 x g for 1 min.
24. Check the filter to make sure the entire sample is eluted from the filter (store samples short-term at 4 °C. Store long-term at -20 °C).
25. Add 20 µl of Human Cot-1 DNA.
26. Add 5 µl of CEP 6.
27. Add 35 µl of hybridization buffer (keep in ice or store at -20 °C).

B. How to prepare the tissue

28. Pretreat FFPE tissue microarrays or FFPE whole tissue sections (7 μ m thick) 10 min in the Xylene-1 min in 100% EtOH-1 min in 90% EtOH-1 min in 70% EtOH-1 min in H₂O.
29. Continue pretreating the tissue sections for 15 min in the pre-warmed paraffin pretreatment reagent VP2000 (80 °C).
30. Immerse the tissue sections for 1 min in the H₂O.
31. Immerse the tissue sections for 2 h into the pre-warmed 37 °C protease buffer (Protease 1 - 250 mg in 500 ml Protease Buffer).
32. Immerse the tissue sections for 1 min in the H₂O.
33. Immerse the tissue sections for 1 min in the 70% EtOH-1 min in the 90% EtOH-1 min in the 100% EtOH-1 min in the H₂O.
34. Let the tissue section air-dry at room temperature for approximately 10 min.

C. Hybridization

35. Apply 10-20 μ l of the probe on the tissue sections and cover it.
36. Apply the rubber cement to prevent the probe from leaking.
37. Leave the tissue sections for 20 min at 75 °C.
38. Incubate the tissue sections over-night at 37 °C.
39. Uncover the tissue sections.
40. Wash the tissue sections in the wash buffer at room temperature 5 min.
41. Wash the tissue sections in the wash buffer, pre-warmed at 48 °C between 2-5 min (when you have background wash longer and with higher temperature).
42. Immerse the tissue sections 1 min in the H₂O.
43. Let the tissue sections dry protecting it from the light.
44. Add 10-20 μ l of DAPI solution (1,000 ng/ml) to the tissue sections.
45. Cover the tissue sections with cover glass 24 x 50 mm.
46. Place the tissue sections 15 min at 4 °C.
47. Evaluate the tissue sections using a fluorescence microscope (63x objective).
48. Count a minimum of 100 tumor nuclei signals in four separate regions of the tissue section independently.

Representative data

FISH results (representative pictures are shown in Figure 1) were interpreted according to: (1) absolute Chr6 copy number and (2) the ratio VEGFA gene/Chr6 copy number. We classified as not amplified samples with a VEGFA/Chr6 ratio of <1.8 and an average VEGFA copy number <4.0 signals per cell; equivocal with a VEGFA/Chr6 ratio <2.0 and an average VEGFA copy number ≥ 4.0 and <6.0 signals per cell and amplified with a VEGFA/Chr6 ratio of ≥ 2.2 and an average VEGFA copy number of ≥ 6.0 signals per cell or a VEGFA/Chr6 ratio ≥ 2.2 with an average VEGFA copy number of ≥ 4.0 signals per cell, as

proposed by the ASCO/CAP guidelines for HER2 amplification in breast cancer (Wolff *et al.*, 2013). Polysomy of Chr6 was defined as an average of the Chr6 copy number. When the average was included between 2.26 and 3.75, the polysomy 6 was defined as low whereas, when the average was >3.75 the polysomy 6 was defined high (Andreozzi *et al.*, 2014; Sauter *et al.*, 1995; Wolff *et al.*, 2007; Salido *et al.*, 2005; Torrisi *et al.*, 2007; Tapia *et al.*, 2007; Ma *et al.*, 2005).

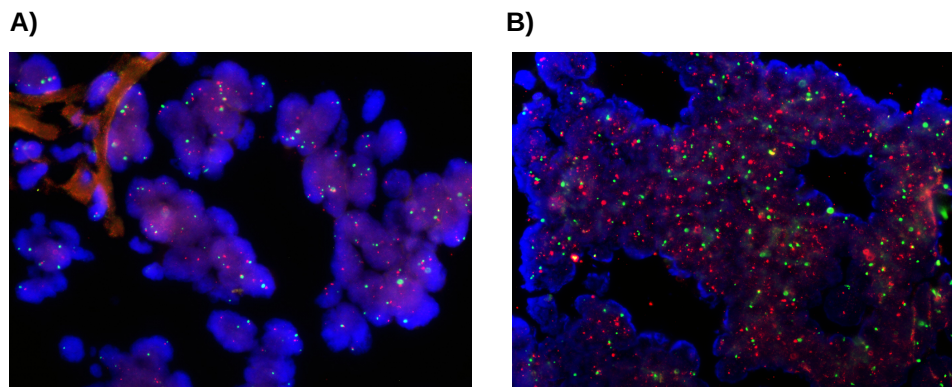


Figure 1. Fluorescence *in situ* hybridization (FITC+Rhodamine+DAPI) of the VEGFA gene locus 6p12 in colorectal cancer (CRC), A) non-amplified CRC sample, B) amplified CRC sample. Red dots highlight VEGFA gene, green dots highlight Chr6 centromere.

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