Technical Manual

SILVER SEQUENCE™ DNA Sequencing System

INSTRUCTIONS FOR USE OF PRODUCTS Q4130, Q4131 AND Q4132.

NOTICE OF PACKAGING CHANGE

Due to U.S. federal regulations, the Silver Nitrate component of the SILVER SEQUENCE™ staining reagents must be shipped in a separate container from the rest of the components. Therefore, SILVER SEQUENCE™ DNA Sequencing System (Cat.# Q4130) will ship as three boxes (Cat.# Q4131, Q4133 and Q4134), and the SILVER SEQUENCE™ DNA Staining Reagents (Cat.# Q4132) will ship as two boxes (Cat.# Q4133 and Q4134).

If you have questions, please contact your local Promega representative or Promega Technical Services by phone: 800-356-9526 or e-mail: techserv@promega.com

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SILVER SEQUENCE™ DNA Sequencing System

All technical literature is available on the Internet at: www.promega.com/tbs/
Please visit the web site to verify that you are using the most current version of this Technical Manual. Please contact Promega Technical Services if you have questions on use of this system. E-mail: techserv@promega.com

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I. Description

The SILVER SEQUENCE™ DNA Sequencing System[a–d] is a non-radioactive alternative for enzymatic sequence analysis (1). The SILVER SEQUENCE™ System combines thermal cycle sequencing with a sensitive silver staining protocol to detect bands in a DNA sequencing gel (2). Silver staining is a rapid, inexpensive alternative to radioactive, fluorescent and chemiluminescent detection approaches. Unlike conventional radioactive sequencing protocols, results are obtained the same day; sequence may be read 90 minutes after completion of electrophoresis.

The system takes advantage of the intrinsic properties of the DNA polymerase isolated from *Thermus aquaticus* (*Taq* DNA polymerase). *Thermus aquaticus* is an extremely thermophilic microorganism whose DNA polymerase exhibits thermal stability to 95 °C (3,4). The SILVER SEQUENCE™ DNA Sequencing System includes a patented Sequencing Grade *Taq* DNA Polymerase[b], which is modified to give superior results on double-stranded DNA (dsDNA) templates. Sequencing Grade *Taq* DNA Polymerase produces uniform band intensity, low background and a high degree of accuracy.

Because the system uses thermal cycling sequencing, several advantages are realized over conventional sequencing strategies (5–9):

- The protocol yields linear amplification of the template DNA, generating sufficient material to achieve a detectable sequence ladder using silver staining technology. The sequencing reactions require between 0.03–2 pmol of template DNA depending on the template type.
- The high temperatures employed during each denaturation cycle eliminate the requirement for alkaline denaturation and ethanol precipitation of dsDNA templates. The denaturation cycles also help to circumvent the problems associated with rapid re-annealing of linear dsDNA templates such as PCR products.
- High annealing temperatures increase the stringency of primer hybridization.
- The high polymerization temperature decreases the secondary structure of DNA templates, permitting polymerization through highly structured regions (10).

The SILVER SEQUENCE™ System contains deaza nucleotide mixes that substitute 7-deaza-dGTP[d] for dGTP. The deaza mixes resolve band compressions associated with GC-rich regions (11,12).

The SILVER SEQUENCE™ System staining procedure consists of just a few steps. The sequencing gel is fixed in acetic acid to remove electrophoresis buffer and urea from the gel and to prevent diffusion of small extension products. Excess acetic acid and urea/Tris-borate are removed from the gel with water. Next, the gel is stained in a solution containing Silver Nitrate and Formaldehyde. Subsequently, the gel is rinsed very briefly in water to remove excess silver and immediately developed in an alkaline Sodium Carbonate solution containing Formaldehyde and Sodium Thiosulfate. Under these conditions, the silver ions are reduced to metallic silver by the Formaldehyde. The speed of the reaction is temperature-dependent and is stopped by the addition of acetic acid. The gel is then rinsed in water to remove excess reactants. After staining, the DNA sequencing fragments can be viewed directly in the gel with the aid of a white light box. Permanent copies of the sequencing gel can be produced in approximately ten minutes using Automatic Processor Compatible (APC) Film (Cat.# Q4411; see Related Products, Section XI.B).

II. Product Components and Storage Conditions

Product	Cat. #
SILVER SEQUENCE™ DNA Sequencing System	Q4130

Each system contains sufficient reagents for 100 sets of sequencing reactions and staining reagents for 10 gels. Includes:

Sequencing reagents (Q4131):

- 500 U Sequencing Grade *Taq* DNA Polymerase
- 1 ml DNA Sequencing 5X Buffer
- 800 µl 200 µl each of 4 SILVER SEQUENCE™ d/ddNTP Mixes (Deaza)
- 1.5 ml DNA Sequencing Stop Solution
- 2 × 20 µg pGEM®-3Zf(+) Control DNA (1 µg/µl)
- 1 µg pUC/M13 Forward Primer (24-mer)

Staining reagents (Q4132) packaged in two boxes:

Q4133:

- 20 g Silver Nitrate (10 × 2 g)

Q4134:

- 500 µl Bind Silane
- 60 ml Formaldehyde, 37% (20 × 3 ml)
- 10 ml Sodium Thiosulfate, 10 mg/ml (10 × 1 ml)
- 600 g Sodium Carbonate (10 × 60 g)
- 1 Protocol

(!) Formaldehyde MUST be stored at room temperature.

Product	Cat. #
SILVER SEQUENCE™ DNA Sequencing System	Q4131

Contains the same **sequencing** reagents as Cat.# Q4130, sufficient for 100 sets of sequencing reactions. No staining reagents are supplied with this product.

II. Product Components and Storage Conditions (continued)

Storage Conditions: Store all **sequencing components** at –20 °C and keep on ice when thawed for use. Store **staining reagents** at room temperature.

Use a Fume Hood. The Stop Solution contains 95% formamide, which is a teratogen. Silver nitrate is highly toxic in the powder form. Silver staining and developing solutions contain low levels of formaldehyde, a suspected carcinogen.

Regulations for the handling and disposal of silver ion differ among institutions. Consult your institution for policies regarding the handling and disposal of silver ion.

Materials to Be Supplied by the User

- thermal cycler
- SigmaCote® solution (Sigma Cat.# SL-2)
- Nuclease-Free Water (Cat.# P1193)
- glacial acetic acid
- orbital shaker or rocking platform
- 2–3 high-density polyethylene trays with dimensions slightly larger than the glass plate

III. Before You Begin

The following are critical factors that affect the results obtained with the SILVER SEQUENCE™ System. Poor results will be obtained if these considerations are not followed.

III.A. Template Considerations

Spectrophotometric methods fail to yield reliable estimation of DNA concentration in many DNA preparations. Artificially high DNA concentration estimates may result from contaminating chromosomal DNA, protein, RNA, ribonucleosides liberated from RNase digestion of DNA, and chemicals because they all contribute to absorbance at 260 nm.

Always perform a positive control reaction with the supplied pGEM®-3Zf(+) Vector DNA.

Template DNA concentration MUST be estimated by agarose gel electrophoresis or fluorescent detection methods before performing the sequencing reactions.

III.B. Primer and Profile Considerations

Optimal results will be obtained using a >24-mer primer that has a GC content of approximately 50%. Use a primer with a relatively high primer annealing temperature. High annealing temperatures inhibit strand re-annealing, reduce template secondary structure and improve the stringency of primer hybridization.

Use a 42 °C annealing temperature when the primer is less than 24 bases, has a GC content less than 50% or if you are unsure of the appropriate annealing temperature. Although the incidence of false pauses may increase at lower annealing temperatures, the lower temperature should yield sequence data, verifying that the template purity and concentration and the primer are acceptable. The optimal annealing temperature should then be determined empirically to reduce the incidence of false pauses.

The sequencing profile for each template may differ and should be determined empirically.

III.C. Staining Considerations

The water used for the staining procedure must be ultrapure (e.g., NANOpure® or Milli-Q® purified) or double-distilled. If the water contains contaminants, especially halogen or metal ions, the sequencing signal may not develop or only sequence bands in the top half of the gel may be visible.

Use only the provided Sodium Carbonate or the ACS reagent grade equivalent.

The length of the rinse step following staining is critical. The total time taken to rinse the gel and place it into developing solution *must be no longer than 5–10 seconds.* If the gel is rinsed for too long, the silver will be removed from the DNA and little or no sequencing signal will develop.

The developing solution must be prechilled to 10 °C to minimize background staining. The rate of development is very temperature-dependent.

Add the Formaldehyde and a 400 µl aliquot of the Sodium Thiosulfate to the developing solution immediately before use. Do not use the entire vial of Sodium Thiosulfate.

Prepare the staining solution just before use.

IV. Sequencing Protocol

Please review Section III before performing the sequencing reactions.

1. For each set of sequencing reactions, label four 0.5 ml microcentrifuge tubes (G, A, T, C). Add 2 µl of the appropriate d/ddNTP Mix to each tube. Add 1 drop (approximately 20 µl) of mineral oil to each tube. Cap the tubes and store on ice or at 4 °C until needed.

2. For each set of four sequencing reactions, mix the following reagents in a microcentrifuge tube:

Sample Reaction	
plasmid template DNA (for other templates, see Table 1)	1–2 pmol
DNA Sequencing 5X Buffer	5 µl
primer	4.5 pmol
Nuclease-Free Water to final volume	16 µl

Control Reaction	
pGEM®-3Zf(+) Control DNA (4 µg)	4.0 µl
DNA Sequencing 5X Buffer	5 µl
pUC/M13 Forward Primer (4.5 pmol)	3.6 µl
Nuclease-Free Water to final volume	16 µl

Table 1. Recommended Amounts of Template DNA.

Template Length (Type)	Amount of Template
200 bp (PCR product)	16 ng (120 fmol)
3,000–5,000 bp (supercoiled plasmid DNA)*	2–4 µg (1–2 pmol)
48,000 bp (lambda, cosmid DNA)	1 µg (31 fmol)

*Because supercoiled plasmid yields a weaker signal than relaxed dsDNA, we recommend using greater amounts of supercoiled plasmid template to obtain the same signal intensity that you would obtain with nonsupercoiled templates.

Note: 1 pmol = 1,000 fmol.

In general:

for dsDNA: ng of template = fmol of template × 6.6 × 10^{-4} × N,

where N is the number of base pairs in the template

for ssDNA: ng of template = fmol of template × 3.3 × 10^{-4} × N,

where N is the number of bases in the template

Example: How many ng of a 300 bp PCR product is equivalent to 120 fmol?

ng of template = 120 × 6.6 × 10^{-4} × 300 = 24

3. Add 1.0 µl of Sequencing Grade *Taq* DNA Polymerase (5 u/µl) to the primer/template mix (Step 2, above). Mix briefly by pipetting.
4. Add 4 µl of the enzyme/primer/template mix from Step 3 to each labeled tube containing d/ddNTP. Mix briefly.
5. Briefly centrifuge in a microcentrifuge.
6. Place the reaction tubes in a thermal cycler that has been preheated to 95 °C and with the following cycling profiles as a guideline, start the cycling program. The optimal annealing temperature should then be determined empirically to reduce the incidence of false pauses. The following programs routinely allow sequence to be read from near the primer up to 350 bases from the primer.

Annealing temperatures need to be optimized for each primer/template combination. If you are uncertain which profile to use, we suggest **starting with Profile 1.**

Note: It is important to preheat the thermal cycler to 95 °C to prevent nonspecifically annealed primers from being extended (and thus stabilized) by Sequencing Grade *Taq* DNA Polymerase. Ramp times should be as rapid as possible. The following cycling times do not include ramp time. The recommended times are for thermal cyclers that require an oil overlay.

Profile 1: for a primer <24 bases or with a GC content <50%:

95 °C for 2 minutes, then

95 °C for 30 seconds (denaturation)
42 °C for 30 seconds (annealing)
70 °C for 1 minute (extension)
For a total of **45–60 cycles**, then

4 °C soak

Profile 2: for a primer ≥24 bases or shorter primers with a GC content ≥50%:

95 °C for 2 minutes, then

95 °C for 30 seconds (denaturation)
70 °C for 30 seconds (annealing/extension)
For a total of **45–60 cycles**, then

4 °C

Note: The samples may be left overnight at 4 °C before adding the Stop Solution.

IV. Sequencing Protocol (continued)

7. After the thermal cycling program has been completed, add 3 µl of DNA Sequencing Stop Solution to the inside wall of each tube. Briefly centrifuge in a microcentrifuge to terminate the reactions.

8. Heat the reactions at 70 °C for 2 minutes immediately before loading 3.0–3.5 µl of each reaction on a 4–6% polyacrylamide (19:1 acrylamide: bisacrylamide) sequencing gel with 0.4 mm spacers, prepared as described in Section V.C.

   Note: It is not necessary to remove the mineral oil overlay, but be careful to draw up only the blue sample **below** the mineral oil when pipetting. If APC film is to be used, some users prefer to load their samples in the reverse order to compensate for the mirror image effect of this type of film.

V. Preparation of the Sequencing Plates and Gel

The glass plates must be meticulously clean. Clean the plates with warm water and a detergent such as Liqui-Nox® detergent. Common household-type detergents will lead to a high background. Rinse washed plates thoroughly with deionized water to remove detergent residues and perform a final ethanol wash of the plates. Detergent microfilms left on the glass plates may result in a high (brown-colored) background upon staining the gel.

The short glass plate described below is treated with a binding solution to chemically crosslink the gel to the glass plate (13). **This step is essential to prevent tearing of the gel during the silver staining protocol.** Because the gel is mechanically stabilized by the glass plate, it is possible to prepare 4% polyacrylamide/urea gels to maximize the length of read.

Materials to Be Supplied by the User

- 95% ethanol, 0.5% glacial acetic acid
- SigmaCote® (Sigma Cat.# SL-2)

Both the Bind Silane and SigmaCote® solutions are toxic and should be used in a fume hood. Wear gloves when handling these solutions.

V.A. Short Glass Plate Preparation

Treat the short glass plate with binding solution each time a gel is prepared.

1. Prepare fresh binding solution by adding 3 µl of Bind Silane to 1 ml of 95% ethanol, 0.5% glacial acetic acid.

2. Wipe a scrupulously cleaned plate using a Kimwipes® tissue saturated with 1 ml of freshly prepared binding solution. Make sure the plate is completely covered with solution.

3. After 4–5 minutes, apply approximately 2 ml of 95% ethanol to the plate and wipe with a Kimwipes® tissue in one direction and then perpendicular to the first direction using gentle pressure.

   Note: Rubbing hard will remove too much of the Bind Silane and the gel may not adhere as well.

   Repeat this wash three times, using a fresh Kimwipes® tissue each time, to remove excess binding solution. **This is essential to prevent the binding solution from contaminating the long glass plate, which could result in a torn gel.**

   Note: A used gel may be removed by scraping the rehydrated gel from the plate with a razor blade or plastic scraper. The plate should be cleaned thoroughly with detergent. Alternatively, the gel may be removed by soaking in 10% NaOH. **Keep all cleaning utensils (including sponges) for the short plates separate from those for the long plates to prevent cross-contamination of the long glass plates.** If cross-contamination occurs, subsequent gels may tear or become loose.

V.B. Long Glass Plate Preparation

Treatment of the long plate with SigmaCote® solution each time is unnecessary if water beads on the surface of the plate.

1. Change gloves before preparing the long glass plate to prevent cross-contamination with binding solution.
2. If necessary, wipe a scrupulously cleaned plate using a tissue saturated with SigmaCote® solution.
3. After 5–10 minutes, remove the excess SigmaCote® solution by wiping the plate with a Kimwipes® tissue. Excess SigmaCote® may cause inhibition of staining.

   Note: If the plate becomes contaminated with Bind Silane, soak it in 10% NaOH for 30–60 minutes.

V.C. Preparation of the Sequencing Gel

Prepare a 4–6% polyacrylamide gel (19:1 acrylamide:bisacrylamide) in 7 M urea in TBE buffer with 0.4 mm spacers.

Tips

1. Use freshly prepared acrylamide solutions—premixed solutions may give higher background.
2. Gels thinner than 0.4 mm may give weak signals.
3. Gels thicker than 0.4 mm or higher than 6% polyacrylamide may crack during drying.
4. Do not use wedge gels—they stain unevenly.

VI. Silver Staining the Sequencing Gel

Materials to Be Supplied by the User
(Solution compositions are provided in Section XI.A.)

- fix/stop solution
- staining solution
- developing solution, prechilled
- orbital shaker or rocking platform
- 2–3 high-density polyethylene trays, with dimensions slightly larger than the glass plate

Please review Section III before performing the silver staining procedure.

The staining procedure requires that the gel be incubated in plastic trays. We recommend using a minimum of two trays with similar dimensions to the plate. Rinse the trays with ultrapure water before adding fresh solutions to the trays.

Note: Handle the gel by the plate edges with gloved hands to avoid fingerprints.

VI.A. Preparation of Solutions

1. Prepare the three solutions as follows.

   Fix/stop solution: (10% glacial acetic acid): Add 200 ml of glacial acetic acid into 1,800 ml of ultrapure or double-distilled water. Do not reuse fix/stop solution.

   Staining solution: Combine 2 g (1 packet) of Silver Nitrate ($AgNO_3$) and 3 ml (1 vial) of 37% Formaldehyde in 2 L of ultrapure water.

Developing solution: Dissolve 60 g (1 packet) of Sodium Carbonate (Na_2CO_3) in 2 L of ultrapure water. Chill to 10 °C in an ice bath. **Immediately before use** (Section VI.B, Step 5a), add 3 ml (1 vial) of 37% Formaldehyde and a **400 µl aliquot** of the provided Sodium Thiosulfate (10 mg/ml). Discard the remaining Sodium Thiosulfate in the vial.

VI.B. Staining Procedure

1. **Separate the plates:** After electrophoresis, carefully separate the plates using a plastic wedge. The gel should be attached strongly to the short glass plate.
2. **Fix the gel:** Place the gel (plate) in a shallow plastic tray, cover with fix/stop solution and agitate well for 20 minutes or until the tracking dyes are no longer visible. The gel may be stored in fix/stop solution overnight (without shaking). Save the fix/stop solution to terminate the developing reaction (Step 8). If the developing solution has not been chilled yet, place it on ice at this time.
3. **Wash the gel:** Rinse the gel 3 times (2 minutes each) with ultrapure water using agitation. Lift the gel (plate) out of the wash and allow it to drain 10–20 seconds before transferring it to the next wash.
4. **Stain the gel:** Transfer the gel to staining solution and agitate well for 30 minutes.
5. a. Complete preparation of the developing solution by adding 3 ml (1 vial) of the provided 37% Formaldehyde and a **400 µl aliquot** of Sodium Thiosulfate (10 mg/ml) to the **prechilled** (10 °C) Sodium Carbonate solution. **Pour 1 L (half) of the prechilled developing solution into a tray and set it aside.** Keep the remaining developing solution on ice.

   b. Remove the gel from the staining solution and set it aside. Transfer the staining solution into a flask or beaker for silver recovery (see the Note on recovering silver at the end of this section). Rinse the tray and fill it with ultrapure water.

   Warning: The timing of the next (rinse) step, Step 6, is very important. Total time from when the gel is placed in ultrapure water to the time it is placed in developing solution should be no longer than **5–10 seconds**. Longer rinses result in weak or no signal. If the rinse proceeds too long, repeat Steps 4, 5b and 6 with the staining solution.
6. **Rinse the gel:** Dip the gel *briefly* into the tray containing ultrapure water, drain and place the gel *immediately* into the tray of chilled developing solution. The time taken to dip the gel in the water and transfer it to developing solution should be no longer than **5–10 seconds**.

VI.B. Staining Procedure (continued)

7. **Develop the gel:** Agitate the gel well by rocking until the template band starts to develop or until the first bands are visible. Transfer the gel to the remaining 1 L of chilled developing solution and continue developing for an additional 2–3 minutes or until all bands become visible.

   Note: The developed bands will appear fairly light. Prolonged development times result in high background. It is better to stop development early than to overdevelop the gel. The sequence ladder will darken upon gel drying and exposure to APC Film.

8. **Stop development:** To terminate the developing reaction and fix the gel, add 1 L of fix/stop solution (from Section VI.A) directly to the developing solution and incubate with shaking for 2–3 minutes. Longer incubations will fade the stain.

9. **Rinse the gel twice:** Rinse for 2 minutes each time in ultrapure water.

10. **Dry the gel:** Leave the gel at room temperature or use convection heating. View the gel on a light box at visible wavelengths or place it against a bright white or yellow background (e.g., paper). For permanent records, proceed to Section VII, APC Film Development. Discard all solutions according to institutional policies.

    Note: Waste silver can be recovered from the used staining solution for recycling. Precipitate silver by adding approximately 10 g of NaCl. Collect the AgCl precipitate by filtration or allow it to settle out by gravity.

VII. APC Film Development

VII.A. General Considerations

APC Film is a convenient format for making a permanent record of silver- or Coomassie®-stained gels. APC Film produces a direct positive, mirror image of the original on a white opaque background. Processing can be done either manually or with an automatic film processor using standard autoradiographic film developing chemicals (such as Kodak® GBX Developer and Fixer).

VII.B. Exposure with a White Fluorescent Light Box

Note: Light boxes differ in their ability to diffuse light evenly. Some models have "hot spots" or localized regions of high light intensity in the area of the bulbs. These light boxes produce images that are washed out in the center and very dark along the edges. An incandescent light box may be used if a suitable diffuser plate is present. We **do not** recommend the use of overhead fluorescent lighting to expose the APC Film.

Note: The gel must be dried completely before APC Film development. Handle all plates with gloved hands to avoid fingerprints.

1. In the darkroom with a safelight on, place the dry, stained gel attached to the plate (gel side up) on a white fluorescent light box.
2. Position the APC Film, emulsion side down, over the gel to be copied. The emulsion side of the film can be identified as the glossy white surface; the nonemulsion side has a gray tint.
3. Place a clean glass plate on top of the film to maintain contact between the gel and the film. Turn on the light box and expose the film for 1–2 minutes.

   Note: The optimal exposure time may vary with different light sources. Optimize the exposure time for your particular light source by exposing strips of APC Film for various time intervals. In general, exposure times of 1–2 minutes produce good results.
4. If the film appears clear and there is little signal, decrease the white light exposure time. If the film appears brown or black, increase the white light exposure time.

VII.C. Processing the Exposed APC Film

APC Film may be processed manually or with an automatic film processor. For automatic film processors, follow the manufacturer's instructions. For manual development, we recommend the following reagents and conditions:

1–5 minutes in Kodak® GBX Developer
1-minute wash with deionized water
3 minutes in Kodak® GBX Fixer
1-minute wash in deionized water

VIII. Troubleshooting

VIII.A. Troubleshooting Sequencing Problems

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**

Symptoms	Causes and Comments
Bands at the same position in two or three lanes throughout the gel sequence	DNA sample contains two different templates, generating overlapping products: • Prepare new template DNA starting with a single plaque or colony. • Gel-purify PCR products if you suspect nonspecific amplification products.
	Primer hybridized to a secondary site. Increase stringency of annealing or make a new primer.
	Priming at nicks or gaps in template or on contaminating DNA fragments. Prepare new template DNA.
Short read length or faint signal in isolated lanes	Inaccurate pipetting. Use only high-quality pipette tips and spin tubes briefly after adding reagents to ensure that no liquid remains on the tube walls.
Bands in the same position in all four lanes throughout the gel	DNA template is nicked or contaminated with PEG: • Remove nicked DNA by acid-phenol extraction. • Remove excess PEG by precipitating with ethanol. • Resuspend pellet in 10 mM Tris-HCl (pH 7.6) and extract with chloroform, then ethanol precipitate the aqueous phase.
	Dirty template DNA or contaminating RNA serving as a primer. Prepare new template or remove contaminating RNA.
Anomalous spacing of bands, missing bands and bands at the same position in two or three lanes, occurring only at specific regions	Dissociation of enzyme from DNA template due to secondary structure in template: • If possible, increase the primer annealing temperature to 70–74 °C. • We strongly recommend selecting 24-mer primers with a GC content of approximately 50%.

Symptoms	Causes and Comments
Anomalous spacing of bands, missing bands and bands at the same position in two or three lanes, occurring only at specific regions (continued)	When nonsequencing grade *Taq* DNA polymerase is used, residual 5´→3´ exonuclease activity can lead to ghost bands. Use sequencing grade *Taq* DNA Polymerase (Cat.# M2031), which has been modified specifically to remove 5´→3´ exonuclease activity.
	Band compression; a newly synthesized DNA strand is forming secondary structure during gel electrophoresis, leading to anomalous migration. • Increase the gel electrophoresis temperature. • Sequence the complementary strand. • The SILVER SEQUENCE™ DNA Sequencing System contains 7-deaza-dGTP to eliminate most compressions. • We do not recommend the use of dITP.
	Strong secondary structure within specific regions. Use the highest possible annealing temperature to reduce secondary structure.
Bands in all four lanes, occurring at specific regions	Dissociation of enzyme from DNA template due to secondary structure in template: • If possible, increase the primer annealing temperature to 70–74 °C. • We strongly recommend selecting 24-mer primers with a GC content of approximately 50%.
Faint or no sequence bands (also see Section VIII.B)	Dirty template DNA. Confirm presence of contaminants by agarose gel electrophoresis. If impure, re-extract with phenol:chloroform or purify with the appropriate Wizard® product. To confirm DNA purity, add the template DNA in question to the positive control reaction. Impure DNA will cause the control to fail.
	Insufficient template. Estimate the DNA concentration by agarose gel electrophoresis. Use the recommended amount of template DNA.
	Insufficient enzyme activity. Check the expiration date on the tube label.
	Samples not denatured before loading onto gel. Heat samples to 70 °C for 2 minutes immediately prior to loading.

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VIII.A. Troubleshooting Sequencing Problems (continued)

Symptoms	Causes and Comments
Faint or no sequence bands (continued; also see Section VIII.B)	Poor annealing of primer to template: • Verify that the primer sequence is correct for the template DNA. • Make sure that the primer does not self-anneal or form hairpin structures. • Reduce the annealing temperature. Virtually all primers (≥17-mer) will anneal at 42 °C. Although the ladder may contain more false stops, the lower temperature will verify primer annealing. • Redesign the primer if possible. Optimal results will be obtained using a ≥24-mer primer that has a GC content of approximately 50%.
	Contamination of sequencing reaction with salt. Excess salt can be removed by precipitating with ethanol and then washing the pellet with 70% ethanol before drying.
	Electrophoresis temperature too high. Run the gel at lower temperatures (40–60 °C).
Weak signal in some lanes, especially the "G" lane	Nonspecific adsorption of nucleotides onto the microcentrifuge tube surface. Use a different type of microcentrifuge tube.
Low band intensity at bottom of gel (also see Section VIII.B)	DNA concentration is too low: • Estimate the DNA concentration by agarose gel electrophoresis. • Increase the template DNA concentration twofold. • The addition of more enzyme will not increase signal strength.
Bands are fuzzy throughout the lanes	Poor-quality polyacrylamide gel. • Commercially available premixed solutions have a limited shelf life. Prepare fresh acrylamide and buffer solutions using high-quality reagents. • Store acrylamide solutions in the dark.
	DNA sample contains two templates, generating overlapping sequences. Prepare new template DNA from a single plaque or colony. Gel purify PCR products if nonspecific products are suspected.

VIII.B. Troubleshooting Staining Problems

Symptoms	Causes and Comments
Faint or no sequence bands (also see Section VIII.A)	Improper rinsing following the staining. Rinse step was performed for more than 5–10 seconds. Longer rinses remove the silver deposited on the DNA. Shorten rinse time.
	Poor-quality water. Use ultrapure water (e.g., NANOpure® or Milli-Q® purified) or double-distilled water.
	Incorrect amount of Sodium Carbonate added to the developing solution. Add 1 packet (60 g) of the provided Sodium Carbonate.
	Too much Sodium Thiosulfate added to the developing solution. Add only a 400 µl aliquot of Sodium Thiosulfate to the developing solution.
	Methanol present in the fix/stop solution. The fix/stop solution should contain only 10% acetic acid.
Low band intensity at bottom of gel (see also Section VIII.A)	Poor-quality water. Use ultrapure water (e.g., NANOpure® or Milli-Q® purified) or double-distilled water.
High background staining	Developing solution too warm. Cool the developing solution to 10 °C in an ice bath.
	Development performed too long. Stop the development reaction after the bands near the bottom of the gel **first** appear. The bands should be light gray. They darken upon gel drying and exposure to APC film.
	Insufficient fixation. Fix the gel until the bromophenol blue and xylene cyanol bands are no longer visible.
	Detergent residues present on glass plates may result in a brown background. • Rinse the cleaned plates thoroughly with deionized water to remove detergent microfilms. • Change detergents or acid-wash plates.
	Poor-quality polyacrylamide gel. Use freshly prepared acrylamide solutions. Premixed solutions may give higher background.

(continued next page)

VIII.B. Troubleshooting Staining Problems (continued)

Symptoms	Causes and Comments
High background staining (continued)	Decomposition of solutions. Use freshly prepared fix/stop solution, staining solution and developing solution.
	Poor-quality Sodium Carbonate was used. Use the Sodium Carbonate provided with the system. If preparing your own reagents, use only fresh, ACS reagent-grade Sodium Carbonate.
Dark, swirling patterns on the gel surface	Inadequate agitation during the staining steps. The gel must be agitated during all staining steps.
	Inadequate rinsing before the development step. Rinse the gel for 5–10 seconds prior to transferring it to development solution.
Clear, unstained areas in the gel	Excess SigmaCote® on the long glass plate. Remove excess SigmaCote® with ethanol-saturated paper towels.
Yellow gel	Improper gel fixing. Fix the gel for 20 minutes. To ensure that the urea is completely removed, fix the gel soon after electrophoresis.
	Poor-quality Sodium Carbonate. Use the Sodium Carbonate provided with the system. If preparing your own reagents, use only newly prepared, ACS reagent grade Sodium Carbonate.
Gray gel	Sodium Thiosulfate was not added to the developing solution. Add a 400 µl aliquot of Sodium Thiosulfate to the developing solution. Discard remaining vial contents. Always use a new vial for each gel.
Bands stain yellowish-brown with poor contrast, as opposed to dark gray	Dirty template DNA. Confirm presence of contaminants by agarose gel electrophoresis. If impure, re-extract with phenol:chloroform or purify with the appropriate Wizard® product. To confirm DNA purity, add the template DNA in question to the positive control reaction. Impure DNA will cause the control to fail.
Gel adheres to both plates	Long glass plate contaminated with binding solution, or inadequate treatment of the long plate with SigmaCote® solution. Wipe excess binding solution from the short plate. Avoid contaminating the long plate.

Symptoms	Causes and Comments
Gel peels off the plate when dried	Buildup of binding solution (Bind Silane) after multiple treatments. Wash plates as described, then soak the plates in 10% NaOH for 30–60 minutes.
	Acrylamide percentage of the gel was too high. Use a 4–6% polyacrylamide gel (19:1 acrylamide:bisacrylamide).

IX. Preparation of Template DNA

A wide variety of templates may be sequenced with this system, including plasmid DNA, single-stranded M13 or phagemid DNA, lambda DNA and PCR products. The Wizard® and Wizard® *Plus* DNA Purification Systems provide rapid and reliable methods to conveniently purify template DNA. See Related Products (Section XI.B) for available Wizard® products.

Alternatively, standard protocols for small-scale purification of plasmid DNA (minipreps) and the preparation of single-stranded template are provided in the *Protocols and Applications Guide* (14).

X. References

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XI. Appendix

XI.A. Composition of Buffers and Solutions

DNA Sequencing 5X Buffer

250 mM	Tris-HCl (pH 9.0 at 25 °C)
10 mM	$MgCl_2$

DNA Sequencing Stop Solution

10 mM	NaOH
95%	formamide
0.05%	bromophenol blue
0.05%	xylene cyanol

fix/stop solution

10%	glacial acetic acid

staining solution

1 g/L	silver nitrate ($AgNO_3$)
0.056%	formaldehyde (HCOH) (1.5 ml of 37% HCOH/liter)

developing solution

30 g/L	sodium carbonate (Na_2CO_3)
0.056 M	formaldehyde (HCOH) (1.5 ml of 37% HCOH/liter)
2 mg/L	sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$)

XI.B. Related Products

Additional SILVER SEQUENCE™ Products

Product	Cat. #
SILVER SEQUENCE™ Staining Reagents	Q4132

Contains the same staining reagents as Cat.# Q4130, sufficient to stain 10 gels. No sequencing reagents are supplied with this product.

Product	Size	Cat.#
Automatic Processor Compatible (APC) Film	25 sheets	Q4411

Additional Sequencing Products

Product	Size	Cat.#
Acrylamide, Molecular Grade	100 g	V3111
	500 g	V3115
Ammonium Persulfate	25 g	V3131
Bisacrylamide	25 g	V3141
fmol® DNA Cycle Sequencing System*	100 reactions	Q4100
Urea, 99% Pure	1 kg	V3171
	5 kg	V3175

*For Laboratory Use.

DNA Isolation Products

Product	Size	Cat.#
Wizard® SV Gel and PCR Clean-Up System	50 preps	A9281
	250 preps	A9282
Wizard® PCR Preps DNA Purification System	50 preps	A7170
	250 preps	A2180
Wizard® *Plus* SV Minipreps DNA Purification System	50 preps	A1330
	250 preps	A1460
Wizard® *Plus* SV Minipreps DNA Purification System plus Vacuum Adapters	50 preps	A1340
	250 preps	A1470
Wizard® SV 96 Plasmid DNA Purification System	1 × 96 preps	A2250
	5 × 96 preps	A2255
Wizard® DNA Clean-Up System	100 preps	A7280
Wizard® Genomic DNA Purification Kit	100 preps × 300 µl	A1120
	500 preps × 300 µl	A1125

For Laboratory Use.

XI.B. Related Products (continued)

DNA Isolation Products (continued)

Product	Size	Cat.#
PureYield™ Plasmid Midiprep System	25 preps	A2492
	100 preps	A2495
PureYield™ Plasmid Maxiprep System	10 preps	A2392
	25 preps	A2393

Cloning and Sequencing Vectors

Product	Size	Cat.#
pGEM®-T Easy Vector System I*	20 reactions	A1360
pGEM®-T Easy Vector System II*	20 reactions	A1380
pGEM®-T Vector System I*	20 reactions	A3600
pGEM®-T Vector System II*	20 reactions	A3610
pTARGET™ Mammalian Expression Vector System	20 reactions	A1410

*For Laboratory Use.

XI.C. Composition of Nucleotide Mixes

Component	G Nucleotide Mix	A Nucleotide Mix	T Nucleotide Mix	C Nucleotide Mix
ddGTP	45 µM	–	–	–
ddATP	–	525 µM	–	–
ddTTP	–	–	900 µM	–
ddCTP	–	–	–	300 µM
7-deaza-dGTP	30 µM	30 µM	30 µM	30 µM
dATP	30 µM	30 µM	30 µM	30 µM
dTTP	30 µM	30 µM	30 µM	30 µM
dCTP	30 µM	30 µM	30 µM	30 µM

XI.D. Sequencing Primers

The primers described in this section are designed for sequencing inserts in a variety of vectors. The SP6 and T7 sequencing primers anneal to the SP6 and T7 promoter sites flanking the multiple cloning region of all pGEM® Vectors. For sequencing the pSI, pCI and pCI-neo Vectors, the T7 EEV Promoter Primer (Cat.# Q6700) should be used instead of the T7 Promoter Primer. The pUC/M13 primers are designed for sequencing inserts cloned into the M13 and pUC vectors developed by Vieira and Messing (13) and also can be used for sequencing other *lac*Z-containing plasmids such as the pGEM®-Zf(+/–) Vectors.

Primer	Sequence	Size	Cat.#
RNA Polymerase Promoter Primers			
SP6 Promoter (19-mer)	5′-d(TATTTAGGTGACACTATAG)-3′	2 µg	Q5011
T7 Promoter (20-mer)	5′-d(TAATACGACTCACTATAGGG)-3′	2 µg	Q5021
T3 Promoter (20-mer)	5′-d(ATTAACCCTCACTAAAGGGA)-3′	2 µg	Q5741
T7 EEV Promoter (22-mer)	5′-d(AAGGCTAGAGTACTTAATACGA)-3′	2 µg	Q6700
pUC/M13 Primers			
pUC/M13 Forward (17-mer)	5′-d(GTTTTCCCAGTCACGAC)-3′	2 µg	Q5391
pUC/M13 Reverse (17-mer)	5′-d(CAGGAAACAGCTATGAC)-3′	2 µg	Q5401
pUC/M13 Forward (24-mer)	5′-d(CGCCAGGGTTTTCCCAGTCACGAC)-3′	2 µg	Q5601
pUC/M13 Reverse (22-mer)	5′-d(TCACACAGGAAACAGCTATGAC)-3′	2 µg	Q5421
Luciferase Vector Primers			
GLprimer1 (cw, 23-mer)	5′-d(TGTATCTTATGGTACTGTAACTG)-3′	2 µg	E1651
GLprimer2 (ccw, 23-mer)	5′-d(CTTTATGTTTTTGGCGTCTTCCA)-3′	2 µg	E1661
RVprimer3 (cw, 20-mer)	5′-d(CTAGCAAAATAGGCTGTCCC)-3′	2 µg	E4481
RVprimer4 (ccw, 20-mer)	5′-d(GACGATAGTCATGCCCCGCG)-3′	2 µg	E4491

cw = clockwise
ccw = counterclockwise

[a]U.S. Pat. No. 5,523,206 has been issued to Promega Corporation for non-radioactive DNA sequencing.

[b]U.S. Pat. No. 5,108,892 has been issued to Promega Corporation for the use of a modified *Taq* DNA polymerase to determine DNA sequence and amplify DNA sequence.

[c]DNA silver staining is licensed under U.S. Pat. Nos. 5,492,810 and 5,567,585.

[d]The 7-deaza-dGTP component is licensed from Roche Diagnostics GmbH under U.S. Pat. No. 5,480,980.

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