phoresis. Because of potential problems in the assay of MC, especially problems due to antigen excess (6), we compared the results of assays of non-MC-containing samples and of MC-containing samples separately, as well as the overall results (Fig. 1). The ratios (Beckman/Behring) of the mean values for samples and the variances within results from a given assay and reference material have remained the same for all samples during the 5-year period evaluated here.

The ratios of results for all three immunoglobulins are substantially closer to unity (1.0) with the standardization referenced to RPPHS than was the case previously. The persisting differences may relate to combinations of several factors, including the following: (a) Either or both manufacturers did not use the recommended method of value transfer (1); (b) the reactivities of the different antisera are different, i.e., the antiserum from one manufacturer recognizes different epitopes, or possibly complex mixtures, from those recognized by the other; or (c) matrix differences exist between the samples and the manufacturers’ calibrants. That at least the second factor is likely is supported by the different ratios of concentrations obtained for IgM in samples containing IgM MC and for IgA in samples containing IgA MC, compared with samples with no MC. A similar difference was not seen in samples with IgG MC (Fig. 1).

The changes in values secondary to changes in the manufacturers’ assigned values for the three immunoglobulins before and after the introduction of RPPHS are shown in Table 1. Each manufacturer’s change is expressed as the ratio of the value after conversion to RPPHS to the value before conversion, using only survey samples without MC. That the Beckman values changed relatively little is not surprising; previous CAP reference materials (RPSP1–RPSP4) were referenced against the US National Reference Preparation (2), as is RPPHS (1). In addition, values were assigned to RPSP4, the CAP reference material that immediately preceded RPPHS, using the same protocol that was used for RPPHS. In contrast, Behring previously referenced against in-house purified proteins.

Although the observed differences in concentration between the manufacturers are small (2–5%), they are statistically significant (two-tailed P values by paired t-test were all <0.0005). If “universal” reference intervals for plasma proteins are to be used (7), it is critical for manufacturers to assign values to calibrants and controls as accurately as possible, preferably using the recommended method (1).

### Table 1. Ratio of concentrations in CAP survey samples containing no MC.

<table>
<thead>
<tr>
<th>Immunoglobulin</th>
<th>Beckman</th>
<th>Behring</th>
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<tbody>
<tr>
<td>IgG</td>
<td>0.9774 ± 0.0084b</td>
<td>0.8533 ± 0.0081</td>
</tr>
<tr>
<td>IgA</td>
<td>0.9946 ± 0.0059</td>
<td>0.8345 ± 0.0078</td>
</tr>
<tr>
<td>IgM</td>
<td>0.9756 ± 0.0162</td>
<td>0.6734 ± 0.0155</td>
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</table>

* Values referenced to RPPHS divided by values referenced to the manufacturers’ own materials (see text).

* Ratio ± SD.

### References


### PCR Conditions and DNA Denaturants Affect Reproducibility of Single-Strand Conformation Polymorphism Patterns for BRCA1 Mutations, Lori K. Hennessy,1* John Teare,1,2 and Christopher Ko1 1Amersham-Pharmacia Biotech, Inc., 654 Minnesota St., San Francisco, CA 94107. 2Present address: Chiron Corp., Chiron Diagnostics, 4560 Horton St, Emeryville, CA 94608; * address for correspondence: Hyseq, Inc., 670 Almanor Ave., Sunnyvale, CA 94086; fax 408-524-8140; e-mail lorih@sbh.com)

Single-strand conformation polymorphism (SSCP) is a mutation screening method based on changes in the secondary structure(s) in a defined single-stranded DNA fragment caused by a change in sequence (1). These structural changes are detected as alterations in the fragment mobility by native gel electrophoresis. The method is one of the easiest screening procedures to perform, is very cost effective, requires a minimum amount of equipment, and has a reportedly high mutation detection rate (95% for some genes (2)). Although SSCP is fast and simple, problems with the reproducibility of the technique have been a major hindrance. Several variables of gel electrophoresis conditions have been tested (2–4), but studies have focused on the detection of mutations and not on the reproducibility of the SSCP band pattern. Analytical steps performed before electrophoresis may affect this reproducibility. The variables can be classified into two groups: PCR reaction conditions and post-PCR conditions. Neither of these variables have been adequately investigated. We investigated the concentrations of MgCl₂, the DNA template, and the PCR primers in the PCR reaction, as well as the choice and concentrations of chemical denaturants.

Eleven DNA samples from patients with characterized mutations in the breast cancer gene BRCA1 were obtained.
from Coriell Cell Repositories (Camden, NJ). The Coriell catalog numbers for the DNA samples were NA13708–15, NA14090, NA14093, and NA14097. Samples of DNA specimens from women with no known history of breast cancer were used as controls and were collected according to institutional guidelines approved by the Committee on Human Ethics.

PCR products were obtained using Ready-To-Go PCR beads (Amersham-Pharmacia Biotech). In our standard 25-μL reaction volume, 50 ng of genomic DNA and 50 ng of each primer were used. To amplify the BRCA1 mutation Glu1250ter, we used the primer set 11.15 of Castilla et al. (5). All other primers were as specified by Friedman et al. (6). Amplification was performed on a PTC 200 thermal cycler (MJ Research), using 35 cycles of 94 °C for 30 s, 72 °C for 1 min.

For SSCP analysis, aliquots of the PCR products (1–2 μL) were mixed with 3–4 μL of 24% formamide–0.025% bromphenol blue–0.025% xylene cyanol for a total volume of 5 μL. Samples were heated at 100 °C for 3 min, snap-chilled on ice, and loaded onto a 12.5% polyacrylamide gel (GeneGel Excel 12.5/24 kit, Amersham-Pharmacia Biotech). Electrophoresis was carried out at 10 °C, with maximum settings of 600 V, 25 mA, and 15 W for 2–2.5 h on the GenePhor System (Amersham-Pharmacia Biotech). The gels were silver stained with the Hoefer Automated Gel Stainer and the Plus One DNA Silver Staining kit (Amersham-Pharmacia Biotech) according to the manufacturers’ procedures.

Three variables in our PCR reactions were changed as follows. The MgCl₂ concentration was increased from 1.5 mmol/L to 3, 4.5, or 9 mmol/L. The DNA template concentration was either 10, 25, 100, or 500 ng. Primer concentrations were either 25, 50, or 100 ng of each primer. The results from these experiments are summarized in Table 1.

Increasing concentrations of MgCl₂ had no substantial effect on the SSCP banding pattern, even at a concentration of 9 mmol/L. Although the band pattern did not change at high MgCl₂ concentrations, the SSCP bands were much sharper at lower concentrations of MgCl₂. The result still suggests, however, that the MgCl₂ concentrations commonly used in PCR reactions do not affect SSCP band patterns. SSCP band patterns were also unchanged even when the DNA template concentration was increased 50-fold, which suggests that this variable also does not have an effect on SSCP patterns.

In contrast to the results of the MgCl₂ and DNA template experiments, SSCP band patterns were markedly different at all three primer concentrations. On the basis of this observation, we performed a series of experiments to examine the effect of excess primers in SSCP analysis in more detail. To eliminate unincorporated primers, we purified the PCR products with a QIAquick PCR kit (Qiagen). The purified product was eluted in 1× TE (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0). Forward or reverse primers were added back to the sample before SSCP analysis in multiples of the concentration of primer in the standard aliquot taken for analysis. Thus, the concentration of primer added back to the purified sample is expressed as 0.5×, 1×, or 3×. An unrelated primer (i.e., primer used to amplify a different exon of the BRCA1 gene) and oligo(dT)₁₈ were also tested. We analyzed three BRCA1 mutations: Ser1040Asn, Lys1443Gly, and Glu1250ter. The results of the experiment with BRCA1 mutation Lys1443Gly are shown in Fig. 1A. The addition of forward or reverse primers had different effects on the SSCP band pattern (the addition of PCR buffer without primers had no effect). This suggests that the effect of excess primer is sequence-dependent. The change in the SSCP band pattern probably results from the interaction between the primer and one or more of the multitude of conformers within the denatured sample. Similar results were seen with the other two BRCA1 mutations studied. Interestingly, in the experiments with the Ser1040Asn and Glu1250ter mutations, we noticed that removal of unincorporated primers before SSCP produced band patterns that were not as sharp as when primers were present. Addition of primers back to the sample, even at 0.5×, generated sharper bands. The presence of unincorporated primers may shift the equilibrium of unstable conformations to more stable biomolecular complexes, reducing or eliminating minor or metastable bands. One could hypothesize that excess primers may improve SSCP pattern formation and potentially increase the ability to differentiate wild types from mutants.

To rule out the possibility that the conformation of primers was mediating the altered mobility, we synthesized a 6-mer and a 12-mer with sequences derived from the 5' end of the forward primer. These primers were adjusted to the same molar concentration and added back to the purified product. When these primers were used, only the 12-mer yielded the same pattern as that of the forward primer. These results further support the conclusion that primer-amplicon interaction is an important factor in SSCP analysis.

Although it is clear that excess primers in the PCR reaction can affect SSCP band pattern, other post-PCR variables may affect SSCP band pattern. The use of several denaturants for SSCP has been reported in the literature; each report suggests better results with one class of denaturant compared with another. To investigate whether denaturant concentration and class had any influence on SSCP patterns, we tested serial dilutions of several denaturants: 95% formamide, 100 mmol/L NaOH + 10 mmol/L EDTA, methylmercuric hydroxide, dimethyl sulfoxide (DMSO), and 1× TE. We found that the choice of denaturant had a minimal effect on the SSCP

<table>
<thead>
<tr>
<th>Variable tested</th>
<th>Effect on SSCP pattern</th>
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<tbody>
<tr>
<td>MgCl₂ concentration</td>
<td>–</td>
</tr>
<tr>
<td>DNA concentration</td>
<td>–</td>
</tr>
<tr>
<td>Primer concentration</td>
<td>+</td>
</tr>
<tr>
<td>Type of denaturant</td>
<td>–</td>
</tr>
<tr>
<td>Snap chilling</td>
<td>–</td>
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Fig. 1. Results of experiments comparing effects of primers and snap chilling.

(A) The effect of primers on SSCP band pattern was sequence-dependent. Forward, reverse, or unrelated primers or an oligo(dT)18 primer were added back to the purified PCR product. The concentration of primer added is expressed as a multiple of the concentration in our standard PCR reaction as indicated. Samples were loaded onto a 12.5% polyacrylamide gel and electrophoresed at 600V, 25 mA, and 10 W for 2.5 h. (B) Omission of the snap-chill step after heat denaturation had no effect on SSCP band pattern of six BRCA1 mutations tested. The PCR products were mixed with 24% formamide–0.025% bromophenol blue–0.025% xylene cyanol and either denatured at 100 °C for 3 min and snap chilled for 5 min or denatured and loaded directly onto a 12.5% polyacrylamide gel. Samples were electrophoresed at 600V, 25 mA, and 10 W for 2.5 h. (C) SSCP-heteroduplex analysis of 11 BRCA1 mutations. PCR products were obtained using Ready-To-Go PCR beads (Amersham-Pharmacia Biotech). In the 25-μL reaction, 50 ng of genomic DNA and 75 ng of each primer were used. One or two microliters of each PCR reaction were mixed with 24% formamide–0.025% bromophenol blue–0.025% xylene cyanol, denatured at 100 °C, and electrophoresed on a 12.5% polyacrylamide gel at 10 °C for 2 h 15 min. For mutations Tyr1564ter and 5382insC, the electrophoresis run was extended to 3 h because of the larger size of the fragments. The mutations, exon location, and primer set used are indicated at the top. Lane 1 is the φX-174 Haelll DNA molecular weight marker.
patterns of BRCA1 mutations. In fact, the SSCP patterns did not differ from the pattern obtained with 1× TE, suggesting that strong denaturants are not necessary for sample denaturation. A similar result using a low ionic strength buffer for denaturation before SSCP analysis has also been reported (7). It should be noted that at high concentrations of methylmercuric hydroxide and DMSO over-denaturation of the DNA occurred and caused smeared bands.

The final variable that we investigated was snap-chilling after denaturation. This step is found in 99% of all SSCP protocols published, suggesting that it is essential for successful SSCP analysis. We tested this hypothesis, using six known BRCA1 mutations. For each mutation, the product was either heated for 3 min at 100 °C and snap-chilled on ice or heated and loaded directly onto a polyacrylamide gel. Contrary to popular methodology, we found that snap-chilling the denatured sample on ice was unnecessary. No effect on SSCP patterns was seen in the six BRCA1 mutations tested (Fig. 1B). Similar results were also found when we examined p53 and cystic fibrosis mutations. These results suggest that the refolding of single-stranded DNA occurs rapidly and that it is more stable than previously thought. In addition, we tested the effect of leaving the samples at room temperature for 2.5 h after denaturation in 24% formamide with no snap-chilling. No change was seen in the SSCP band patterns, providing further support that the refolding of single-stranded DNA occurs rapidly and is quite stable.

On the basis of our findings in this study, we analyzed 11 DNA samples containing known BRCA1 mutations. We were able to detect mobility shifts in all 11 samples (Fig. 1C). In seven of the samples, we could also detect heteroduplexes. Because some double-stranded DNA molecules usually remain in samples prepared for SSCP analysis, they can be used as a confirming feature when both forms of DNA are present in the same sample.

Our data demonstrate that the concentration of primers in the PCR reaction has the largest effect on the formation of specific SSCP patterns and that the effect is sequence-dependent. All of the other factors tested had minimal effects on SSCP pattern reproducibility. Thus, our results suggest that variations in primer concentrations in PCR reactions are the most likely cause of nonreproducible SSCP patterns.

**Measurements of Total and Desialylated Sex Hormone-binding Globulin in Serum by ELISA, Jenny Vaysse,1,2 Michel Beaugrand,2 and Michel Pontet1 (1 Laboratoire de Biochimie and 2 Service d’Hépato-Gastroentérologie, Hôpital Jean Verdier, Avenue du 14 juillet, 93143 Bondy, France; *author for correspondence: fax 33 01 4802 6831; e-mail jenny.vaysse@jvr.ap-hop-paris.fr)

The sex steroid-binding protein, also called sex hormone-binding globulin (SHBG), is an androgen- and estradiol-carrier protein in human serum. The two identical subunits of this glycoprotein both bear three oligosaccharide chains, two N-linked at Asn^51 and Asn^367 and one O-linked at Thr^7. The role of this carbohydrate moiety in the biological function of SHBG is not known; however, the complete enzymatic deglycosylation of human SHBG has very little effect on the steroid-binding activity (1). Because estrogens regulate SHBG synthesis, this protein is usually measured to evaluate estrogen status, especially in the biochemical assessment of hirsutism. Other indications for SHBG measurement are promising because high SHBG concentrations are associated with a decreased risk of breast cancer in postmenopausal women (2) and with an increased incidence of hepatocellular carcinoma in patients with cirrhosis (3). Furthermore, in the latter situation, it has been suggested that increased androgen transport through the hepatocyte membrane, via the asialoglycoprotein receptor, might be involved in hepatic carcinogenesis (3). Determination of desialylated SHBG (dSHBG) is needed to investigate this hypothesis and could be useful in tracking this disease.

Total SHBG is usually measured with a radiometric method using tritiated dihydrotestosterone or with immunoradiometric assay (4, 5). To our knowledge, procedures for dSHBG determination have never been described. The present study was thus designed to propose two easy ELISA methods for the parallel measurement of SHBG and dSHBG.

Total SHBG was determined with a classical ELISA sandwich assay. Calibration was performed with purified human SHBG obtained from Calbiochem or Eurodiagnostica. Controls were run with sera from Eurodiagnostica and with dSHBG prepared by treatment of SHBG obtained from Calbiochem with agarose-linked Clostridium perfringens neuraminidase (EC 3.2.1.18) from Sigma (6). The sera, collected from in-patients included in a screening program (3) and from healthy donors, were stored at −25 °C. Informed consent was obtained, and blood sampling was performed in accordance with ethical standards. The microtiter plates (Nunc) were coated overnight at 8 °C with 100 μL of a 1.500 dilution of a 3.4 g/L purified

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**References**