CANCER GENOMICS & PROTEOMICS *1*: 117-124 (2004)

Review

Real-time Quantitative PCR Arrays for Virally-associated Cancers

DIRK P. DITTMER, WOLFGANG VAHRSON, MICHELLE R. STAUDT, JAMES F. PAPIN, REBECCA HINES-BOYKIN and FARNAZ D. FAKHARI

Department of Microbiology and Immunology, The University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, U.S.A.

Abstract. *Virally-associated cancers are unique in that their origin is typically well defined and suitable to genomic analysis on a smaller scale. We recently reported the transcription profile of Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) in Kaposi's sarcoma (KS) using a real-time quantitative PCR (QPCR) array. This review explores the advantages and limitations of such an approach as well as the possibilities of extending PCR-based profiling to human cancers. Since real-time QPCR records a truly quantitative transcription profile, this technology will improve statistical analysis and solidify clinical decision-making.*

One quarter to one third of human cancers are associated with infectious agents (1). Generally, prolonged exposure of a particular microorganism leads to chronic inflammation, tissue remodeling and eventual neoplastic transformation. Such is the case in *Heliobacter pylori*-induced ulcers and gastric carcinoma. Chronic inflammation activates signaling pathways, such as the p53 pathway, that have traditionally been associated with spontaneous or virally-induced cancers (2). In most virally-associated cancers, for instance human papilloma virus (HPV)-associated cervical cancer, intracellular expression of the viral oncogenes alters these cellular signaling pathways (3). Viral gene products interfere with the host cells' ability to respond to genotypic or environmental stress, leading–eventually–to cell autonomous transformation. During the initial stages of tumor progression, clearance of virally-infected cells by the immune system or inhibition of viral oncogenes may stop the progression to a fully transformed cancer cell. This rationale drives the development of therapeutic anti-cancer vaccines and virus-specific, anti-cancer drugs (4). Hence, it is crucial to identify the viral proteins that are expressed in human tumors and to determine their mechanism of action.

Cancers of infectious etiology follow characteristic and statistically recognizable patterns. Figure 1, panel A plots the annual incidence rates for Kaposi's Sarcoma (KS) in the San Francisco/Bay area (USA) as collected in the Surveillance, Epidemiology and End Results (SEER) repository of the National Cancer Institute (USA). In the mid-nineteen eighties, incidence rates for KS showed a greater than exponential increase while incidence rates for other human cancers, in this example lung cancer (Figure 1, panel B), remained largely level. This marked increase in KS incidences, of course, signified the onset of the AIDS epidemic in the United States. KS is one of the original AIDS-defining conditions (5). One important characteristic for cancers of infectious etiology is that the incidence rate in any single year depends on the incidence rates in the preceding years, which correlates with the rate of spread of the infectious agent. During the observation period of the SEER study, KS incidence rates changed only in men, not in women. In contrast, the incidence rate changes over time for lung cancer were similar in men and women, even though the overall rate for lung cancer was higher in men. As seen in Figure 1, panel C, KS incidence rates per age group follow a bimodal distribution that peaks at ages 30-36 and increases again at ages >70 years. In contrast, incidence rates for most spontaneously occurring cancers increase exponentially with age as the number of spontaneous mutations accumulates (6). Cancers with a strong association to one genetically predisposing mutation,

Correspondence to: Dirk P. Dittmer, Department of Microbiology and Immunology, The University of Oklahoma Health Sciences Center, 940 Stanton L. Young Boulevard, Oklahoma City, OK 73104, U.S.A. Tel: 405-271-2133 ext 46645, Fax: 405-271-3117, e-mail: dirk-dittmer@ouhsc.edu

Key Words: Kaposi's Sarcoma, real-time PCR, transcription profiling, review.

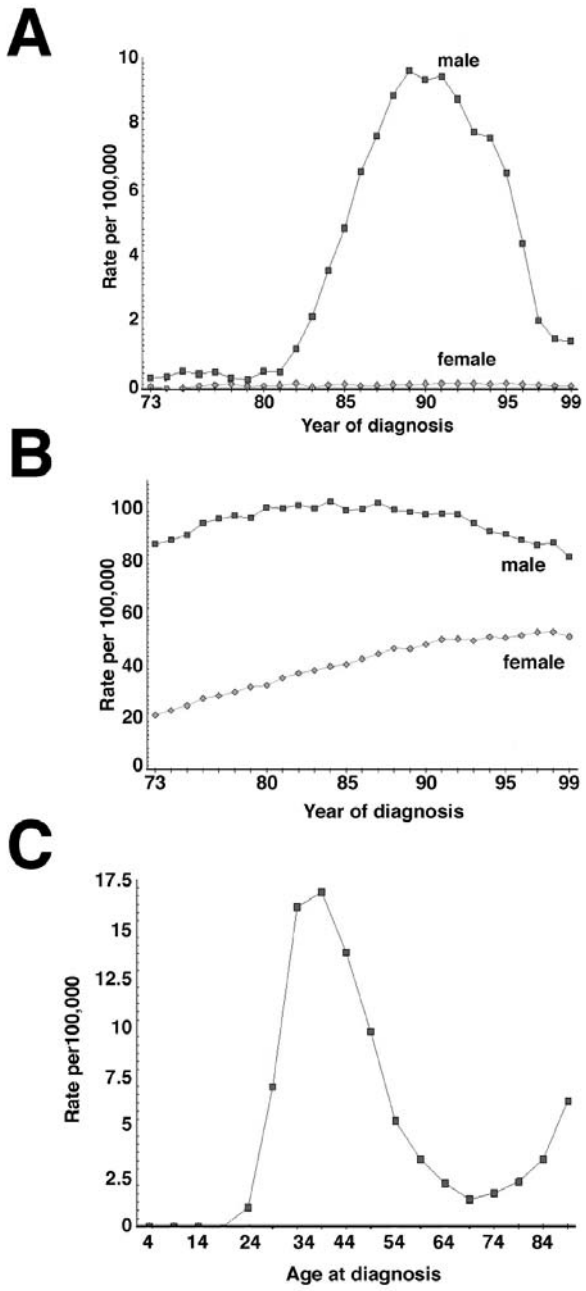

Figure 1. *A. Incidence rates of Kaposi's sarcoma in the US; B. Incidence rates of lung cancer in the US; C. Age-specific incidence rates of Kaposi's sarcoma in the US.*

such as loss of *Rb* alleles in the case of retinoblastoma (7), also exhibit a bimodal age distribution. However, in cases of genetic predisposition, a familial pattern is also present and the typical age of onset is early childhood. In contrast the age distribution of many virus-associated cancers correlates with sexual maturity. In the case of Kaposi's sarcoma-associated herpesvirus (KSHV), KS incidence rates correlated significantly with the lifetime number of male sexual partners (8), which immediately suggested a sexually-transmitted agent as the cause of this cancer.

Attempts to culture an infectious agent directly from KS tumors failed, but in 1994 Chang *et al.* (9) used representational difference analysis (RDA) to demonstrate the presence of a novel human herpesvirus in KS lesion that was not present in the normal skin of the same patient nor in KS-negative patients. The new virus was named Kaposi's sarcoma-associated herpesvirus (KSHV) or human herpesvirus 8 (HHV-8). The cloning of KSHV established a new paradigm for the discovery of uncultivatable agents (10, 11). Traditionally, infectious causes of diseases had to fulfill Koch's postulates: (a) to be found exclusively in the affected patients (cases), but not in unaffected control individuals (controls); (b) to be isolated and grown clonally in the laboratory and (c) the pure isolate should also be able to induce disease in the original or an experimental host. These rules have to be relaxed for the discovery of infectious causes or co-factors of cancer to succeed due to the long latency period between initial infection and clinically recognized malignancy. In contrast to acute infections, such as influenza or tuberculosis, people may carry an oncogenic virus for years prior to disease manifestation, although the KSHV viral load typically rises in the months that precede lesion formation (12, 13). We still require all cases of virally-induced cancers to contain the infectious agent, though more often at the early stages of tumor progression. At advanced stages, mutations in cellular genes may substitute for the viral oncoprotein, as for instance in EBV-negative Burkitt's lymphoma. KSHV was never successfully isolated from KS lesions and tumor explants routinely lose the virus after passage in culture, since *ex vivo* conditions select for aneuploid, highly neoplastic clones that no longer depend on viral oncogenes (14). Coincidentally, lymphoproliferative diseases, such as primary effusion lymphoma (PEL) or multicentric Castleman's disease (MCD) (5, 15), often accompany KS in AIDS patients. The lymphatic effusions harbor KSHV and maintain the virus upon continuous growth in culture (16-18).

The development of experimental models of cancers is essential for diagnostic, exploratory and drug-related research (19), yet few research endeavors are more time-consuming and less predictable. With some exceptions, human tumor viruses have homologous viruses in non-human primates (20, 21). Sometimes smaller experimental animals can be developed into a suitable model as well (22-24). Similar to transgenic mouse models of genetic alterations in human cancer (25), extensive validation is needed to establish animal models for virus-associated cancers.

High-throughput genomic profiling offers the chance to accelerate our investigations into viral-associated cancers as much as it has benefited research into cancers of hereditary

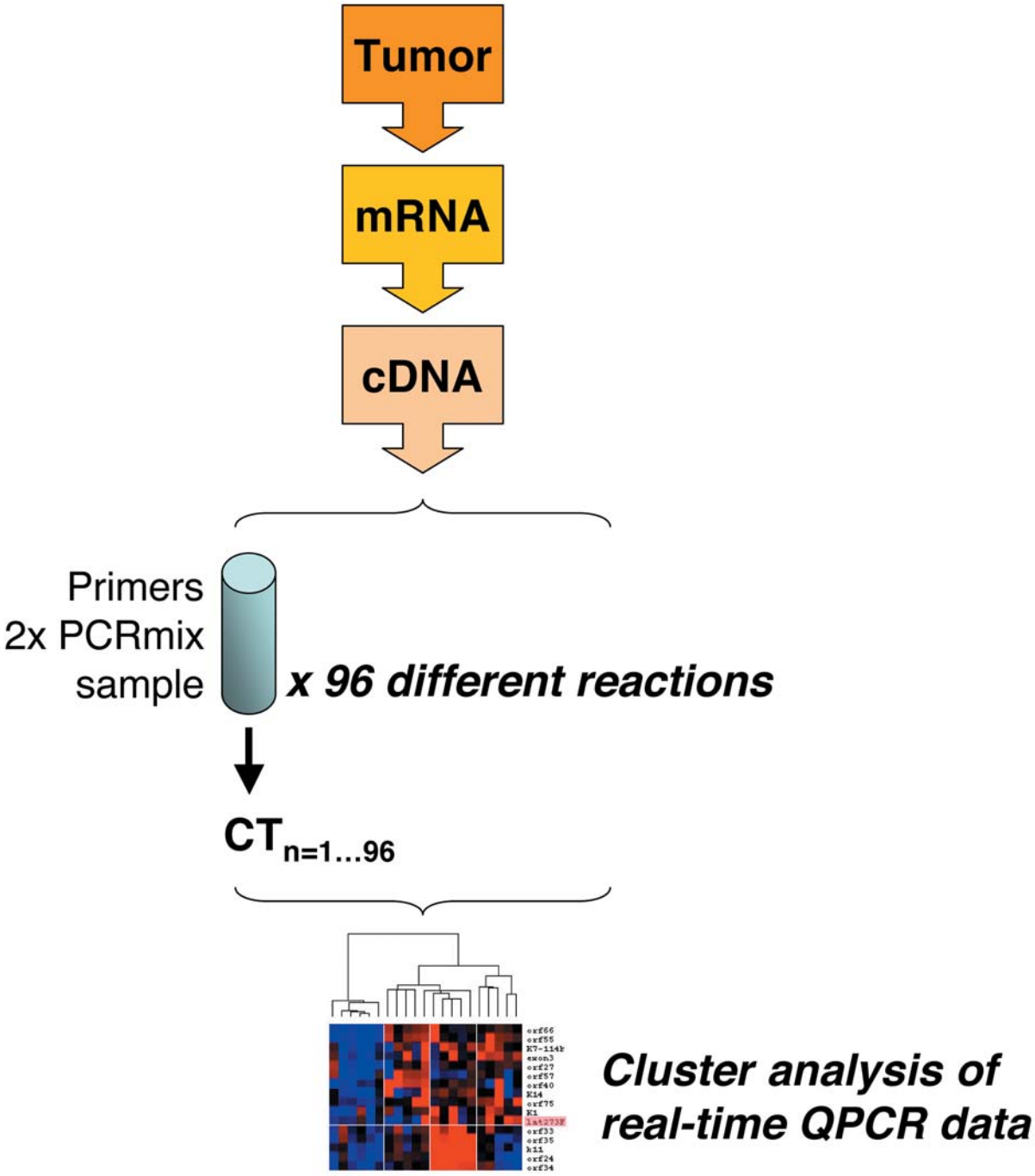

Figure 2. *Schematic of real-time QPCR set-up and array analysis.*

or spontaneous origin (26). When a virus (*e.g.* KSHV) has been established as a causative agent of a particular cancer (*e.g.* KS), the viral genome presents a well-defined target for genomic explorations. Since viral genomes are orders of magnitude smaller than the human genome, we have developed whole genome arrays based upon real-time quantitative PCR (QPCR) rather than hybridization to probes on solid support matrixes (27, 28). Instead of using real-time quantitative RT-PCR only as a means to confirm selected "hits" from large-scale cDNA-based array, oligonucleotide-based array, or Serial Analysis of Gene Expression- (SAGE) based screens, viral real-time quantitative PCR arrays

combine whole genome screening with truly quantitative determination of individual mRNA levels. This novel technique resulted in spectacular increases in throughput, sensitivity and specificity, which we detail here.

Many technological platforms are in use to measure changes in mRNA levels (reviewed in (29)). Most were developed with the long-term goal of assessing the transcription pattern of every gene for a particular organism. This, of course, is of utmost importance for cancer research where, in particular, changes in hitherto uncharacterized human genes or ESTs may identify novel biological pathways and novel targets for cancer therapy (26, 30, 31). Real-time quantitative PCR (QPCR) arrays, as we developed for KSHV, compress conventional array methodology into a technologically robust, rapid and inexpensive system for routine profiling. Today even smaller clinical and research centers have extensive experience in real-time QPCR, which has become routine for viral diagnostics and they are in a position to use QPCR-based expression arrays (32).

Performance parameters of real-time quantitative PCR arrays. The primary achievement of real-time QPCR is that, for the first time, PCR delivers reliable quantitative information without the need for dilution series or internal competitors, *etc.* The quantitative information can be extracted since the QPCR reaction is monitored in real-time. The reaction product is quantified at every cycle and only data points during exponential amplification are used to compute the initial target concentration (33).

Generally, transcriptional profiling by real-time QPCR proceeds as follows (see Figure 2): Real-time quantitative PCR measures the amount of PCR product at each cycle of the reaction either by binding of a fluorescent, double strand-specific dye (SYBRgreen™) or by hybridization to a third sequence-specific, dual-labeled fluorogenic oligonucleotide (molecular Beacon, TaqMan™). Since the introduction of real-time QPCR, many applications have arisen using this technology. Its kinetics and chemistries of real-time QPCR are covered in detail by Mackay *et al.* (32). The first step in setting up the reaction is creating the primer mix. The primer mix consists of both the forward and reverse primers at a concentration of 1pmol/µl. This primer mix is then combined with the SYBR Green 2xPCR mix (Applied Biosystems, CA, USA) to create the master mix. Often a final volume of 15µl will be sufficient and we have previously demonstrated its efficacy (27, 28). Depending on the individual equipment, smaller volumes may be possible, but we found that without automation the pipetting error becomes substantial. In comparison to the volume originally recommended by many manufacturers a smaller volume lowers the cost of QPCR per reaction by 70%. A single set of universal cycling conditions is used for all primers in the real-time QPCR array. 10 minutes at 95.0˚C, is used to activate the polymerase within the PCR mix (hot-start PCR). Phase two is the cycling or amplification phase of the reaction. During this phase the first step is a denaturing phase, 15 seconds at 95.0˚C and the sec step is a combined annealing and elongation phase, 1 min at 60.0˚C. This second phase is iterated for 40 cycles.

In adopting real-time QPCR to comparative transcription profiling for KSHV, we realized that we could feed the real-time QPCR output (the so-called "CT value") for all primers in the array directly into existing cluster analysis programs (34). In fact, the initial step in hybridization-based analysis is to compute the logarithm of the signal intensity in order to improve statistical performance. The real-time QPCR output (CT) already represents a logarithmic measure of the target concentration and can therefore be used directly.

In conventional PCR, different primer pairs perform with different amplification efficiencies and require different annealing temperatures for successful target amplification. In contrast, real-time QPCR primers are designed to fit very narrow performance criteria. This eliminates the need for computing actual mRNA levels or for recording an external standard curve for each primer pair. The primers in the KSHV-specific real-time QPCR arrays exhibited an average amplification efficiency (E) for each primer pair of $E=1.94\pm0.12$ ($n=91$), which is very close to the ideal amplification efficiency of $E=2$. The associated standard error across primers that are directed against the same target (purified viral linear genomic DNA) was 6% (unpublished observation). By contrast, cellular mRNA levels typically change several fold in response to specific stimuli. Hence, we conclude that for any target in the array, the biological variation associated with clinical specimens is well above the experimental error for this technology. In sum, real-time QPCR is a truly quantitative assay with a linear range of quantification over six orders of magnitude.

PCR is the most sensitive DNA and RNA detection method available today. It is inherently more sensitive than hybridization-based RNA detection methods. Thus far, we have been able to quantify 96 different viral mRNAs from a 2x2 mm fine-needle KS biopsy (28) or from as little as 5000 FACS-sorted cells (22). Real-time QPCR from a single cell or oocyte has been reported (35) and this technology has emerged as the assay of choice for single cell analyses after laser capture microscopy (36). Non-specific amplification methods are in use for conventional arrays (37). Conceivably, these genome-wide pre-amplification steps will boost the sensitivity of real-time QPCR arrays even higher. When using fluorescent hybridization probes (TaqMan™ or Beacon technology) to quantify real-time QPCR products non-specific signals were rarely encountered (22). Recent developments have further increased the signal-to-noise ratio of real-time QPCR (38-41). Another feature of real-

time QPCR lies in the ability to select primers that can detect splice-site-specific isoforms (22, 42). This is important, for instance, in evaluating the profile of VEGF in human cancer since different VEGF isoforms correspond to different malignant phenotypes (43-45).

It is unclear how many markers are needed to predict cancer phenotypes. For example, a single event–loss of p53–is sufficient to predict Li-Fraumini-Syndrome or in the case of MDR resistance to chemotherapy. In human lymphomas, changes in 10-60 mRNAs can predict long-term survival (for examples see (30, 46)). For most common cancers, changes in the expression of multiple genes are responsible for the cancer phenotype. Real-time QPCR arrays are easily scalable to analyze any number of mRNAs in parallel or multiples thereof per sample. This is in contrast to printed arrays, which always analyze the fixed number of genes in the array regardless of the experimental need. Real-time QPCR arrays can be used to investigate specific hypotheses with regard to the relevant predictors on study, rather than canvassing one standardized set of genes in all instances.

Application of real-time quantitative PCR arrays. The relatively small genome size ($\leq$100 open reading frames) of most cancer-associated viruses implies that the entire genome can be queried by real-time QPCR arrays. We have conducted such an inquiry with regard to KS and KSHV (27, 28) to address the same questions that others investigated in KSHV-associated lymphomas using hybridization-based cDNA arrays (47, 48) or Northern blots (49, 50): Which genes are actively transcribed in the tumor? How much do they vary between individuals? Can molecular profiling identify subclasses among tumors that are grouped based upon pathology review? What is the transcriptional response to a particular drug?

Because KS is characterized by high levels of inflammation and angiogenesis, prior attempts by us and others to extract enough RNA from KS biopsies for conventional analysis had failed. We were, however, able to extract sufficient RNA quantities from KS biopsies for real-time QPCR, since only minute amounts of input samples ($<$1 ng) are required and since 100-nucleotide amplification targets generally do not require long intact RNA molecules. Thus, we were able to successfully transfer real-time QPCR technology from the laboratory setting, which is characterized by unlimited sampled material, to the analysis of clinical biopsies (28). After analyzing 21 frozen 2x2-mm KS biopsies using our viral real-time QPCR arrays and public-domain cluster software (35), we were able to group otherwise identical KS tumors into molecularly distinct clusters based upon the degree of viral lytic gene expression.

The application of real-time QPCR array technology is not limited to small viral genomes but can be extended to the transcriptional profiling of human cancers; not as a primary, comprehensive tool for screening, but for follow-up and rapid diagnosis. A cursory review of existing micro-array studies revealed that $<$0.1 to 1% of genes in any given array exhibit $>$2 fold variation and were able to predict disease outcome, such as responder *vs*. non-responder or low-grade *vs*. high-grade tumor. For instance, Bohen *et al.* (52) evaluated 24 patients with follicular lymphoma with regard to their response to rituximab. After examining $>$30,000 mRNAs, they focused on 2037 mRNAs which showed $>$2-fold variation. Further comparison to normal spleen and tonsil tissue revealed that most mRNAs were, as expected, lymphoid-specific. In the end, only 98 genes showed a significant ($p \leq 0.005$ by Wilcox sum-rank test) correlation with clinical outcome. In this study the response to rituximab was determined by the gene expression profile at time of initial diagnosis, suggesting how array analysis can be incorporated in treatment decisions or in the assignment of patients to a particular clinical trial. Such small sets of "informative" mRNA markers are clearly different for each tumor type. No one would expect that the same genes that affect tumor progression in follicular lymphoma would necessarily be crucial to the development of breast cancer. However, we can envision custom-tailored real-time QPCR arrays (*e.g.* a "rituxomab array") in routine clinical diagnosis or for large-scale, multicenter phase III studies where comprehensive microarray analysis may not be economically feasible and, as exemplified above, may not yield any more statistically significant information.

Acknowledgements

Work on this project was supported by NIH grants CA97951 and EB00983 and a subcontract from the AIDS malignancy consortium (AMC), U.S.A.

References

1 Goedert JJ: Infectious Causes of Cancer. Totowa: Humana Press, 2000.
2 Cordon-Cardo C and Prives C: At the crossroads of inflammation and tumorigenesis. J Exp Med *190*: 1367-1370, 1999.
3 Werness BA, Levine AJ and Howley PM: Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science *248*: 76-79, 1990.
4 Lee RK, Cai JP, Deyev V, Gill PS, Cabral L, Wood C, Agarwal RP, Xia W, Boise LH, Podack E and Harrington WJ Jr: Azidothymidine and interferon-alpha induce apoptosis in herpesvirus- associated lymphomas. Cancer Res *59*: 5514-5520, 1999.
5 Antman K and Chang Y: Kaposi's sarcoma. N Engl J Med *342*: 1027-1038, 2000.
6 Fearon ER and Vogelstein B: A genetic model for colorectal tumorigenesis. Cell *61*: 759-767, 1990.
7 Knudson AG: Two genetic hits (more or less) to cancer. Nat Rev Cancer *1*: 157-162, 2001.
8 Martin JN, Ganem DE, Osmond DH, Page-Shafer KA, Macrae D and Kedes DH: Sexual transmission and the natural history of human herpesvirus 8 infection. N Engl J Med *338*: 948-954, 1998.

9 Chang Y, Cesarman E, Pessin MS, Lee F, Culpepper J, Knowles DM and Moore PS: Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma [see comments]. Science *266*: 1865-1869, 1994.

10 Relman DA: The identification of uncultured microbial pathogens. J Infect Dis *168*: 1-8, 1993.

11 Moore PS: New directions in molecular techniques for pathogen identification. Trends Microbiol *6*: 180, 1998.

12 Whitby D, Howard MR, Tenant-Flowers M, Brink NS, Copas A, Boshoff C, Hatzioannou T, Suggett FE, Aldam DM, Denton AS and *et al*: Detection of Kaposi sarcoma associated herpesvirus in peripheral blood of HIV-infected individuals and progression to Kaposi's sarcoma [see comments]. Lancet *346*: 799-802, 1995.

13 Yang J, Tao Q, Flinn IW, Murray PG, Post LE, Ma H, Piantadosi S, Caligiuri MA and Ambinder RF: Characterization of Epstein-Barr virus-infected B cells in patients with posttransplantation lymphoproliferative disease: disappearance after rituximab therapy does not predict clinical response. Blood *96*: 4055-4063, 2000.

14 Gallo RC: The enigmas of Kaposi's sarcoma. Science, 282: 1837-1839, 1998.

15 Ablashi DV, Chatlynne LG, Whitman JE Jr and Cesarman E: Spectrum of Kaposi's sarcoma-associated herpesvirus, or human herpesvirus 8, diseases. Clin Microbiol Rev *15*: 439-464, 2002.

16 Renne R, Zhong W, Herndier B, McGrath M, Abbey N, Kedes D and Ganem D: Lytic growth of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in culture. Nat Med *2*: 342-346, 1996.

17 Boshoff C, Gao SJ, Healy LE, Matthews S, Thomas AJ, Coignet L, Warnke RA, Strauchen JA, Matutes E, Kamel OW, Moore PS, Weiss RA and Chang Y: Establishing a KSHV+ cell line (BCP-1) from peripheral blood and characterizing its growth in Nod/SCID mice. Blood *91*: 1671-1679, 1998.

18 Cannon JS, Ciufo D, Hawkins AL, Griffin CA, Borowitz MJ, Hayward GS and Ambinder RF: A new primary effusion lymphoma-derived cell line yields a highly infectious Kaposi's sarcoma herpesvirus-containing supernatant [In Process Citation]. J Virol *74*: 10187-10193, 2000.

19 Teicher B: (ed.) Tumor models in cancer research. Totowa: Humana Press, 2002.

20 Whitby D, Stossel A, Gamache C, Papin J, Bosch M, Smith A, Kedes DH, White G, Kennedy R and Dittmer DP: Novel Kaposi's sarcoma-associated herpesvirus homolog in baboons. J Virol *77*: 8159-8165, 2003.

21 Damania B and Desrosiers RC: Simian homologues of human herpesvirus 8. Philos Trans R Soc Lond B Biol Sci *356*: 535-543, 2001.

22 Dittmer D, Stoddart C, Renne R, Linquist-Stepps V, Moreno ME, Bare C, McCune JM and Ganem D: Experimental Transmission of Kaposi's Sarcoma-associated Herpesvirus (KSHV/HHV-8) to SCID-hu Thy/Liv Mice. J Exp Med *190*: 1857-1868, 1999.

23 Virgin HW t, Latreille P, Wamsley P, Hallsworth K, Weck KE, Dal Canto AJ and Speck SH: Complete sequence and genomic analysis of murine gammaherpesvirus 68. J Virol *71*: 5894-5904, 1997.

24 Usherwood EJ, Stewart JP and Nash AA: Characterization of tumor cell lines derived from murine gammaherpesvirus-68-infected mice. J Virol *70*: 6516-6518, 1996.

25 Van Dyke T and Jacks T: Cancer modeling in the modern era: progress and challenges. Cell *108*: 135-144, 2002.

26 Staudt LM: Gene expression profiling of lymphoid malignancies. Annu Rev Med *53*: 303-318, 2002.

27 Fakhari FD. and Dittmer DP: Charting Latency Transcripts in Kaposi's Sarcoma-Associated Herpesvirus by Whole-Genome Real-Time Quantitative PCR. J Virol *76*: 6213-6223, 2002.

28 Dittmer DP: Transcription profile of Kaposi's sarcoma-associated herpesvirus in primary Kaposi's sarcoma lesions as determined by real-time PCR arrays. Cancer Res *63*: 2010-2015, 2003.

29 Bowtell D and Sambrook J DNA Microarrays: A molecular Cloning manual, p. 712. Cold Spring Harbor: Cold Spring HarborLaboratory Press, 2003.

30 Rosenwald A, Wright G, Chan WC, Connor JM, Campo E, Fisher RI, Gascoyne RD, Muller-Hermelink HK, Smeland EB, Giltnane JM, Hurt EM, Zhao H, Averett L, Yang L, Wilson WH, Jaffe ES, Simon R, Klausner RD, Powell J, Duffey PL, Longo DL, Greiner TC, Weisenburger DD, Sanger WG, Dave BJ, Lynch JC, Vose J, Armitage JO, Montserrat E, Lopez-Guillermo A, Grogan TM, Miller TP, LeBlanc M, Ott G, Kvaloy S, Delabie J, Holte H, Krajci P, Stokke T and Staudt LM: The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. N Engl J Med *346*: 1937-1947, 2002.

31 Alter O, Brown PO and Botstein D: Singular value decomposition for genome-wide expression data processing and modeling. Proc Natl Acad Sci USA *97*: 10101-10106, 2000.

32 Mackay IM, Arden KE and Nitsche A: Real-time PCR in virology. Nucleic Acids Res *30*: 1292-1305, 2002.

33 Heid CA, Stevens J, Livak KJ and Williams PM: Real time quantitative PCR. Genome Res *6*: 986-994, 1996.

34 Eisen MB, Spellman PT, Brown PO and Botstein D: Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA *95*: 14863-14868, 1998.

35 Steuerwald N, Cohen J, Herrera RJ and Brenner CA: Quantification of mRNA in single oocytes and embryos by real-time rapid cycle fluorescence monitored RT-PCR [In Process Citation]. Mol Hum Reprod *6*: 448-453, 2000.

36 Barozzi P, Luppi M, Facchetti F, Mecucci C, Alu M, Sarid R, Rasini V, Ravazzini L, Rossi E, Festa S, Crescenzi B, Wolf DG, Schulz TF and Torelli G: Post-transplant Kaposi sarcoma originates from the seeding of donor-derived progenitors. Nat Med *9*: 554-561, 2003.

37 Matz, M, Shagin, D, Bogdanova, E, Britanova, O, Lukyanov, S, Diatchenko, L and Chenchik, A. Amplification of cDNA ends based on template-switching effect and step-out PCR. Nucleic Acids Res *27*: 1558-1560, 1999.

38 Kutyavin IV, Afonina IA, Mills A, Gorn VV, Lukhtanov EA, Belousov ES, Singer MJ, Walburger DK, Lokhov SG, Gall AA, Dempcy R, Reed MW, Meyer RB and Hedgpeth J: 3'-minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. Nucleic Acids Res *28*: 655-661, 2000.

39 Afonina I, Zivarts M, Kutyavin I, Lukhtanov E, Gamper H and Meyer RB: Efficient priming of PCR with short oligonucleotides conjugated to a minor groove binder. Nucleic Acids Res *25*: 2657-2660, 1997.

40 Buhr CA, Wagner RW, Grant D and Froehler BC: Oligodeoxynucleotides containing C-7 propyne analogs of 7-deaza-2'-deoxyguanosine and 7-deaza-2'-deoxyadenosine. Nucleic Acids Res *24*: 2974-2980, 1996.

41 Wagner RW, Matteucci MD, Lewis JG, Gutierrez AJ, Moulds C and Froehler BC: Antisense gene inhibition by oligonucleotides containing C-5 propyne pyrimidines. Science *260*: 1510-1513, 1993.

42 Dittmer D, Lagunoff M, Renne R, Staskus K, Haase A and Ganem D: A cluster of latently expressed genes in Kaposi's sarcoma-associated herpesvirus. J Virol *72*: 8309-8315, 1998.
43 DePrimo SE, Wong LM, Khatry DB, Nicholas SL, Manning WC, Smolich BD, O'Farrell AM and Cherrington JM: Expression profiling of blood samples from an SU5416 Phase III metastatic colorectal cancer clinical trial: a novel strategy for biomarker identification. BMC Cancer *3*: 3, 2003.
44 Ancelin M, Buteau-Lozano H, Meduri G, Osborne-Pellegrin M, Sordello S, Plouet J and Perrot-Applanat M: A dynamic shift of VEGF isoforms with a transient and selective progesterone-induced expression of VEGF189 regulates angiogenesis and vascular permeability in human uterus. Proc Natl Acad Sci USA *99*: 6023-6028, 2002.
45 Olofsson B, Pajusola K, von Euler G, Chilov D, Alitalo K and Eriksson U: Genomic organization of the mouse and human genes for vascular endothelial growth factor B (VEGF-B) and characterization of a second splice isoform. J Biol Chem *271*: 19310-19317, 1996.
46 Shipp MA, Ross KN, Tamayo P, Weng AP, Kutok JL, Aguiar RC, Gaasenbeek M, Angelo M, Reich M, Pinkus GS, Ray TS, Koval MA, Last KW, Norton A, Lister TA, Mesirov J, Neuberg DS, Lander ES, Aster JC and Golub TR: Diffuse large B-cell lymphoma outcome prediction by gene-expression profiling and supervised machine learning. Nat Med *8*: 68-74, 2002.
47 Paulose-Murphy M, Ha NK, Xiang C, Chen Y, Gillim L, Yarchoan R, Meltzer P, Bittner M, Trent J and Zeichner S: Transcription program of human herpesvirus 8 (kaposi's sarcoma-associated herpesvirus). J Virol *75*: 4843-4853, 2001.
48 Jenner RG, Alba MM, Boshoff C and Kellam P: Kaposi's sarcoma-associated herpesvirus latent and lytic gene expression as revealed by DNA arrays. J Virol *75*: 891-902, 2001.
49 Sarid R, Flore O, Bohenzky RA, Chang Y and Moore PS: Transcription mapping of the Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) genome in a body cavity-based lymphoma cell line (BC-1). J Virol *72*: 1005-1012, 1998.
50 Zhong W, Wang H, Herndier B and Ganem D: Restricted expression of Kaposi sarcoma-associated herpesvirus (human herpesvirus 8) genes in Kaposi sarcoma. Proc Natl Acad Sci USA *93*: 6641-6646, 1996.
51 Bohen SP, Troyanskaya OG, Alter O, Warnke R, Botstein D, Brown PO and Levy R: Variation in gene expression patterns in follicular lymphoma and the response to rituximab. Proc Natl Acad Sci USA *100*: 1926-1930, 2003.

Received November 5, 2003
Revised February 25, 2004
Accepted February 28, 2004