# Short Technical Reports

## Nylon Membrane-Immobilized PCR for Detection of Bovine Viruses

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**ABSTRACT**

*Bridge Technology™ is an amplification technique in which pairs of primers are immobilized on a solid support, allowing amplification only at the location of the primer pair spot. The technique has diagnostic potential since an array of primer pairs, each specific for a different pathogen, can be used with a diagnostic sample without inter-pair interactions that plague the development of multiplex PCRs. As a result, one assay should be able to determine which of multiple pathogens are present and which are absent in each sample. As test material, we examined the specificity of detection of the RNA-containing bovine viral diarrhea virus (BVDV) and two DNA-containing bovine herpesviruses 1 and 2 (BHV-1 and BHV-2). Nylon membranes with two spots of UV-immobilized primer pairs—one for BVDV and one for BHV—were used in amplification with both corresponding templates, with each template singly and with no template. When amplification was assayed by chemiluminescent detection of incorporated DIG-nucleotides, the expected amplification patterns were obtained.*

**INTRODUCTION**

Oligonucleotide-based techniques for the detection of pathogens are basically of two types: hybridization and PCR. Individual labeled oligonucleotides may be hybridized to nucleic acids from the test material. In a more specific approach, labeled nucleic acid molecules can be hybridized to arrays of immobilized oligonucleotides. Oligonucleotide arrays should have particular potential in the screening of samples for the presence of pathogens and for the measurement of the biodiversity of organisms or viruses in samples (11). Though detecting hybridization to multiple specific oligonucleotides increases confidence, over that with single oligonucleotides, in the presence of pathogen-derived nucleic acid, it does not necessarily establish that hybridization of multiple oligonucleotides is to the same molecule in the population of targets.

In contrast, a major contributor to the high specificity of PCR is the requirement that each of two oligonucleotides bind sequence specifically to the same template molecule. As a result, an increase in the specificity of oligonucleotide arrays could be achieved using immobilized oligonucleotide primer pairs to prime PCR. The gain in specificity by dual recognition of the template over single recognition is lost in oligonucleotide microarray hybridization applications.

Lockley et al. (7) immobilized single primers on nylon membranes. The second primer of a PCR pair was present in solution. During PCR, the immobilized and non-immobilized primers annealed to template strands and were elongated. Then, elongated products from immobilized primers annealed to the soluble primer whose elongation produced an unattached strand. On the other hand, unattached elongated strands annealed to the immobilized primer whose elongation produced strands attached on the nylon membrane. Thus, after many cycles, half of the amplified PCR product strands should not be attached to the membrane.

Since only a single primer of a PCR pair was immobilized in the Lockley et al. (7) method, extension of their method to multiple spots will suffer from the same problems as soluble multiplex PCR. These problems are due to the unwanted interaction among the non-immobilized primers and result in the accumulation of mispriming PCR products and reduced amplification caused by the interaction of primers with templates and PCR products in unintended ways despite computer-assisted design (References 1, 2, and 6 and unpublished observation). These problems could be overcome by Bridge Technology™, developed by Abrams et al. (http://www.mostek.com/reports/pdf/Bridge_Report.pdf). In this technology, during the first PCR cycle, an oligonucleotide in a spot of primer pairs anneals to a template in the target population and is elongated, the product remaining attached. In the next cycle, the product anneals to the other oligonucleotide in the primer pair spot, allowing this second oligonucleotide also to be further elongated. Subsequent cycles allow all available oligonucleotides in the spot to become elongated. The primers in Bridge Technology are immobilized on glass surfaces by co-polymerization of acrylamide with an oligonucleotide containing an acrylamide on its 5′ nucleotide (8). The necessity for acrylamide polymerization limits the utility of the technique for the preparation of large arrays of primer pairs. The alternative of attaching 5′ amino-linked oligonucleotides to silylated glass is also unsatisfactory because the oligonucleotides do not remain attached because of the high temperature of the denaturation step (Reference 8 and unpublished observations), which presumably loosens the silylation coating from the glass.

We therefore explored the use of nylon membranes as solid supports for PCR with both primers immobilized. Because the PCR amplification uses virus-specific oligonucleotide sequences, we have called the method virus signature amplification (ViSA).

**MATERIALS AND METHODS**

**Templates**

Bovine herpesvirus type 1 (BHV-1) and 2 (BHV-2) were propagated from clinical tissue samples in primary bovine fetal kidney cells and subsequently in Madin Darby bovine kidney (MDBK) cells (3). DNA was extracted from infected MDBK cells and purified by ethidium bromide-sodium iodide equilibrium gradient centrifugation at $1.2 \times 10^5 \times g$ for 44 h (3). Bovine viral diarrhea virus types 1 (BVDV-1) and 2 (BVDV-2) RNAs were obtained from the supernatant fluid of BVDV-infected cells by mixing the fluid with guanidine isothiocyanate lysis buffer, sodium acetate, phenol, and chloroform (10). Following centrifugation, the aqueous phase was precipitated with isopropanol, and the resultant precipitate was washed with ethanol and dissolved in water (10).

**Primers**

Table 1 lists primers used in this

**Table 1. Primers Used in This Study**

| Virus | Primer | Primer Sequence (5′→3′) | Genome Position[a] |
|---|---|---|---|
| BHV-1 | 766 | CAACCTCGTCGTGTGCACCCTC (22 bp) | 63775–63796 |
| | 665 | AGAGCTCGCGGCACTTGAGCG (21 bp) | 64079–64059 |
| BHV-2 | 4109 | GCGGCGGCGGAGTCTGGCTTTGAGG (25 bp) | 2416–2440 |
| | 4110 | TCGCTGATGTTGTTCGGAGGGAGGTTGA (28 bp) | 2837–2810 |
| BRD, BVDV-1, BVDV-2 first amplification | P1 | AACAAACATGGTTGGTGCAACTGGT (25 bp) | 1424–1448 |
| | P2 | CTTACACAGACATATTTGCCTAGGTTCCA (29 bp) | 2250–2222 |
| BVDV-2 | TS2 | TGGTTAGGGAAGCAATTAGG (20 bp) | 1802–1821 |
| second amplification | P2 | CTTACACAGACATATTTGCCTACGTTCCA (29 bp) | 2250–2222 |

[a]For 665 and 766 primers, genome positions were based on GenBank® accession no. AJ004801.1. For 4109 and 4110 primers, positions were based on accession no. M21628.1. For P1, P2, and TS2 primers positions were reported (10) to be based on accession no. M31182.1.

study. All primers are designed for soluble PCR. Two 22-bp primers, 665 and 766, were designed (4) from a conserved region of published nucleotide sequences of the BHV-1 thymidine kinase gene. Sullivan et al. (10) designed P1 and P2 to prime amplification from nucleic acids of BVDV-1, BVDV-2, and border disease virus (BDV). TS2 discriminates BVDV-2 from BVDV-1 and BDV by annealing to BVDV-2 sequences. Primers 4109 and 4110 were designed (unpublished data) from the sequence of the BHV-2 thymidine kinase gene (9).

### cDNA Preparation from BVDV Template

BVDV cDNA templates were created by RT-PCR before the ViSA card application. BVDV template was mixed with reagents from a GeneAmp® RNA PCR kit (Applied Biosystems, Foster City, CA, USA): 25 U MuLV reverse transcriptase, 10 U RNase Inhibitor, and 1 µL of 1 mg/mL P2 primer, and 0.32 mM dNTP in 10 µL total volume. This mixture was incubated at 42°C for 15 min and 99°C for 5 min and then cooled to 4°C for 5 min. The product was used as template for BVDV cDNA amplification without further purification.

### PCR Conditions

BVDV cDNA amplification followed a semi-nested soluble PCR procedure (10), in which the first PCR amplified BVDV and BDV, while the second (semi-nested) PCR amplified specifically BVDV-1, BVDV-2, or BDV. The first PCR mixture consisted of 10 µL RT reaction (including primer P2), 2.5 U *Taq* DNA polymerase, 1 µL of 1 mg/mL primer P1, 1× PCR buffer, 1.5 mM $MgCl_2$, and 0.2 mM dNTP. Incubation was at 94°C for 2 min, followed by 35 cycles of 94°C, 55°C, and 72°C for 1 min each, and then 72°C for 10 min. The BVDV second PCR mixture consisted of 1 µL BVDV first PCR mixture diluted 1:500 with water, 2.5 U *Taq* DNA polymerase, 1 µL each of 1 mg/mL primers TS2 and P2, 1× PCR buffer, 1.5 mM $MgCl_2$, and 0.2 mM dNTP. Incubation was at 94°C for 2 min, followed by 30 cycles of 94°C, 50°C, and 72°C for 1 min each, and then 72°C for 10 min in PTC-100™ thermal cycler (MJ Research, Waltham MA, USA).

The 50-µL mixture for BHV-1 or BHV-2 amplification by the ViSA card method consisted of 2.5 U *Taq* DNA polymerase, 1× PCR buffer, 2% DMSO, 1.5 mM $MgCl_2$, and 0.2 mM dNTP. The mixture was heated at 94°C for 3 min, followed by 40 cycles of 94°C, 68°C, and 72°C for 1 min each, and then 72°C for 10 min. For soluble PCRs performed for BHV-1, 1 µL each of 1 mg/mL 665 and 766 primers (Table 1) were added to the 50-µL mixture for BHV-1 ViSA card (see above). Soluble PCR products were analyzed by 1.5% agarose gel electrophoresis, which contained 0.025% ethidium bromide, with a 100-bp ladder (Invitrogen, Carlsbad, CA, USA) as size standard.

### ViSA Card Preparation

Mixed primers (2 µg) were spotted on 2 × 5 mm rectangles of nylon membrane (Hybond-N®, 0.45 µm; Amersham Biosciences, Piscataway, NJ, USA) and treated with 120 mJ/cm$^2$ UV (Stratalinker®; Stratagene, La Jolla, CA, USA). (Alternatively, a vacuum oven was used for 4 h at 80°C.) The nylon membrane was then washed with 0.2% SDS and 0.2× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate) solution twice for 10 min, with 0.2× SSC solution four times for 10 min, and used immediately. For PCR, the spotted membrane was directly fully immersed in a tube containing 50 µL reagents as described above, except that no additional primers were added and 0.7 mM DIG-dUTP (Roche Molecular Biochemicals, Indianapolis, IN, USA) was included to allow production of DIG-labeled products.

Following PCR, nylon membranes were washed in 2× SSC and 0.2% SDS twice for 5 min and in 0.2× SSC and 0.2% SDS twice for 15 min. They were then equilibrated in maleic buffer (100 mM maleic acid, pH 7.5, 150 mM NaCl) for 5 min and incubated in 1% blocking reagent (Roche Molecular Biochemicals) for 30 min. Next, they were incubated with a 1:5000 dilution

of Anti-DIG-AP conjugate (Roche Molecular Biochemicals) in 1% blocking buffer for 30 min and washed in maleic buffer for 15 min twice and in AP buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 100 mM $MgCl_2$) for 2 min. Lastly, binding of the conjugate was detected by X-ray film using CSPD® ready-to-use chemiluminescent substrate (Roche Molecular Biochemicals) according to the manufacturer's instructions.

To check the immobilization of the primers, 1 µL of a 1:1000 dilution of YOYO-1 (Molecular Probes, Eugene, OR, USA) in TAE buffer (4 mM Tris-acetate, 0.1 mM EDTA, pH 8.2) was applied to a nylon membrane on which primers were immobilized. One hour later, the nylon membrane was washed with water briefly and dried. Yellow fluorescence of the primer-bound YOYO-1 was observed with a UV lamp (short wave).

## RESULTS

For nylon membrane-immobilized PCR, spotted primers must be firmly attached to the nylon membrane. YOYO-1 was used to assess attachment. Primers (0.2 µg) were spotted on membranes in circular deposits of about 1.5 mm diameter. UV cross-linking was used to attach the primers on the membranes. Staining of the membrane with YOYO-1 before or after washing revealed a fluorescent spot no larger than the spot made during primer application and that the primers remained attached during the washing steps.

When used in PCR, primer-spotted membranes (ViSA cards) detected the presence of viral nucleic acid (Figure 1). When the primers 665 and 766 (BHV-1-specific primers) were spotted and BHV-1 was used as a template, a definite spot with less intense darkening of the whole membrane was detected on X-ray film after brief exposure to the ViSA card (Figure 1A). The primer-spotted card incubated without BHV-1 template did not produce a spot stable to the post-reaction washing steps. Similar results were obtained for membranes spotted with TS2 and P2 (BVDV-2-specific) primers and incubated with and without a small aliquot of the products of first-step amplification of BVDV-2 cDNA (Figure 1B).

As demonstrated by YOYO-1 staining (see above), primers were confined to small spots by the application procedure used. Thus, no PCR products should accumulate outside of the primer spots. However, most X-ray films showed chemiluminescent reaction product covering the entire membrane. This apparent background required both template and primer, suggesting that PCR products may be released from the spot and reattach elsewhere on the membranes. Indeed, when a soluble PCR was performed with BHV-1 template, soluble primers, and a blank nylon membrane, chemiluminescent reaction product was detected distributed over the membrane. A no-template control lacked this reaction product. These observations indicate that PCR products can attach to the membranes during PCR. However, PCR products were not detected by ethidium bromide staining after agarose gel electrophoresis of the liquid phase of control ViSA card reactions with template present, suggesting that only a small portion of the products detach and reattach.

Whatever the cause of the background problem, it could be controlled by lowering the primer amounts and template concentrations. Membranes were prepared with 2 or 0.2 µg primers (4109 and 4110) or no primers and were submitted to amplification with or without BHV-2 template. After 11 min of exposure to X-ray film (Figure 2), the membrane with 2 µg primers produced a spot on the film corresponding to the center of the ViSA card (where primers had been spotted), and other ViSA cards did not produce a clear image. After 30 min, the membrane with 0.2 µg primers also produced a spot in the center of the ViSA card, while the no-template control weakly darkened the film all over the membrane.

ViSA cards with 0.1 µg primers 665 and 766 were used to attempt amplification from a series of diluted BHV-1 templates, ($10^{-3}$, $10^{-4}$, and $10^{-5}$ dilutions). The dilution series was also used in soluble PCR under the same PCR conditions, except that 2% DMSO was used in soluble PCR. The differential use of DMSO was necessary because optimal amplification in soluble PCR experiments occurred with 2% DMSO,

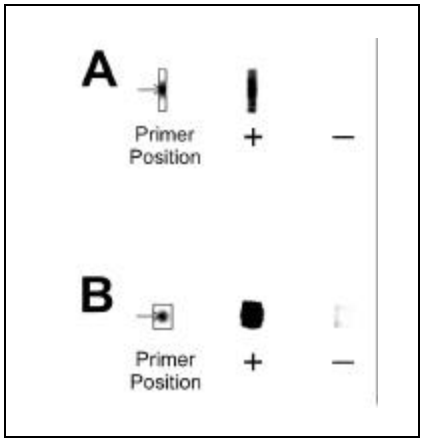


**Figure 1. ViSA card detection with (A) BHV-1 template and (B) BVDV-2 template.** Autoradiographic images of nylon membranes. Membranes in panel A contained 665 and 766 primers and were amplified in the presence (+) or absence (-) of BHV-1 template at 2% DMSO (3 min exposure for panel A). Membranes in panel B contained TS2 and P2 primers and were amplified in the presence (+) or absence (-) of BVDV-2 template at 0% DMSO (15 min exposure for panel B).

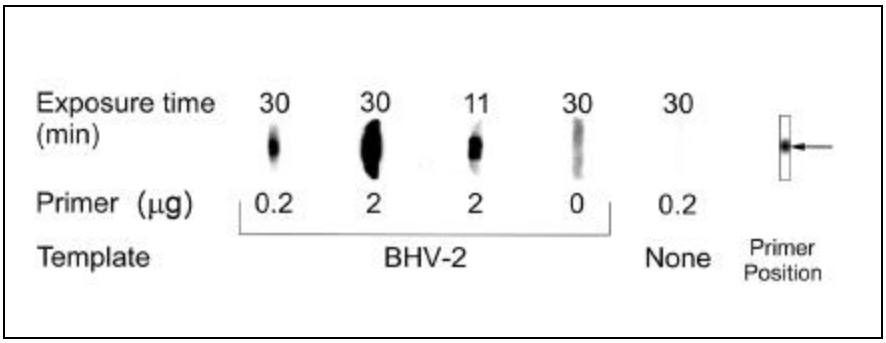


**Figure 2. BHV-2 detection by ViSA card using different primer concentrations.** Autoradiographic images (with indicated exposure times) of nylon membranes containing 0, 0.2, and 2 µg 4109 and 4110 primers and amplified in the presence or absence of BHV-2 template with various primer concentrations.

but DMSO inhibited BHV-2 amplification in ViSA card experiments (data not shown). DMSO was not required for ViSA card amplification of BHV-1 and BVDV. All dilutions produced spots in ViSA cards (Figure 3). However, $10^{-5}$ diluted BHV-1 template produced a spot in the area where the primers were actually applied, while higher concentrations produced a more disseminated reaction. In contrast, soluble PCR produced an agarose gel band detectable by ethidium bromide staining at $10^{-3}$ and $10^{-4}$ dilutions of template, but none at $10^{-5}$ dilution. Thus, under the same conditions, including template and primer pairs, the ViSA card assay did as well or better than soluble PCR using ethidium bromide staining and agarose gel electrophoresis for detection.

Detection of BHV-2 and BVDV-2 sequences in a single ViSA card assay was then attempted. Two spots were applied to nylon membranes: 0.2 µg P2 and TS2 on one half and 0.2 µg 4109 and 4110 on the other. Each PCR tube contained one ViSA card. Tubes had BHV-2 or BVDV-2 templates, both templates or no templates. The BVDV-2 template was a 1:500 dilution of the first amplification product of soluble PCR. After the PCR (Figure 4), the ViSA card with two templates produced two X-ray film spots, one for each primer pair spotted, while those with one template produced one spot at the appropriate place and the one with no template did not produce any spots.

## DISCUSSION

A ViSA card can amplify a fragment of virus DNA with immobilized primer sets such that the immobilized amplified products are detectable by chemiluminescence. With the ViSA card, we detected BVDV-1, BHV-2 (data not shown), BVDV-2, and BHV-1 in single-template PCRs, and a mixture of BHV-2 and BVDV-2 in multiplex PCR. Differentiation between BVDV types was successful but not entirely satisfactory because of high background (not shown). The high background problem was caused by nonspecific binding of DIG-labeled PCR products to the nylon membrane. All ViSA cards produced high background levels when primer or virus template concentrations were high. This problem was partially solved by reducing the primer or template concentration. Routine use of ViSA cards in virus detection and diagnosis requires that this background problem be solved because the experimenter will have no control over the amount of template present. Alternative primer attachment methods and/or alternative support media may provide improved stability of primer, and thus product, attachment.

Both the Bridge Technology (http://www.mostek.com/reports/pdf/Bridge_Report.pdf) and the ViSA card have common benefits: less use of reagents due to the multiplexing made possible by the use of many primer pairs and the ability to avoid cross-contamination and unwanted primer-primer or primer-template interaction due to immobilization. In soluble multiplex PCR, each primer can partner with any other primer in the mixture to give unexpected products. Using the primer sets and soluble amplification conditions presented above, we obtained poorly reproducible results (unpublished observation). The products always contained bands of unexpected sizes, and inhibition of the amplification occurred often. The ViSA card method uses UV cross-linking to immobilize standard primers on a nylon membrane. Thus, nylon-based ViSA cards can be created more easily and less expensively than glass surfaces bearing, as immobilized primers, the acrylamide-modified oligonucleotides of Bridge Technology.

These experiments demonstrate the feasibility of creating an immobilized PCR assay for the simultaneous assay of multiple viruses. Substantially increasing the number of viruses to be assayed requires an automated array fabrication approach. Primers could be applied to nylon membranes using automated high-density stamping devices,

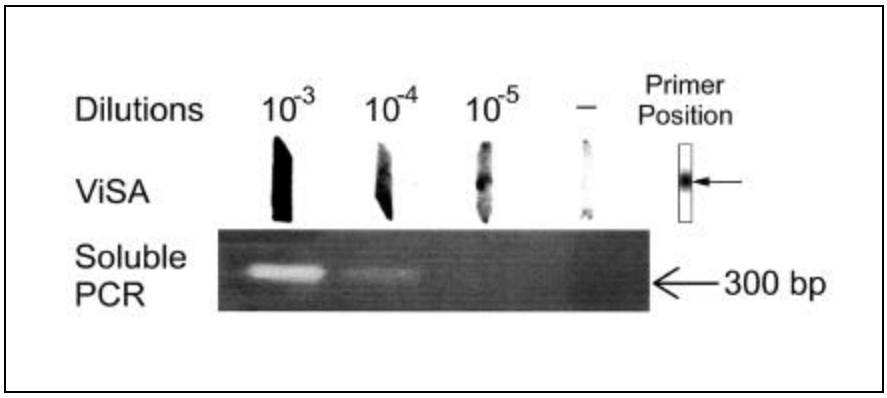


**Figure 3. Comparison of detection of diluted BHV-1 by soluble and immobilized PCR.** Autoradiographic images (upper) of nylon ViSA cards containing 665 and 766 primers and amplified in the presence of, from left to right, $10^{-3}$, $10^{-4}$, and $10^{-5}$ dilutions of BHV-1 template or no template (-). Gel electrophoretic analysis of soluble PCR (lower) performed with the same templates.

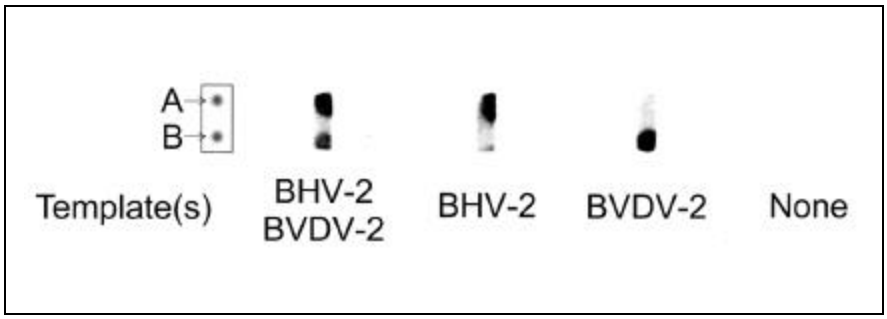


**Figure 4. Multiplex immobilized PCR on nylon ViSA card.** In the diagram (left) showing the placement of primer pairs on the card, A indicates BHV-2 primers, 4109 and 4110, and B indicates BVDV-2 primers, P2 and TS2. Autoradiographic images (right) of cards containing primers placed as in the diagram and amplified in the presence or absence of BHV-2 and BVDV-2 template.

followed by the automated cutting of membrane strips. Alternatively, they could be applied as lines, followed by cutting of the membrane in thin strips perpendicular to the application lines. Glass slides could be used instead of nylon. Photolithographic methods have been developed to generate two different oligonucleotides in one spot on silanated glass (5). However, the instability of the attachment of oligonucleotides attached to coatings on glass slides to the high temperatures of PCR (8) may hamper the application of this method. Less expensive methods of covalent attachment of modified oligonucleotides to derivatized glass surfaces need to be explored to identify an attachment method compatible with PCR amplification conditions. In addition, the flatness of most microarray slides may interfere with the access of the polymerase to the oligonucleotides. Thus, a combination of the attachment method and glass, nylon, or other surface compatible with PCR is key for the automation of the ViSA card method to detect multiple viruses simultaneously and should lead to a marriage of microarray and PCR technologies for many diagnostic applications.

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# Live-Cell Nucleocytoplasmic Protein Shuttle Assay Utilizing Laser Confocal Microscopy and FRAP

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## ABSTRACT

*In eukaryotes, protein trafficking to and from the nucleus, or shuttling, has been demonstrated to be an important function for proteins that have vital roles in one or both subcellular compartments. Current techniques of detecting protein nuclear shuttling are extremely labor intensive and only statically visualize evidence of shuttling. Fluorescence recovery after photobleaching (FRAP), or fluorescence microphotolysis, has proven to be an effective method of analyzing protein dynamics in live cells, especially when coupled to GFP technology. Here, we describe a relatively simple in vivo protein nuclear shuttling assay that utilizes red fluorescent and green fluorescent protein fusions as substrates for FRAP using a laser confocal microscope. This technique is less time consuming than established shuttle assays, is internally controlled, and visualizes nucleocytoplasmic shuttling in living cells of the same species and cell type. This technique can be potentially used to detect the ability of any nuclear protein to shuttle from the nucleus to any other subcellular compartment for any eukaryotic species in which GFP or dsRed1 fusion protein can be expressed.*

## INTRODUCTION

During the normal eukaryotic cell life cycle, every protein in the cell greater than approximately 25 kDa in size must be regulated to either be excluded from or included to the nucleus (2). Several unique protein nuclear localization signals (NLSs) have been described as modular polypeptide sequences that can modulate the nuclear import of any heterologous fused protein (15). There also exist nuclear export signals (NESs) that can signal the export of a protein from the nucleus to the cytoplasm (2,15). Some proteins have vital roles in both subcellular compartments