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Simplified high throughput protocol for Northern hybridization

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The quantitative determination of a particular mRNA species' concentration within a complex population of transcripts is most readily achieved by Northern blot hybridization analysis. In the most widely employed protocol, RNA samples are fractionated by denaturing agarose gel electrophoresis, transferred to a solid support matrix, and hybridized to radiolabelled complementary probe fragments in the presence of formamide at slightly acidic pH (1). Historically, the inclusion of formamide was rationalized by the need to lower the annealing temperature required for the formation of probe:target duplexes and preserve the integrity of the RNA which would otherwise be compromised at high temperature, especially at neutral or alkaline pH (2). Although advantageous in this regard, formamide is not without shortcomings. By retarding the rate of duplex formation (3, 4) formamide limits the sensitivity of target site detection (5). Moreover, this denaturant is unstable, volatile and significantly toxic. We report here that these limitations are overcome if hybridizations are conducted in a modified version of the tripartite buffer (henceforth referred to as HYBSOL) recently described by Budowle and Baechtel (5) for forensic DNA typing. This approach was used to monitor the induction of tumour necrosis factor alpha (TNFα) and interleukin 1 alpha (IL-1α) gene expression in rat alveolar macrophages (RAMs) following *in vitro* exposure to bacterial lipopolysaccharide (LPS) a recognized stimulator of IL-1 and TNF release in this system (6, 7). We demonstrate that HYBSOL enhances signal intensity when compared to a conventional formamide-based hybridization cocktail and consistently produces autoradiograms free of background even after prolonged exposures. In addition, we suggest that hybridization protocols can be streamlined effectively by showing that HYBSOL also facilitates the detection of single copy gene sequences on Southern blots.

RAMs were isolated (8) and incubated (2×10^6 per 35 mm diam. dish) in 1 mL RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum in the presence or absence of 5 μg/ml LPS (Sigma Chemical Co., St Louis, MO) for 18 h at 37°C in a humidified atmosphere consisting of 5% CO_2/95% air. Total cellular RNA was recovered by the procedure of Chomczynski and Sacchi (9). RNA samples were electrophoresed through 1% (w/v) agarose–formaldehyde/MOPS gels (11×14×0.5 cm), transferred by capillarity to Pall Biodyne A nylon membranes (12×15 cm; Pall Canada Ltd, Mississauga, Ontario, Canada) (10) and cross-linked under UV light. Human TNFα (585bp Hind III/Ava I fragment, ATCC No. 39884) and rat IL1α (920bp Bam HI/Hind III fragment) probes (25ng) were labelled with α[^{32}P]dCTP (3000 Ci/mmol) to specific activities $>5\times10^8$dpm/μg by random priming (11). Individual membranes were inserted in 30 cm siliconized glass hybridization bottles (Robbins Scientific Corp., Sunnyvale, CA) and prehybridized for 4 h at 65°C in 15 mL HYBSOL [composed of 1.5×SSPE (0.15 M NaCl, 0.01 M NaH_2PO_4, 0.001 M EDTA)/7%SDS/10% PEG (polyethylene glycol m.w. 8000; Sigma)] and augmented with 100 μg/mL sonicated and heat-denatured herring sperm DNA (average fragmented size 1 kbp) and 250 μg/mL heparin (Sigma Cat. No. H-3125). The mixture was decanted and replaced with 15 mL fresh prewarmed mixture further augmented with 2 ng/ml heat-denatured ^{32}P-labelled probe. Hybridizations were conducted for 18 h at 65°C. The blots were then washed at high stringency (65°C) in 0.1×SSC (0.15 M NaCl, 0.015 M Na_3 Citrate)/0.1% SDS, sealed wet in nuclease-free polypropylene bags and subjected to auto-radiography for 24 h to 5 days at −76°C under Kodak XAR-5 film sandwiched between Dupont Cronex Lightning Plus intensifying screens. Probes were stripped from the membranes by boiling for 2 min. in DEPC water/0.1% SDS and a second round of hybridization was then performed with a human β-actin probe to correct for RNA loading discrepancies between lanes.

The data generated by the HYBSOL protocol are summarized in Figure 1. A representative ethidium bromide stained gel of serially diluted RNA appears on the left. The autoradiographic results appear on the right. Blots processed in HYBSOL produced sharp autoradiographic patterns devoid of background noise. The formulation, therefore, was effective in 'blocking' non-specific probe binding sites. In agreement with recent reports, low levels of TNFα mRNA were detected in unstimulated RAMs (12, 13) but unlike Ulich *et al.* (13), we were also able to detect the presence of IL-1α transcripts, albeit at very low levels. The presence of the latter may, however, constitute a stress response to *in vitro* manipulation rather than reflect constitutive expression in the absence of LPS stimulation. By contrast, exposure to LPS produced a dramatic induction of steady-state levels of TNFα and IL-1α mRNAs (6-fold and 20-fold, respectively) as determined by quantitative laser densitometry (Molecular Dynamics, Sunnyvale, CA). The increase in TNFα expression, however, was not characterized by the appearance of a higher molecular weight transcript (13).

Next, we sought to determine the effect of HYBSOL on the rate of target site detection. To do this, serial dilutions of total

RNA from the LPS stimulated macrophages were applied to duplicate gels, separated by electrophoresis, blotted and probed with TNTα cDNA. One membrane was hybridized at 65°C using HYBSOL while the other was hybridized at 42°C with conventional formamide cocktail (14). The autoradiograms are shown in Figure 2. Quantitative laser densitometry revealed that HYBSOL increased the signal intensity 2.5-fold over that produced by the formamide buffer formulation and also background eliminated.

Finally, we asked if HYBSOL could be adopted to streamline Southern and Northern hybridization protocols. Since HYBSOL effectively detected polymorphic variable number tandem repeat (VNTR) sequences from low amounts (20 ng to 1 μg) of human genomic DNA on Southern blots (5), we elected to confirm this observation and extend the demonstration to include representative single-copy gene sequences. For this purpose, an allelic profile was generated for the polymorphic sequence D10S28 (ATCC No. 59676; 1.9 kbp Eco RI/Hind III fragment) from 250 ng Hae III digested genomic DNA from 5 human fibroblast strains (NIGMS Human Genetic Mutant Cell Repository, Camden, NJ). In addition, Bam HI digested genomic DNA (2 μg) from human pancreatic tissue and two fibroblast strains were probed for the presence of the c-Ha-ras1 (ATCC No. 41001; 6.4 kbp Bam HI genomic fragment), c-fos (ATCC No. 41042; 2.5 kbp Eco RI/Hinc II 5′ specific genomic fragment) and p53 genes (ATCC No. 57254; 2.0 kbp Bam HI cDNA fragment). DNA samples were fractionated by electrophoresis through 1% agarose−TPE gels, transferred to Pall Biodyne B positively charged nylon membranes in the presence of 0.4 N NaOH and processed for hybridization in HYBSOL. The data are presented in Figure 3. Twelve hours of autoradiography were sufficient to reveal the allelic profile of the c-Ha-ras1 locus and of its pseudogene sequences while 5 days of autoradiography were amply adequate to reveal the presence of the c-fos and p53 genes. Informative autoradiograms are routinely obtained within 24 h.

Lee *et al.* (15) have recently reported that Northern hybridizations can be carried out in the buffer formulation described by Church and Gilbert (16) and that the presence of high SDS (20% w/v) and 5% (w/v) bovine serum albumin are sufficient to obtain clear autoradiographic signals. The formulation reported here is considerably more cost effective, simple in composition and compatible with both Northern and Southern hybridization. Omitting non-homologous DNA and heparin frequently leads to background. We have tested nylon membranes from various suppliers and have encountered significant variability with respect to blocking. Subsequent experiments also indicated i) that the prehybridization step is necessary for the first hybridization but expendable during subsequent rounds of hybridization, ii) that hybridization times can be shortened to 6 h without significant loss in signal intensity, iii) that substitution of dextran sulfate for PEG invariably leads to high background and iv) that membranes processed according to our protocol can be re-probed at least 5 times with minimal loss of target.

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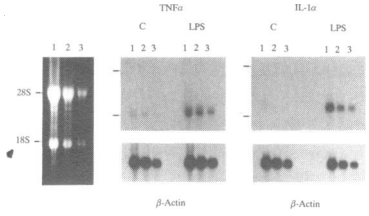

Figure 1. Northern blot hybridization analysis of cytokine mRNA expression in control (C) versus LPS-stimulated RAMs. Lanes 1, 2 and 3 correspond to 10 μg, 5 μg and 2.5 μg total RNA, respectively.

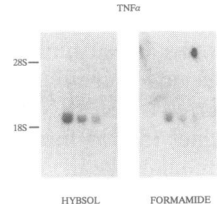

Figure 2. Effect of HYBSOL and conventional formamide-based hybridization cocktail on the rate of detection of TNFα transcripts from LPS stimulated macrophages. Lanes (left to right) correspond to sample loadings of 10 μg, 5 μg and 2.5 μg total RNA. The blots were exposed to X-ray film for 5 days.

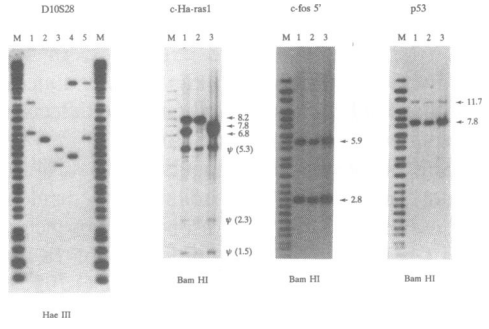

Figure 3. Detection of single copy gene sequences using HYBSOL. Lanes 1 to 5 correspond to fibroblast strains GM10, GM11, GM37, GM38 and GM43, respectively, for the D10S28 panel. Lanes 1 to 3 correspond to strains GM38, GM43 and human pancreatic tissue, respectively for the remaining panels. Lane M corresponds to the autoradiographic signal produced by BRL's Forensic DNA Analysis Marker. Autoradiographic exposures are for 5 days except for c-Ha-ras1 (12 h). Molecular sizes are in kbp. The symbol ψ denotes c-Ha-ras pseudogene sequences.