Protocol

IN-SITU AMPLIFICATION & HYBRIDIZATION

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FORWARD

When the first papers on the polymerase chain reaction were appearing in the mid-1980's, Omar Bagasra says his first reaction was, "I've got to do this inside a cell!" However, at that time the details of the PCR technique were not well-developed, and very few thought that the structure of a cell could possibly survive the brutal treatment inflicted by repeated denaturation. Yet Omar Bagasra and several others—notably Ashley Haase of the University of Minnesota, Gerald Nuovo of SUNY–Stony Brook, and Steve Wolinsky of Northwestern University—persisted. By 1990, successful protocols had been developed, and the technique of *in-situ* amplification-hybridization was born.

Since that time, the company for which I work—a thermal cycler manufacturer—has been steadily receiving requests for information on the *in situ* technique. At first, the requests were few but eventually quite a number started to roll in from Europe, and now, requests—demands, even—come into our offices continuously. However, due to the constraints of patent and copyright law, our company has been frequently unable to provide the detailed information scientists need. Professor Bagasra too has been bombarded by inquiries, but his laboratory is focused on academic and clinical research—not on processing a plethora of faxed inquiries.

Nor has the peer-reviewed scientific press done the best job in keeping researchers informed of the latest work. Legitimate concerns over the validity of the *in situ* amplification technique have mushroomed into delay after delay in the review process. As a result, in a field that is advancing by the hour, the most recent protocols generally available are over a year old. Furthermore, because the format of many journals does not permit sufficient space to detail the actual methodology used to obtain the published results, the reproducibility of the data itself is a matter of controversy.

Therefore, Omar Bagasra and Thikkavarapu Seshamma have decided to utilize the method of communication that has served the scientific community so well during the nineteenth and early twentieth centuries—the privately-published scientific pamphlet. Care has been taken not to publish any data (except for illustrative purposes) or to draw any conclusions—communications of these sorts are best conducted through the peer-reviewed journals. Rather, the purpose of this document is to provide a detailed description of the methods used by Drs. Bagasra and Seshamma to obtain the rather extraordinary results which have been coming from their laboratory at the Thomas Jefferson School of Medicine.

It is our sincere hope that the prompt communication of this information will help bring to researchers worldwide an exciting new tool that has already significantly advanced the scientific understanding of life processes.

John Hansen
Cambridge, Massachusetts

I: PREPARATION OF SLIDES-SILANATION

1) Prepare the following 2% AES solution just prior to use:

• 3-aminopropyltriethoxysilane (AES: Sigma A-3648)	5 ml
• Acetone	250 ml

2) Dip glass slides (further described in the *Materials & Methods* section) in 2% AES (250 ml) for 60 seconds.

3) Dip slides in distilled water (1000 ml) five times.

4) Repeat step #3 three times, changing the water.

5) Air dry in laminar-flow hood overnight, then store slides at room temperature. (*N.B. Do not substitute other silanes for AES!*)

II: PREPARATION OF CELL SUSPENSION

To use peripheral blood leukocytes, first isolate cells on a Ficoll-Hypaque density gradient. Tissue-culture cells or other single-cell suspensions can also be used. Prepare all cell suspensions with the following procedure:

1) Wash cells with 1X PBS twice.

2) Resuspend cells in PBS at 2 X 10^6 cells/ml.

3) Add 10 µl of cell suspension to each well of the slide using a P20 micropipette.

4) Air dry slide in a laminar-flow hood.

III: PREPARATION OF PARAFFIN-FIXED TISSUE

Routinely-fixed paraffin tissue sections can be also be used. This permits the evaluation of individual cells in the tissue for the presence of a specific RNA or DNA sequence. For this purpose, tissue sections are placed on specially-designed slides that have simple wells (described further in the *Materials & Methods* section). In our laboratory, we routinely use placental tissues, CNS tissue, etc., which are sliced to

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1) Place tissue section upon the glass surface of the slide.

2) Incubate the slides in an oven @ 60°C for 2 hours, to melt the paraffin.

3) Dip the slides in Xylene solution for 5 minutes, then in 100% ethanol for 5 minutes.

4) Repeat step three once.

5) Dry the slides in an oven @ 80°C for 2 hours.

IV: *IN SITU* AMPLIFICATION, RNA & DNA

Heat Treatment

The slides prepared above are placed on a heat-block at 105°C for 90 - 120 seconds—***this step is absolutely critical,*** for it stabilizes the cells or tissue on the glass surface of the slide.

Fixation & Washes

1) Place the slides overnight in a solution of 2% paraformaldehyde in PBS (pH 7.4) at room temperature.

2) Wash the slides once with 3X PBS for 10 minutes, agitating periodically with an up and down motion.

3) Wash the slides twice with 1X PBS for 10 minutes each time, agitating periodically with an up and down motion.

If biotinylated probes or peroxidase-based color development are to be used, the samples should further be treated with a 0.3% solution of hydrogen peroxide in PBS, in order to inactivate any endogenous peroxidase activity. Once again, incubate the slides overnight—either at 37° C or at room temperature. Then, wash the slides once with PBS. If other probes are to be used, proceed directly to the following proteinase K treatment, which is a *very* critical step.

Proteinase K Treatment

1) Treat samples with 6 µg/ml proteinase K in PBS for 15 to 60 minutes at room temperature or at 55°C*. To make a proper solution, dilute 1.0 ml of proteinase K @ (1 mg/ml) in 150 ml of 1X PBS.

2) After 15 minutes, look at the cells under the microscope at 400X. If the majority of the cells-of-interest exhibit uniform-appearing, small, round "bubbles" or "blobs" on the cytoplasmic membrane, then stop the treatment immediately with Step #3. Otherwise, continue treatment for another 15 minutes and re-examine *(see Figure 1 on the inside front cover to see typical cytoplasmic membranes before PK treatment, and Figure 2 to see membranes exhibiting "bubbles" after PK treatment).*

3) Heat on a block at 95°C for 2 minutes to inactive the proteinase K.

4) Rinse in 1X PBS for 10 seconds.

5) Rinse in distilled water for 10 seconds.

6) Air dry.

*Footnote: The time and temperature of incubation should be optimized for each cell line or tissue-section type. That way, the results of in situ amplification will be consistent among various types of cells and tissues. Typically, lymphocytes will require 30 - 45 minutes @ 55° or room temperature, CNS tissue will require 10-12 minutes at room temperature, and paraffin-fixed tissue will require between 20 - 40 minutes at room temperature. However, these periods vary widely and the appearance of the "bubble" is the important factor. Unfortunately, the appearance of the "bubble" is less prominent in paraffin sections.

In Situ RNA Amplification:

Note: All reagents for RT-amplification should be prepared with RNase-free water (i.e. DEPC-treated water).

For *in situ* RNA amplification, the cells are treated with a DNase solution after the proteinase K treatment. This step destroys all of the endogenous DNA in the cells so that only RNA survives to provide signals for amplification.

Prepare a RNase-free, DNase solution:

- 40 mM Tris Hcl, pH 7.4
- 6 mM $MgCl_2$
- 2 mM $CaCl_2$
- 1 U/µl final volume of DNase *(RNase free, such as RQ1 DNase from Boehringer)*

1) Add 10 µl of solution to each well.

2) Incubate the slides overnight @ 37° in a humidified chamber.

3) After incubation, rinse the slides with a similar solution that was prepared ***without*** the DNase enzyme.

4) Wash the slides twice with DEPC-treated water.

Reverse Transcriptase Reaction:

Next, one wishes to make DNA copies of the targeted RNA sequence so that the signal can be amplified. The following is a typical cocktail for this reverse-transciptase reaction:

• 10 X Reaction buffer (see below)	2.0 µl
• 10 mM dATP	2.0 ul
• 10 mM dCTP	2.0 ul
• 10 mM dGTP	2.0 ul
• 10 mM dTTP	2.0 µl
• RNasin inhibitor @ 40 u/µl (Promega)	0.5 µl
• 20 µM downstream primer	1.0 µl
• AMVRT 20 U/µl	0.5 µl
• DEPC-water	8.0 µl
Total Volume:	20.0 µl

10X Reaction Buffer: 100 mM Tris pH 8.3, 500 mM KCl, 15 mM $MgCl_2$.

1) Add 10 µl of the cocktail to each well. Carefully place the coverslip on top of the the slide .

2) Incubate @ 42°C or 37°C for 1 hour in a humidified atmosphere.

3) Incubate slides @ 92° C for five minutes.

3) Remove coverslip and wash twice with distilled water. Proceed with the amplification procedure below, which is the same for both DNA- & RNA-based protocols:

Amplification Protocol:

Prepare an amplification cocktail containing the following: 1.25 µM of each primer, 10 µM (each) dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl2, and 0.1 unit/µl Taq polymerase. The

following is a convenient recipe that we use in our laboratory:

• 25 µM forward primer (SK 38)	5.0 µl
• 25 µM reverse primer (SK 39)	5.0 µl
• 1.0 mM dNTP	1.0 µl
• 1.0 M Tris-HCl pH 8.3	1.0 µl
• 1.0 M KCL	5.0 µl
• 100 mM MgCl2	2.5 µl
• H_2O	78.5 µl
• Taq pol (Ampli-Taq 5 u/µl)*	2.0 µl
Total Volume:	100 µl

* Note: Other thermostable polymerase enzymes have also been used quite successfully.

1) Layer 8 µl of PCR solution onto each well with a P20 micropipette so that the whole surface of the well is covered with the solution. Be careful—do not touch the surface of the slide with the tip of the pipette.

2) Add a glass coverslip (20 X 60 mm) and carefully seal the edge of the coverslip to the slide with clear or colored nail polish. The polish must *completely* seal the coverslip-slide assembly in order to form a small reaction "chamber" that can contain the water vapor during thermal cycling. Proper sealing is very important, for this keeps reaction concentrations consistent through the thermal cycling procedure, and concentrations are critical to proper amplification. However, be certain to apply the nail polish very carefully so that none of the polish gets into the actual chamber where the cells or tissue reside. If any nail polish does enter the chamber, discard that slide for the results will be variable.

In the case of tissue sections, it is best to use another identical blank slide for the cover instead of a coverslip. Apply the amplification cocktail to the appropriate well of the blank slide, place an inverted tissue-containing slide atop the the blank slide, and seal the edges completely with nail polish. Invert the slide once again so that the tissue-containing slide is on the bottom.

2) Place slides on a heat-block @ 92° C for 90 seconds.

4) Cover the block of the standard thermocycler with one layer of aluminum foil, in order to better distribute the heat. Fit slides atop the block so that the areas of the slide that

contain the tissue will be heated evenly.

Note: Thermocyclers specifically designed for slide use are now available; our laboratory uses an MJ Research PTC-100-60 (with aluminum foil or a Slide Griddle™) or an MJ Research PTC-100-12MS (which is dedicated to hold twelve slides).

5) If the thermocycler being used is a standard, block-type instrument, insulate the slides with about ten layers of paper towel placed on top, then add a weight of about 0.5 kg to press the slides onto the top surface of the block. With a specially designed thermocycler, follow manufacturer's instructions.

6) Run 30 cycles of the following amplification protocol:

94°C	30 seconds
45°C	1 minute
72°C	1 minute

Note: These temperatures may require optimization for the specific thermocycler being used.

7) After the thermal cycling is complete, dip slides in 100% EtOH for at least 5 minutes, in order to dissolve the nail polish. Pry off the coverslip using a razor or other fine blade—the coverslip generally pops off quite easily. Scratch off any remaining nail polish on the outer edges of the slide, so that fresh coverslips will lay evenly in the subsequent steps.

8) Place slides on a heat-block @ 92°C for 1 minute—this treatment helps immobilize the intracellular signals.

9) Wash slides with 2X SSC (see *Materials & Methods* section) at room temperature for 5 minutes.

The amplification protocol is now complete and one can proceed to the labeling/hybridization protocols.

V. LABELING OLIGONUCLEOTIDE PROBE:

Many laboratories wish to use a radioactively-labeled probe, and the following a typical procedure for the ^{33}P labeling of a probe through a kinase reaction:

• 2 µM probe (SK 19)	1.0 µl
• 10 X kinase buffer	2.0 µl
• ATP γ^{33}P (Amersham 10 µCi/ml)	1.0 µl
• H_2O	15 µl
• Polynucleotide kinase (10 µ/ml)	1 µl
Total Volume:	20 µl

1) Incubate at 37°C for 30 minutes.

2) Apply sample to 0.8 ml Sephadex C-50 column (e.g. QuickSpin™ from Boehringer)

3) Elute with TE buffer.

Fraction #1	300 µl
Fraction #2	100 µl
Fraction #3	100 µl
Fraction #4	100 µl
Fraction #5	100 µl
Fraction #6	100 µl

4) Count the radioactivity in 1.0 µl of each fraction. The labelled probe should be contained in fraction 2 through fraction 4.

Nonradioactive labelling-tailing with DIG-11-dUTP

Many laboratories—including our own—prefer to use non-radioactive probes, for this results in less hazardous waste, less bureaucratic procedures, and lower costs (especially when one considers the perishable nature of radioactive isotopes and probes). The following is a typical labeling procedure:

• 20 µM Probe	10 µl
• 5 X Tailing buffer (see below)	10 µl
• 25 mM $CaCl_2$	20 µl
• 2.5 mM dATP (Tris buffer pH 7.5)	3.5 µl
• 1 mM DIG-11-dUTP	1.0 µl
• H_2O	4.5 µl
• Terminal Transferase (25 u/µl)	1.0 µl
Total Volume:	50 µl

Tailing Buffer (5X): 1 mM potassium cacodylate, 125 mM Tris-HCl pH 6.6 & 1.25 mg/ml BSA

Incubate reaction mixture @ 37°C for 15 minutes, then purify the labelled probe as for ^{33}P probe, using chromogen indicator instead of radioactive detectors.

We prefer to use biotinylated probes, which we purchase already conjugated. These probes are usually not tail-end conjugated; rather, they had been conjugated during the oligo-synthesis procedure. Thus, they may contain multiple molecules of biotin instead of one at the tail end—this makes the probes more sensistive.

Probes are good for up to one year @ -70°C.

VI: HYBRIDIZATION:

Prepare a solution containing: 20 pg/ml of the appropriate probe, 50% deionized formamide, 2X SSC buffer, 10X Denhardts solution, 0.1% sonicated salmon sperm DNA, and 0.1% SDS. The following is a convenient recipe:

• Probe (^{33}P-*labeled, biotinylated, or digoxigenin)*	2 µl
• Deionized formamide	50 µl
• 20X SSC*	10 µl
• 50X Denhardts solution	20 µl
• 10 mg/ml ssDNA*	10 µl
• 10% SDS	1 µl
• H_2O	7 µl
Total Volume:	100 µl

* Footnote: See *Materials & Methods* section for prepartion of 20X SSC buffer; the salmon sperm should be denatured @ 94°C for ten minutes before it is added to the hybridization buffer.

Note: 2% BSA can be added if one is observing non-specific binding. For this purpose, one can add 10 µl of 20% BSA solution and reduce the amount of water.

1) Add 10 µl of hybridization mixture to each well and add coverslips.

2) Heat slides on a block @ 95°C for 5 minutes.

3) Incubate slides @ 48°C for 2 to 4 hours in a humidified atmosphere.

Note: The optimal hybridization temperature is a function of the T_m (melting temperature) of the probe. This must be calculated for each probe (*standard formulae are generally available).*

Post-Hybridization procedure for ^{33}P Probe:

1) Wash slides in 2 X SSC for 5 minutes.

2) Dip slides in 3 X nuclear tract emulsion (Kodak NBT-2, diluted 1:1 with water).

NOTE: Steps 3-5 should be carried out in the dark:

3) Slides are air dried, then incubated for 3-10 days in light proof box with a drying agent.

4) Slides are developed for 3 minutes in Kodak D-19 developer, then rinsed in H_2O.

5) Slides fixed for 3 minutes in Kodak Unifax.

6) Slides are counter stained with May Grunewald Giemsa.

Post-Hybridization for Biotinylated Probe:

1) Wash slides in 1X PBS twice for 5 minutes each time.

2) Add 10 µl of streptavidin-peroxidase complex (100 µg/ml in PBS pH 7.2). Gently apply the coverslips.

3) Incubate slides @ 37°C for 1 hour.

4) Remove coverslip, wash slides with 1X PBS twice for 5 minutes each time.

5) Add to each well 100 µl of 3'-amino-9-ethylene carbozole in the presence of 0.03% hydrogen peroxide in 50 mM acetate buffer (pH 5.0).

6) Incubate slides @ 37°C for 10 minutes to develop the color—this step should be carried out in the dark. After this period, observe slides under a microscope. If color is not strong, develop for another 10 minutes.

7) Rinse slides with tap water and allow to dry.

8) Add 1 drop of 50% glycerol in PBS and apply the

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coverslips.

9) Analyze with optical microscope—positive cells will be stained a brownish red.

Post-Hybridization for Digoxigenin Probe:

1) Wash the slides in buffer A for five minutes *(see note below)*.

2) Wash slides in buffer B *(see note below)* + 2% BSA and 0.3% Triton for 30 minutes.

3) Incubate in buffer A + 1% BSA, 0.1% Triton + alkaline phosphatase labelled anti-digoxigenin antibody for two hours.

4) Wash the slides three times with buffer A for 10 minutes each wash.

5) Incubate slides at room temperature in buffer B for 2 minutes.

6) Incubate the slides ***in the dark*** at room temperature in buffer B to which has been added 0.30 mg/ml NBT, 0.2 mg/ml BCIP for 1 -6 hours

7) Rinse with H_2O.

8) Counter stain with 0.1% methyl-green for 1 - 6 seconds at the dilution recommended by the supplier.

9) Mount slides.

Buffer A: 100 mM Tris-HCI pH 7.5, 150 mM NaCl.
Buffer B: 100 mM Tris-HCI pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$.
NBT: 4-nitro blue tetraazolium chloride.
BCIP: 5-bromo-4-chloro-3-indolyl-phosphate.

VII: VALIDATION & CONTROLS

The validity of *in situ* amplification-hybridization should be examined in every run. To validate the amplification procedure and to confirm the efficiency of amplification, we routinely run at least two sets of experiments simultaneously. Furthermore, we generally use slides that have three individual wells so a single slide can have one experiment with two controls.

In our own laboratory, we frequently work with HIV. We always mix HIV-1 infected cells plus HIV-1 uninfected cells in a known proportion (i.e. 1:10, 1:100, etc.), then we confirm that the results are appropriately proportionate. To examine the efficiency of amplification, we use a cell line which carries a single copy or two copies of cloned HIV-1 virus, then look to see that proper amplification and hybridization has occured.

In addition, in all amplification procedures, we use one slide as a control for nonspecific binding of the probe. Here we hybridize the amplified cells with an unrelated probe. We also use HLA-DQα probes and primers with human peripheral blood mononuclear cells (PBMC) as positive controls, to check various parameters of our system.

We suggest that researchers carefully design and employ appropriate positive and negative controls for their specific experiments. In the case of RT-*in situ* amplification, one can use β-actin, HLA-DQα, and other endogenous-abundant RNAs as the positive markers.

VIII: MATERIALS AND METHODS

2% Paraformaldehyde:

1) Take 12 g paraformaldehyde (Merck ultra pure Art No. 4005) and add to 600 ml 1X PBS.

2) Heat @ 65° C for 10 minutes.

3) When the solution starts to clear add 4 drops 10 N NaOH and stir.

4) Adjust to neutral pH and cool to room temperature.

5) Filter on Whatman's N° 1.

Slides:

Suitable heavy teflon-coated glass slides with 10, 12, or 14 mm diameter wells are available from Cel-line Associates of New Field, New Jersey or Erie Scientific of Portsmouth, New Hampshire. This specific slide design is particularly useful, for the teflon coating serves to form three separate wells, each of which serves as a small reaction "chamber" when the coverslip is attached. Furthermore, the teflon coating helps to keep the nail polish from entering the reaction "chamber", and the multiple wells allow for both a positive and

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negative control on the same slide.

10X PBS stock solution pH 7.2 - 7.4:

Dissolve 20.5 g NaH_2PO_4 H_2O and 179.9 g Na_2HPO_4 $7H_2O$ (or 95.5 g Na_2HPO_4) in about 4 liters of double-distilled water. Adjust to the required pH (7.2 - 7.4). Add 701.3 g NaCl and make up to a total volume of 8 liters.

1X PBS:

Dilute the stock 10 X PBS at 1:10 ratio (i.e. 100 ml 10X PBS and 900 ml of water for 1 liter). Final concentration of buffer should be 0.01 M phosphate and 0.15 M NaCl.

0.3% Hydrogen peroxide (H_2O) in PBS:

Dilute stock 30% hydrogen peroxide (H_2O_2) at a 1:100 ratio in 1X PBS for a final concentration of 0.3% H_2O_2.

Proteinase K :

Dissolve powder from Sigma in water to obtain 1 mg/ml concentration. Aliquot and store at -20°C.

Working solution: Dilute 1 ml of stock (1 mg/ml) into 150 ml of 1X PBS.

20X SSC:

Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml of water. Adjust the pH to 7.0 with a few drops of 10 N solution of NaOH. Adjust the volume to 1 liter with water. Sterilize by autoclaving.

2X SSC:

Dilute 20X SSC. 100 ml of 20X SSC and 900 ml of water.

Streptavidin peroxidase:

Dissolve powder from Sigma in PBS to make a stock of 1 mg/ml. Just before use, dilute stock solution in sterile PBS at a 1:30 ratio.

Color solution:

Dissolve one AEC (3-amino-9-ethyl-carbazole from Sigma) tablet in 2.5 ml of N.N. Dimthyl formamide. Store @ 4°C in the dark.

Working Solution:

- 50 mM Acetate buffer — 5 ml
- AEC solution — 250 µl
- 30% H_2O_2 — 25 µl

Make fresh before each use, keeping solution in the dark.

Preparation of 50 mM acetate buffer pH 5.0:

Add 74 ml of 0.2 N acetic acid (11.55 ml galacial acid/litre) and 176 ml of 0.2 M sodium acetate (27.2 g sodium acetate trihydrate in 1 litre) to 1 litre of dionized water and mix.

***In situ* Hybridization buffer (for 5 ml):**

- Formamide — 2.5 ml
- Salmon Sperm DNA (10mg/ml)* — 500 µl
- 20X SSC — 500 µl
- 50X Denhardts solution — 1 ml
- 10% SDS — 50 µl
- water — 450 µl

Total Volume: 5 ml

* Note: Heat denature ssDNA @ 94°C for 10 minutes before adding to the solution.

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blots were striped and rehybridized for sequences in *Bam*HI W (5'-AGTGGTCCCCCTCCCTAGAACTGACAATTG-3', base coordinate 1588)[10] as described previously.[11] In select cases, PCR products were cloned into a pCRII vector, (TA cloning kit Invitrogen, Carlsbad CA) and sequenced (Sequenase Version 2.0 DNA Sequencing, Amersham Pharmacia Biotech, Inc, Piscataway NJ) as per the manufacturers' protocols.

To delineate what cell type within tumor sections bore defective EBV DNA, small lymphocytes or morpholgically distinct Reed-Sternberg cells, we utilized PCR *in situ* cytohybridization to examine paraffin sections, as described in detail elsewhere.[12,13] Briefly, tissue sections affixed to glass slides were deparaffinized with xylene and digested with proteinase K. 25μl of reaction mix (250 nM of each primer described above, 10μM (each) dNTP, PCR buffer) and 2.5 units Taq polymerase were added beneath glass coverslips and 25 cycles of amplification (1 cycle = 95°C for 1 minute; 45°C for 2 minutes; 72°C for 2 minutes) performed on a Hybaid Omnislide thermocycler (National Labnet Co, Woodbridge, NJ). Slides were washed with 2X SSC, heated to 85°C for 5 minutes, then hybridized overnight at 49°C. The hybridization mixture contained 50% formamide, 0.1% single stranded DNA, 10X Denhardt's solution, 0.1% SDS, and 20pg/ml *Bam*HI W-specific oligonucleotide probe labeled with digoxigenin (Boehringer Mannheim, Indianapolis, IN). Bound probe was detected by anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim).

For select HD biopsies, DNA derived above for standard PCR was also used in a real-time quantitative PCR assay to document presence of the standard EBV genome[14,15], regardless of EBER expression status. Targeting the *Bam*HI K fragment of EBV DNA, present in prototype virus but deleted from het EBV DNA[16,17], allows such a determination. A 106 base-pair (bp) region of EBV EBNA 1 gene in the *Bam*HI K fragment was amplified (primers 5'-CCGGTGTGTTCGTATATGGAG-3' and 5'-GGGAGACGACTCAATGGTGTA-3', base