Chapter 2

Conformation Sensitive Gel Electrophoresis

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Abstract

Conformation sensitive gel electrophoresis (CSGE) is a rapid screening method for the detection of DNA sequence variation, specifically single-base changes or small insertions and deletions. It has been widely used for mutation screening in genetic disorders and for the detection of single nucleotide polymorphisms (SNPs).

CSGE is a simple manual method, based on heteroduplex analysis, and compares well in terms of sensitivity with other screening technologies. CSGE also lends itself to automation and such modifications have been useful in increasing sample throughput and sensitivity. However, manual CSGE remains a low-cost, accessible, and effective approach for mutation screening, which can be carried out with minimal specialist equipment. This chapter describes manual CSGE, and outlines some of the uses, modifications, and limitations of this method.

Key words: CSGE, Heteroduplex, Homoduplex, Mutation screening, Genetic analysis, CSCE, F-CSGE

1. Introduction

Conformation sensitive gel electrophoresis (CSGE) was initially developed by Ganguly et al. (1, 2) as a screening method to minimise the amount of nucleotide sequencing required when investigating large genes for mutations.

This method is used for the detection of single-base changes or small insertions and deletions within PCR products. It specifically detects heterozygous changes, although homozygous and hemizygous changes are readily detectable when samples are mixed with an equivalent "normal" control.

CSGE was initially used to improve the analysis of multiple genes associated with collagen disorders (3), and has been shown to be a highly sensitive tool in the analysis of a large range of inherited genetic disorders (4–13). CSGE has also been widely

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used in the analysis of sequence variations in cancer susceptibility genes such as BRCA1 and BRCA2 (14, 15) and in the study of the MEN1 gene in endocrine neoplasia type 1 (16).

CSGE has been compared with other screening methods in a number of studies. Markoff et al. (17) reported that CSGE was better than single-stranded conformation polymorphism (SSCP) for the analysis of mutations in BRCA1 and although these two methods were comparable in a study by Eng et al. (18), they concluded that denaturing high-pressure liquid chromatography (dHPLC) was more sensitive. However, under optimum conditions, manual CSGE has been shown to detect up to 100% of mutations (3) and has the advantage that it can be carried with a minimum of specialist equipment.

The requirement for higher throughput testing, particularly for single nucleotide polymorphism (SNP) analysis, has facilitated a number of modifications to the basic CSGE method, to improve both speed and sensitivity (19–21).

Higher throughput and sensitivity has also been achieved by the use of fluorescent labels and the automation of CSGE on genetic analysers. Fluorescent-CSGE (F-CSGE) offers improved resolution and reproducibility, and has been developed on both a gel and capillary format (14). Hashemi Soteh et al. (13) compared manual and fluorescent CSGE in the study of mutations in the VWF gene, concluding that F-CSGE was a more sensitive method, allowing higher throughput analysis. The transfer of CSGE screening to multicapillary genetic analysers has increased potential throughput significantly (22–24). Such approaches to mutation screening have been comprehensively evaluated by the UK National Genetics Reference Laboratory, Wessex (25)

It is apparent that automated approaches allow higher throughput screening and are more reproducible and sensitive than the manual method, however, they do require specialised equipment and increased cost (14). In addition, the availability of a genetic analyser may make direct nucleotide sequencing a cost-effective alternative.

2. Materials

2.1. Samples and Controls for CSGE Analysis

1. CSGE is carried out on PCR products, the design of which is critical to the success of this method (see Notes 1–4). The PCR product must be of good quality and sufficient concentration to be clearly visible on electrophoresis.
2. It is important to include suitable controls when screening, a previously sequenced equivalent PCR product with no sequence variation should always be included as a “normal”

control, for comparison of band patterns. A previously sequenced "positive" control should also be included. This may be a sample with the same sequence variation when screening for a known change, or may be an unrelated variable.

2.2. Gel Preparation and Electrophoresis

Manual CSGE involves the use of a non-proprietary polyacrylamide gel for electrophoretic separation. These gels are typically manual sequencing format (e.g. 30×45 cm) and 1 mm thick.

1. 20× stock TTE buffer: 1.78 M Tris, 570 mM taurine, 4 mM EDTA, pH 9.0.
2. 40% Acrylamide solution.
3. BAP (1,4 bis (acrolyl) piperazine)*.
4. Ethylene glycol*.
5. Formamide*.
6. Ammonium persulphate (10%, freshly prepared)*.
7. TEMED (*N*,*N*,*N*,N tetramethylethylenediamine)*.
8. DNA loading dye: e.g. 30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol FF.
9. DNA stain (e.g. ethidium bromide (1 mg/ml) or Gelstar)*.

Electrophoresis/ultra-pure grade reagents should be used and all solutions should be prepared in high quality distilled, deionised water.

Note: *These chemicals are potentially harmful. Refer to Material Safety Data Sheets (MSDS) and use appropriate handling and disposal procedures.

3. Method

CSGE is based on the ability to distinguish between homoduplex and heteroduplex DNA fragments by electrophoresis under partially denaturing conditions. DNA homoduplexes consist of double-stranded DNA fragments in which all the bases are paired correctly with their complementary base on the opposite stand. Heteroduplex DNA contains mismatched bases, and in PCR products that originate from a patient with a heterozygous mutation, both homoduplexes and heteroduplexes can form when double-stranded DNA is allowed to dissociate then reanneal with the complementary strand originating from a different allele (Fig. 1).

The presence of mismatched bases induces subtle conformational changes in the heteroduplex compared with the homoduplex as the misaligned bases do not conform to the typical Watson–Crick base-pairing rules.

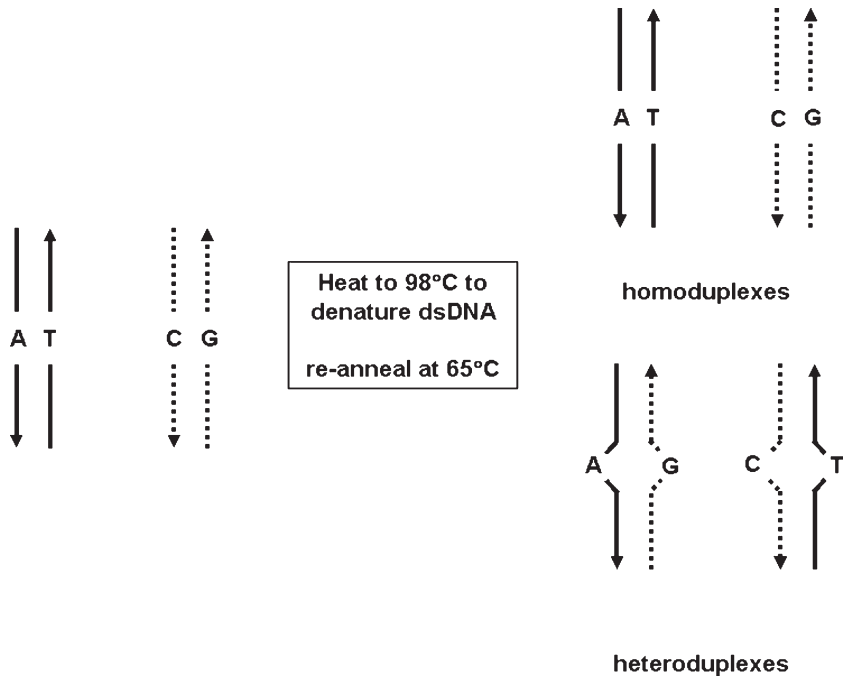

Fig. 1. Illustration of heteroduplex and homoduplex generation. A PCR product from a patient who is heterozygous (A/C) at a specific nucleotide position will contain two species of double-stranded DNA. Heating to 98°C will dissociate the double-stranded DNA, incubation at 65°C allows the strands to reanneal. Heteroduplexes are formed when a strand from one allele reanneals with the complementary strand from the other allele, and will contain a mismatched base pair.

The CSGE protocol involves the generation of heteroduplexes and/or homoduplexes in PCR products through heating and slow reannealling, followed by gel electrophoresis on a large format polyacrylamide gel, cross-linked with BAP (1,4 bis (acrolyl) piperazine) – which greatly improves gel strength and increases conductivity (1). The gel also contains ethylene glycol and formamide, which act as mild denaturants. Under these conditions, heteroduplexes can be resolved from homoduplexes as they generally migrate more slowly through the gel matrix.

Bands are visualised by staining with a DNA stain such as ethidium bromide. Multiple bands will be detectable in samples containing "heterozygous" changes, while a single-homoduplex band is generally visible in samples containing no sequence variation.

Like most screening methods, CSGE gives limited information on the nature of the sequence variation, and further analysis, usually by nucleotide sequencing is essential for identification.

3.1. Sample Mixing and Heteroduplex Formation

1. In order to also detect homozygous changes (or e.g. hemizygous changes in X-linked disorders), the PCR product must be mixed with an equivalent "normal" control. Mix the test sample 1:1 with a previously sequenced male control sample for the detection of hemizygous changes in X-linked disorders,

or a 2:1 mix (test:normal control) in autosomal disorders. Where a number of different fragments are to be screened, PCRs may be multiplexed, or products pooled, and then analysed simultaneously – providing the different PCR products can be easily differentiated.

2. For heteroduplex formation, incubate the PCR product mix (10 µl) in a thermocycler at 98°C for 5 min then 65°C for 30 min, followed by a slow cool down to room temperature. This should be done immediately before electrophoresis.

3.2. Gel Preparation

1. Ensure that gel electrophoresis plates are clean and grease-free.
2. Prepare a gel solution consisting of:

   44 ml of 99:1 acrylamide:BAP (1,4 bis (acrolyl) piperazine) (10% final concentration)

   17.5 ml of ethylene glycol (10% final concentration)

   26 ml of formamide (15% final concentration)

   4.4 ml of TTE buffer

   81 ml distilled, deionised water
3. Initiate polymerisation by adding 1.75 ml of 0.1% ammonium persulphate and 100 µl of TEMED; pour the gel immediately.
4. Allow a minimum 1 h for polymerisation.

3.3. Electrophoresis and Staining

1. Add 2 µl loading dye to 10 µl PCR product mix and load samples onto the gel in a standard loading buffer.
2. Carry out electrophoresis, 0.5× TTE buffer, typically for 16 h at 400 V (1).
3. After electrophoresis, carefully remove one of the glass plates and place the gel (on the remaining plate) in an appropriate container. Pour ethidium bromide solution (1 mg/ml in 0.5× TTE) onto the gel, and allow up to 30 min to stain.
4. To transfer the gel to the UV imaging system, blot with Whatman filter paper, carefully wet the filter paper with water to release from the gel when in position.
5. Visualise the separated products under UV illumination.

3.4. Analysis

Homoduplexes are generally detected as a single band, one or more additional bands representing the Co-Migrating heteroduplexes may be seen if a mismatch is present. Comparison to a "normal" control is important to avoid false-positive results, as additional bands may also be seen due to secondary structure. A detectable and consistent CSGE band pattern should be noted in the positive controls used. As changes in band pattern may be subtle, experience is invaluable for the interpretation of results and optimum PCR product design (see Note 5).

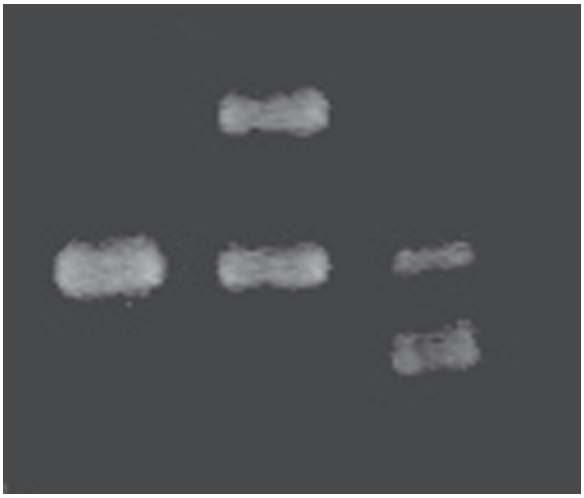

Fig. 2. Illustration of variation in band patterns seen in CSGE. Investigation of *F11* gene, exon 9. Lane 1: normal control, lane 2: patient with a single-base deletion (g), lane 3: patient with t→c substitution.

Figure 2 illustrates the CSGE band patterns associated with two different mutations (1 bp deletion (–g) and a t→c substitution) in exon 9 of the *F11* gene. Although the heteroduplex band/s is commonly seen above the homoduplex band, this type of pattern is not always seen, as illustrated in lane 3. The pattern of bands is highly variable depending on the type of mutation and the sequence context although insertions and deletions tend to produce the largest band separation as they effect a larger conformation change.

4. Notes

1. The exact nature of the mismatch, size of the PCR product, the location, and sequence surrounding the mismatch (sequence context) will all affect the sensitivity of CSGE. The reported detection rate of this method has ranged from 60% of BRCA1 mutations in a study co-ordinated by Eng et al. (18) to 100% in a number of studies, including those of Korkko et al. (3).
2. Optimal size for the PCR product is 200–500 bp, although sequence mismatches have been detected in products up to 800 bp in length. Size is limited by the inherent flexibility of DNA, which may mask any conformation change due to mismatch (26). Korkko et al. (3) suggested that PCR products should be limited to below 450 bp for optimum sensitivity, and demonstrated that a single-base polymorphism in the

COL1A2 gene which could not be detected in a 755 bp product was clearly seen when PCR primers were redesigned to reduce the size to 276 bp.

3. The band pattern seen is dependent on the nature of the mismatch and the surrounding nucleotide sequence (sequence context). Ganguly et al. (27) analysed the ability of CSGE to distinguish specific mismatches within the same sequence context, and found the following order of sensitivity

   G:G = G:T = T:G > G:A = A:G = T:T > A:A > C:T > C:C = C:A = A:C = T:C.

   Initial studies with CSGE by Ganguly et al. (1) suggested that mismatches within high temperature melting domains may be particularly difficult to resolve. However, the same mutations were detectable in later studies when primers were redesigned further away (3). It is generally accepted that CSGE is more sensitive to mismatches within an AT-rich sequence context than a GC-rich region (27).

4. Mismatches which are close to the end of the PCR product are less easily detected. Ganguly et al. (1) initially failed to detect a mismatch that was located 51 bp from one end of the PCR fragment. This became detectable when the primers were redesigned to position the mismatch 81 bp from the end. It is therefore advisable to allow for 50–100 bp of additional sequence at either end of the region of interest when designing primers for CSGE. A tagged primer system can be used (e.g. add 5′ M13 universal sequence tags to primers), which will facilitate sequencing reactions where multiple regions are being analysed (25).

   Figure 3 illustrates the effect of mismatch position on detection. Primers were redesigned to position a c→a substitution within exon 5 of the *F11* gene at 174, 70, and 42 bp from the end of the PCR product. Size was maintained at 290–292 bp. The ability to detect the mismatch is lost as the position of the mismatch approaches the end of the PCR product.

5. CSGE has some limitations as it can only detect single-base changes and small insertions or deletions. However, this method has been successfully used as part of a screening protocol for disorders associated with a wide range of mutation types (28). In common with most screening methods, CSGE provides limited information on the nature of a previously unknown sequence variation or its significance, and may not distinguish between two closely positioned sequence variations. Although band patterns associated with specific mismatches within the same PCR product are reproducible, it is advisable to sequence to confirm the nature of the mismatch.

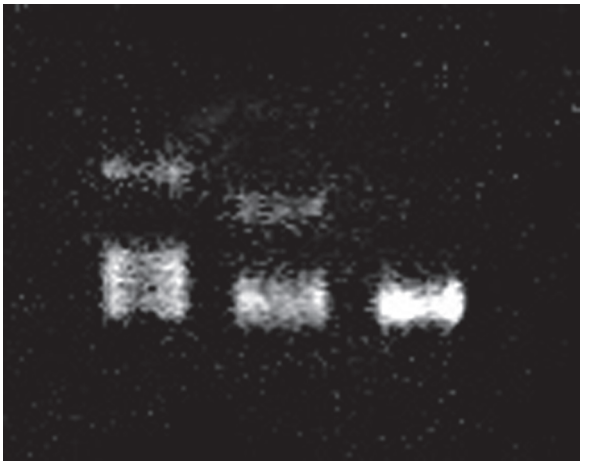

Fig. 3. Illustration of effect of position on the ability of CSGE to detect mismatches. Investigation of *F11* gene, exon 5. Primers were redesigned to position a c → a substitution at different points from the end of the PCR product. Size was maintained at 290–292 bp. Lane 1: mismatch is 174 bp from the end, lane 2: mismatch is 70 bp from the end, and lane 3: mismatch is 42 bp from the end.

CSGE is particularly useful for genes that are not very polymorphic such as *F8*. The sequencing load may increase significantly where highly polymorphic genes such as BRCA1 are analysed but it remains a powerful tool as it greatly reduces the amount of sequencing required during investigation of such genes (2).

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