In situ hybridisation of EBV DNA-DNA hybrids using wet heat in polypropylene containers

L G Labrecque

Abstract

Aims: To explore procedures designed to optimise DNA-DNA in situ hybridisation, using cells infected with Epstein-Barr virus (EBV) and tissues and subfragments of the EBV DNA as probes.

Methods: The denaturation step occurred in a polypropylene container, using wet heat generated by a hot water container, the pressure cooker, or the microwave oven, without coverslips, reaching a temperature of 121°C or more in these two last systems. Two different visualisation systems were used.

Results: Fixed cells and tumours harbouring a high and medium to low copy number (a few hundreds to 33 copies per cell), were clearly labelled, using a simple reiterated subfragment (*Bam*W) of the EBV DNA, and fresh frozen cells, harbouring a very low copy number (one to two on average) labelled using *Bam*W as well as *Bam*H (single non-reiterated 6 kilobase subfragment).

Conclusion: This is a valuable alternative technique for DNA-DNA ISH that can be used in fresh frozen samples as well as fixed samples.

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Since its first applications in 1969,¹⁻³ in situ hybridisation (ISH) has gained a progressively important place in science, initially in virology and pathology (endocrinology mainly), but now in a wide field of research and in some clinical laboratories. Its sensitivity and specificity, coupled with the more recent use of non-radioisotopic markers,⁴⁻⁵ have contributed to this increasing popularity. This technique remains problematic in that reproducibility can be unsatisfactory and it is quite labour intensive. Automation of ISH, or parts of it, should provide a solution in future.⁶⁻¹² The denaturation step in ISH procedures, typically done by heating cells or tissue sections in a dry oven with a hybridisation mix placed between slide and coverslip, can present difficulties: heating on a hot plate can lead to an important loss in cell morphology; use of a coverslip sealed with rubber solution can lead to cell loss when removed. (The use of a siliconised or vinyl sheering coverslip or of a plastic autoclavable polypropylene bag can, however, reduce this risk.) Some studies (including one done in our laboratory)¹³⁻¹⁵ have shown that increasing the

temperature produces a noticeable increase in sensitivity of DNA-DNA ISH, particularly on formalin fixed, paraffin wax embedded sections or when the copy number of the target DNA is low. In these cases a temperature more than 100°C seems optimal, but produces heat associated problems. In 1987 Coates *et al* used a microwave oven to accomplish the same effect as that obtained by high temperature¹⁴; this also increased the sensitivity of the ISH.

Now a technique has been devised that allows denaturation of the sample to a temperature of 100-121°C or greater without loss of morphology or cell number, and without material drying. A sealed coverslip is not required. The denaturation and hybridisation steps are carried out in polypropylene boxes used to store cytopathology slides. This technique can be used in a hot water container as well as in a microwave oven or pressure cooker. Cytospun and paraformaldehyde fixed cells, or formalin fixed, paraffin wax embedded cells, have been successfully analysed using this technique, assaying for the presence of Epstein-Barr virus (EBV) DNA using *Bam*HI W (repetitive) and *Bam*HI H (unique) subfragments of the genome.

Methods

EBV positive B cells, B95-8, Daudi, P3HRI, M81, Raji, AW Ramos, Namalwa, and EBV negative Ramos and SF9 cell lines (negative controls) were used. All cells grown in suspension, in supplemented RPMI 1640, were deposited by cytopinning on to slides (Shandon Cytospin 2), as described before.¹⁵ SF-9 (ovary cells from the night butterfly, *spodoptera frugiperda*) propagated in supplemented GRACE insect medium,¹⁶ were similarly deposited.

Cells spun at $200 \times g$ ^{17 18} for five minutes were washed twice in PBS (phosphate buffered saline) and fixed at a concentration of 10^6 cells per ml in 10% formalin (three hours at 4°C). They were spun again, then washed three times in PBS. The cell pellet was loosened with one to two drops of PBS and mixed with a 1% agar solution in PBS at 55°C in 100-200 μ l (enough to give a turbid clot). The mix was allowed to set, wrapped in cigarette paper, and soaked in 10% formalin for a minimum of two hours before being embedded in an automatic vacuum processor.

Sections were cut from the block at a thickness of 5 μ m and applied to poly-L-lysine coated slides,¹⁹ two sections per slide. Samples

Department of Virology, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London W12 0NN

L G Labrecque

Correspondence to: Dr Louise Labrecque

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dried overnight in an oven at 37°C were stored in a dust free container at room temperature.

A nasopharyngeal carcinoma (NPC), isolated from a young Tunisian girl and propagated in nude mice,²⁰ was used. Tumour biopsy specimens, frozen at -70°C, were cut with a cryostat into 5 µm thick sections, applied, two sections per slide, on to poly-L-lysine covered glass slides and stored at -70°C. Before use, they were thawed, fixed for 30 minutes in 4% paraformaldehyde, washed twice in PBS, and dehydrated in serially concentrated ethanol baths (30%, 60%, 80%, 95%, absolute).

The tumour was put in 10% formalin overnight, then paraffin wax embedded in an automatic vacuum processor. Sections were cut to 5 µm thickness, applied to poly-L-lysine covered slides, two per slide, and dried overnight at 37°C.

Biotinylated probes of the B95-8 *Bam*HI W EBV DNA subfragment ligated in pAT 153 vector and cloned in *Escherichia coli*, were biotinylated by nick translation (BRL) according to the manufacturer's instructions. The final concentration of DNA was adjusted to 20 ng/ml, in 10mM TRIS-HCl, 1 mM EDTA (pH 8). The *Bam*HI W subfragment, 3.1 kilobases in length, is represented a variable number of times in the viral genome (overall target length 10-40 kilobases). Namalwa cells were hybridised with a cloned *Bam*HI H subfragment, which is present as a unique 6.0 kilobase fragment in the viral DNA.

HYBRIDISATION PROTOCOL

Prehybridisation (Adapted from Brigati *et al*²¹ and from Hopman *et al*).²² Briefly, cytopun cells were treated in 0.02M HCl, washed in PBS, then treated in 0.01% Triton X-100. Resulting material was washed in PBS, digested with Pronase in TRIS-HCl 0.05M and EDTA 5 mM, pH 7.6 (at a concentration of 0.1 to 0.25 mg/ml for three to six minutes, according to the cell line), washed in PBS-glycine 2 mg/ml, incubated with RNase A 100 mg/ml for one hour at 37°C, washed in PBS and PBS glycine, dehydrated in ethanol baths (30%, 60%, 80%, 95%, absolute) and air dried.

The fresh frozen C15 tumour was treated in the same manner, but after the last wash in PBS glycine it was immersed in 3% H₂O₂ in methanol for 30 minutes (to remove endogenous peroxidase activity if horseradish peroxidase was used), washed in H₂O, and dehydrated in ethanol baths, as above.

Formalin fixed, paraffin wax embedded cells were dewaxed in xylene, washed in absolute ethanol, and air dried. They were digested with 4 mg/ml pepsin in HCl 0.01M at 37°C for 60 minutes, then washed with H₂O, digested with RNase A for one hour at 37°C, washed in PBS, dehydrated in ethanol baths as above, and air dried.

Paraffin wax embedded, formalin fixed C15 tumour sections were immersed in 3% H₂O₂ in methanol for 30 minutes after the absolute ethanol wash, then washed in H₂O, digested in pepsin-HCl for 60 minutes at 37°C, washed in

H₂O, dehydrated in ethanol baths and air dried.

Hybridisation

The mix, freshly prepared, was made up of 50% formamide (stock solution kept at -70°C, in small aliquots, after deionisation on Amberlite monobed resin MB-3 (BDH Chemicals, Poole, UK), 2 × SSC (20 × stock is 3M NaCl/330 mM sodium citrate), 2 × Denhardt's solution (50 × stock, kept at -20°C, is 1% polyvinylpyrrolidone, 1% Ficoll, and 1% bovine serum albumin, 250 µg/ml calf thymus DNA) 2.5 mM sodium phosphate 10% (w/v), dextran sulfate (50% freshly prepared stock before adding to the mix), and biotinylated DNA probe (used at a concentration of 0.2-0.4 µg/ml). A separate control mix was prepared with biotinylated vector (that is, without EBV DNA) only.

A ring was drawn around the cells or tissue with rubber solution, the latter allowed to dry, and 8 µl-12 µl of mix was added to each sample; slides were introduced carefully into a polypropylene box (Histo-Lab, Hertz, England) below a "blank" slide, placed on the top row to collect the water condensation. No coverslip was added (but one can be added, if desired, without rubber solution). The lid was closed and denaturation was carried out according to one of three following protocols: (1) The covered box was put on a rack in a container of boiling water for five to 10 minutes (SSC could be added to increase the temperature), then removed, and put on ice to cool. (The rate of cooling does not seem to influence the result of ISH). Hybridisation was then carried out in a water bath at 37°C overnight.

(2) The box was put on a tray in a pressure cooker containing boiling water, and pressure applied with a light (5 lbs→108°) or heavy (15 lbs→121°) weight, and left for two to six minutes. The pressure cooker was cooled quickly under cold tap water, then the box removed, and manipulated as in (1).

(3) Water (125-150 ml) in a large Petri dish, was brought to the boil in a microwave oven. A plastic container and the polypropylene box were put in this, the lid of the Petri dish (pressing on the top of the plastic container) was replaced, and the microwave (Toshiba, model ER-644) put on at cook (1½ to 2½ minutes) and reheat (3-4 minutes) settings. Hybridisation was then carried out as described above.

After hybridisation, the rubber solution was removed and the slides were washed for 10 minutes with agitation (magnetic stirrer) in 2 × SSC three times at room temperature, and for 30 minutes in 0.1 × SSC at 42°C.

Cytochemical visualisation

Two detection procedures were used: streptavidin alkaline phosphatase (SAP) (Bluegene, BRL (A)), and indirect immunoperoxidase (B).

The SAP technique was used in accordance with the manufacturer's instructions. Slides were first soaked for 10 minutes in a blocking

Figure 1A B95-8 cells (cytospun). Heavy labelling is observed in a small proportion of the cells; most of the others show a fine, particulate pattern (alkaline phosphatase).

Figure 1B B95-8 cells (cytospun). Results with a preparation kept at room temperature for a period of more than two years, showing heavy labelling, typical of the producer cell lines and a lighter pattern in many other cells (alkaline phosphatase).

Figure 1C Raji cells (cytospun). Cells show a particulate pattern with some variation of density and intensity (alkaline phosphatase).

buffer (buffer 2), composed of 3% bovine serum albumin (BSA) fraction V, in buffer 1: 0.1M TRIS-HCl, pH 7.5, and 0.15M NaCl. The excess liquid was removed and the SAP mix (2 µg/ml) in incubating buffer (0.1M TRIS-HCl + 0.15M NaCl) applied for 10 minutes. The slides were then washed for 10 minutes in buffer 1, and for 10 minutes in developing buffer (buffer 3: 0.1M TRIS-HCl, pH 9.5, 0.1M NaCl, 0.05M MgCl₂); the excess liquid was removed and the developing mix, consisting of NBT (nitroblue tetrazolium) 330 mg/ml and DPIC (5-Bromo-4-chloro-

Figure 1D Ramos cells. No labelling observed on this preparation (negative control), hybridised with BamHIW probe. (a) Namakwa cells, hybridised with BamHIH probe. (b) Same cells, hybridised with BamHIH probe. Most of the signals are on the nuclear margin of the cells, clearly different from the signal we would expect to see if the nucleolus was shown up by the technique. (c) Namakwa cells, hybridised with biotinylated vector only. No signal is evident. In these three cases visualisation was performed using the direct PA technique; it was allowed to develop overnight.

3-indoyl-phosphate) 170 mg/ml in buffer 3, was applied to the cells. Development was carried out in the dark, for a period varying from one to four hours or more. The sensitivity of visualisation of the very low copy number can be increased by a longer incubation (even overnight), but this can increase the background. The speed of visualisation appears to be quite variable, and the best way is to monitor it at regular intervals. Care has to be taken to avoid drying of the slides. An indirect technique can be applied (goat anti-biotin antiserum, and biotinylated anti-goat IgG as in

Figure 1E Ramos cells. No labelling observed on this preparation (negative control), hybridised with BamHIW probe. There is some variation in the intensity of counterstaining (horseradish peroxidase).

B, followed by the standard protocol (A)). This procedure seems to speed up the reaction, as well as increase the strength of the signal; a concentration of 0.2 µg/ml of probe was used in these cases (to decrease the background).

The reaction was stopped by soaking the slides in 20 mM TRIS-HCl, pH 7.5, and 5 mM EDTA. Slides were counterstained with 0.25% Safranin, washed under tap water, dried and mounted with Glycergel (Dako). This procedure was used most of the time.

The indirect avidin-biotin-peroxidase technique was carried out according to the following protocol¹⁵: slides, after washing in SSC, were incubated in PBS buffer containing 0.1% Triton X-100 and 5% BSA (PBSAT), then in goat anti-biotin antiserum, diluted 1 in 10 000, for one hour, in biotinylated anti-goat IgG, 1 in 200, for 30 minutes, and in avidin DH-biotinylated horseradish peroxidase reagent (Vectastain Elite ABC kit, Vector), for 30 minutes. Incubations were carried out in a 37°C warm room under Nescofilm (Nippon Shoji Kaisha Ltd, Japan) and between each incubation, slides were washed in PBSAT for 15 minutes. After the final incubation, slides were washed in PBS containing 0.1% Tween 20 for 30 minutes, in PBS for 5 minutes, and finally treated with 0.1% 3,3 diaminobenzidine tetrahydrochloride (DAB) and 0.02% H₂O₂ for 5 minutes, then washed in PBS twice.

Figure 2 C15 tumour (fresh frozen). A particulate pattern is seen in most of the cells present in this preparation (alkaline phosphatase).

If amplification with silver and gold was used,¹⁸ slides were incubated sequentially in the following solutions (after washing in distilled water): 2.5 mM gold chloride; 0.1 mM sodium sulfite, pH 7.5²³; developer, composed of 0.24M sodium carbonate, 13 mM ammonium nitrate, 6 mM silver nitrate, 1.5 mM dodecatungstosilicic acid and 0.6 µl/ml 40% formaldehyde.

After 10–15 minutes, slides were washed in water, 0.17M acetic acid (twice), 15 minutes each, countercoloured with haematoxylin or haemalum, washed in tap water, dehydrated in ethanol, washed in xylene, and mounted in DPX.

Results

Initially, cytopun cells and paraffin wax embedded sections of pellets were used to investigate optimal conditions for substrate denaturation, before detection of EBV BamHI W DNA. The question was whether the unusual mode of denaturation would lead to improved results (sensitivity and specificity) and allow a full preservation of morphological detail at the temperatures needed for efficient denaturation, and whether the use of coverslips was critical. In cytopun, paraformaldehyde fixed cells it proved possible to visualise the DNA in EBV virus-producing cell lines (B95-8, M81, P3HR-1) using the hot water container, as well as the pressure cooker (used mainly with the light weight, reaching a temperature of 108°C), and either the alkaline phosphatase or horseradish peroxidase detection system. Figure 1A shows B95-8 cells, using the first of these. Heavy labelling in a few cells is typical of productively infected cells. The bulk of cells show a more discrete, particulate staining. The same labelling pattern was observed with all the productive cell lines studied. Interestingly, the same protocols gave satisfactory results on slides that had been kept for two years at room temperature after fixation (fig 1B). With virus producer cell lines, a denaturation time of five minutes was sufficient to show the typical heavy labelling. Raji non-productive cell lines harbour between 50 and 100 copies of episomal EBV genomes per cell. These show a particulate pattern of staining in most of the cells (fig 1C). Here again the cells survived temperatures of 100°C or greater for 10 minutes. In Namalwa and AW Ramos cell lines, which contain an average of one to two copies per cell, most of the cells show very low density labelling (fig 1D(a)), but in this case, a unique subfragment (*Bam*HI H), 6 kilobases in size was used as probe. *Bam*W was used successfully as well (fig 1D(b)). Thus, in spite of a small target, it was possible to visualise the EBV genome in cells that have a very low copy number. The cells hybridised with the biotinylated vector (fig 1D(c)) did not show any clinically important labelling, after the same incubation time in the substrate, allowing a noticeable difference between the probe and the vector slides to be demonstrated, and showing that the labelling is not due to non-specific revelation of the nucleolus. A

Figure 3A B95-8 (formalin fixed, paraffin wax embedded). A heavy pattern of labelling is seen in many cells, with a sparse pattern in others (alkaline phosphatase).

Figure 3B Raji (formalin fixed, paraffin wax embedded). Most of the cells present a particulate labelling with a discrete, minimal background observed (horseradish peroxidase, with silver-gold amplification).

Figure 3C C15 tumour (formalin fixed, paraffin wax embedded). Microwaved, irradiated. Labelling, inside the nuclear boundaries, can be seen in numerous cells present in this preparation (alkaline phosphatase).

Figure 3D C15 tumour (formalin fixed, paraffin wax embedded) using biotinylated vector only, where there is no evident labelling (alkaline phosphatase).

concentration of probe at 0.4 µg/ml gave good results, without relevant background, as well as a concentration of 0.2 µg/ml, revealed by the indirect SAP technique with the hybridisation carried out in a hot water container, as well as in the pressure cooker. Two cell lines (Ramos, a lymphoid cell line derived from an EBV negative Burkitt's lymphoma and SF-9, an insect cell line) were used as negative controls. Neither showed clinically important labelling when hybridised with *Bam*HI W probe (fig 1E).

The C15 NPC tumour was used as a model for tissue harbouring only episomal EBV DNA (in this case 30 copies per cell). Freshly frozen, paraformaldehyde fixed, biopsy specimens showed a particulate pattern of staining with *Bam*HI W, confined to the nucleus of cells (fig 2). A tissue sample hybridised with the biotinylated vector only (data not given), showed no evidence of hybridisation.

A second set of experiments involved using formalin fixed, paraffin wax embedded cells. In such processed samples, cross-linking between molecules decreased the efficacy of DNA-DNA hybridisation by blocking hybridisation sites on the target DNA, a problem that could partly be overcome by increased denaturation temperatures. B95-8, P3HR1, M81 and Daudi cell lines did not really present a detection difficulty, due to the high copy number of EBV DNA (averaging several hundred per cell line) and the ability of some cells to produce virus, giving dense staining. For such cells, a denaturation temperature of 100°C, applied for five to 10 minutes, permitted the identification of many heavily labelled cells (fig 3A); in addition (data not shown), formalin fixed paraffin embedded samples survived high temperature very well, that is, 127°C reached in the pressure cooker (heavy weight) and 132–138°C in the microwave oven (using

thermal recording strips). Furthermore, Raji (a non-producer line) showed particulate staining in most cells, with minimal background, when treated for six to eight minutes in the pressure cooker or in the microwave oven (fig 3B). However, if denaturation was carried out in a hot water container for five minutes, or in the pressure cooker at full temperature (light weight) for two to three minutes only, no signals were observed. Finally, paraffin wax embedded C15 tumour samples were submitted to the same procedure. Tumour tissue denatured at 100°C was negative, but a clear particulate pattern was observed when the microwave procedure was used (fig 3C). Experiments using vector only were negative (fig 3D), as were formalin fixed, paraffin wax embedded Namalwa cells, where no significant differences were seen between samples hybridised with the EBV probe or with vector alone.

Discussion

The usual procedure for denaturation of DNA prior to DNA-DNA hybridisation experiments is to heat cells or tissue, placed between a slide and coverslip, together with a hybridisation mix, in a dry oven or a hot plate. In my experience this process frequently leads to loss of cells and altered morphology of survivors, making interpretation of the results of the ISH difficult, if not impossible. Wet heat has sometimes been used in microwave irradiation of material,^{14 15} as well as in a recently described automatised ISH machine¹² where the slides, incubated upside down, were exposed to a hybridisation mix by positive pressure from a peristaltic pump and by capillary action. I examined the feasibility of using wet heat, without a coverslip, in a widely available polypropylene container. It had not been clear

whether the ISH, without a coverslip, in a moist atmosphere, would allow the hybridisation mix to remain on the slide and, if so, be diluted beyond usefulness with water formed by condensation. In the procedure ultimately devised, my findings proved that the use of a polypropylene box containing a blank ("roof") slide greatly reduced condensation, with any water produced during the procedure remaining mainly on the latter, sparing the experimental slides. Moreover, the creation of a wet atmosphere avoided sample drying and preserved cell morphology. To ensure that the hybridisation mix remained precisely over the "spot" of cells or tissue target during the procedure a ring of rubber solution was spread a few millimetres away from the sample and allowed to dry before applying the mix. This was sufficient to maintain the contact between the probe and substrate; an atmosphere saturated with water vapour permitted good preservation of cells. Cytospin paraformaldehyde fixed cells generally tolerated temperatures as great as 127°C and over. Using no coverslip avoided a step that can lead to a loss of cells (a process that can also be minimised by use of siliconised coverslips). The high heat reached with a pressure cooker or microwave oven notably increased the sensitivity of the ISH technique. For example, in boiling water, paraffin wax embedded, EBV positive Raji cells gave a negative result, whereas a clear signal, without loss of cell morphology was observed when the cells were submitted to a higher temperature. The polypropylene boxes adopted for these purposes melt at 167°C, and although they "soften" at lower temperatures (100°C), this has presented no problems. Boxes have been reused several dozen times with no trouble.

Unlike the procedures that necessitate the complete coverage of slides with hybridisation mixes,^{7,8,12} this technique uses a very small amount of mix (and probe) in each sample, allowing considerable economy of reagents. Moreover, various hybridisations can be carried out on the same slide using different probes, provided that samples are separated by rubber solution to avoid any possible mixing of reagents. In contrast to earlier experiments,¹⁵ the streptavidin-alkaline phosphatase procedure, using a kit with improved sensitivity (Blue Gene, Bethesda Research Laboratories) allowed as little as 0.25 pg of target DNA to be detected. With the very low copy number cells (Namalwa and A-W Ramos), the use of a probe at a concentration of 0.4 µg/ml (or 0.2 µg/ml if indirect SAP technique was used), gave good results, even with a single 6 kilobase probe. The visualisation step has to be monitored, however, particularly with the indirect technique, to avoid background staining. If a large tumour section was analysed and the quantity of probe used increased to cover it, acceleration of the heating process sometimes caused a drying of the mix after exposure in the microwave oven. In such cases the exposure time was reduced (a calibration before use is recommended, or a coverslip is added) to avoid this problem: the pressure cooker does not present this difficulty. In the protocol developed each box permits the processing of four

slides, and three boxes per experiment can normally be manipulated, analysing 12 samples or more (according to the number of samples per slide). The polypropylene container technique provides good conditions of denaturation and hybridisation for ISH in a simple, versatile, and economical way. In the wet conditions it generates, good preservation of the histological morphology is maintained, without the damages sometimes created by coverslips, and this in spite of the very high temperature required for optimising DNA-DNA ISH in a low copy number gene setting.

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