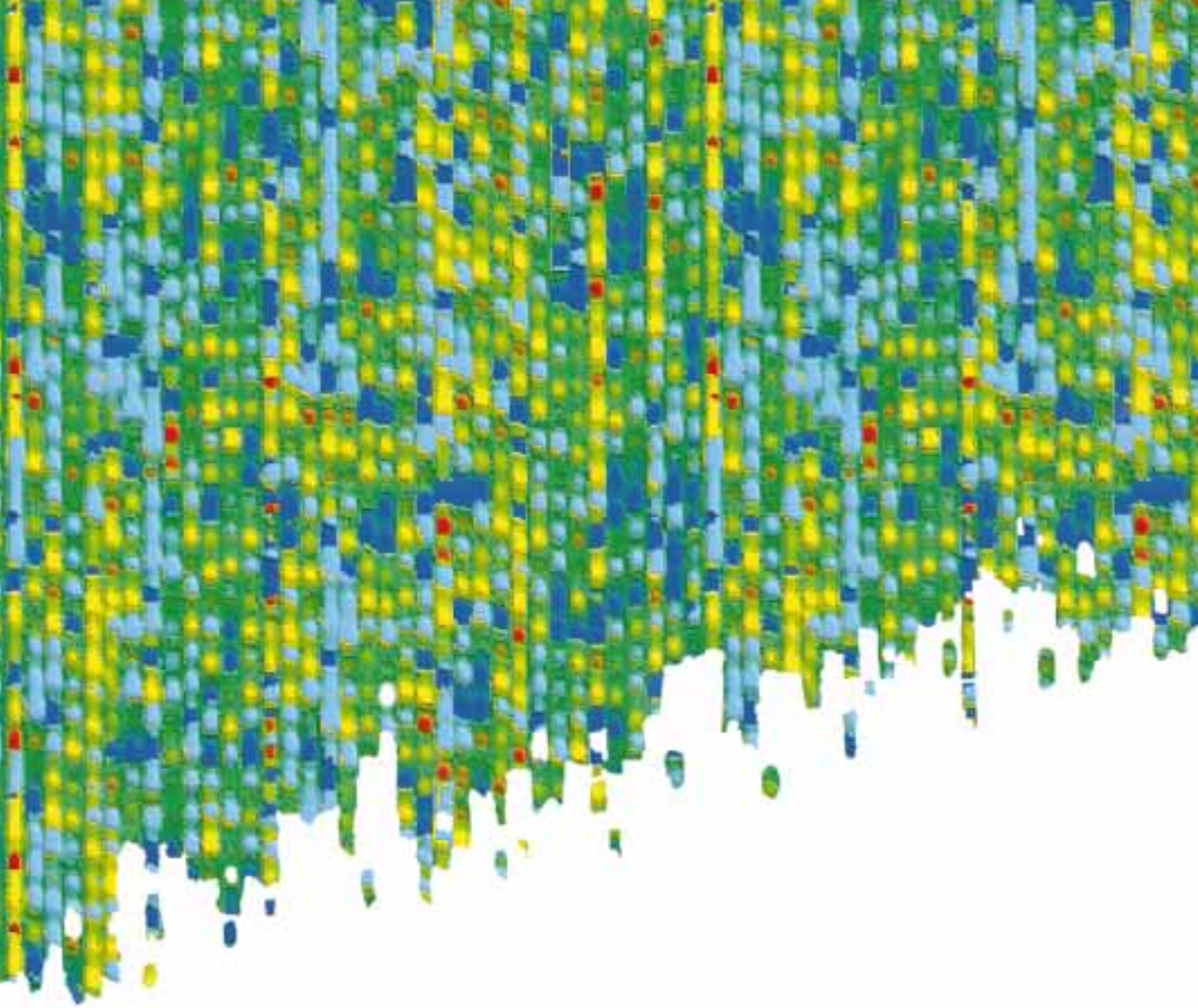

The QIAGEN Guide to Template Purification and DNA Sequencing (2nd Edition)

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I. Preface

Since the publication of the first edition of *The QIAGEN Guide to Template Purification and DNA Sequencing*, advances in sequencing chemistry and instrumentation have significantly improved the capacity, sensitivity, and reliability of DNA sequencing technologies. In this, the second edition of the guide, much of the original text has been revised and new sections have been added to accommodate recent developments[1]. The guide also contains sequencing-related protocols and technical information, as well as helpful technical and troubleshooting tips to improve your template purification and DNA sequencing.

The DNA Sequencing and Genomics Group, in conjunction with the Scientific Instruments Group at QIAGEN, is continually developing new products and instrumentation to simplify template purification and DNA sequencing. These products have enabled us to generate and assemble more than one megabase of final DNA sequence data from various model organisms for publicly funded genome projects. The DNA sequencing team at QIAGEN also performs extensive in-house quality control, and offers contract QIAGEN Sequencing Services for medium- to large-scale projects.

At QIAGEN we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in molecular biology and the use of QIAGEN products. If you have any questions or experience any difficulties regarding sequencing, template purification, or QIAGEN products in general, please do not hesitate to contact us.

Our customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

We hope you find this guide to be a useful tool for understanding and optimizing your DNA sequencing results and look forward to working with you in the years ahead to satisfy your DNA sequencing needs.

Hilden, May 1998

Dr. Andreas Düsterhöft

Business Unit Manager
DNA Sequencing and Genomics
QIAGEN GmbH

[1] *The scope of this guide is limited to those methods currently in common use. We welcome comments from our customers on the use of QIAGEN products in emerging DNA sequencing-related technologies.*

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II. Sequencing Methods

Most DNA sequencing methods presently in use are variations of the chain-termination method developed by Sanger and coworkers in the late 1970s (1). In this method, the DNA to be sequenced acts as a template for the enzymatic synthesis of new DNA starting at a defined primer binding site. A mixture of both deoxy- and dideoxynucleotides is used in the reaction, at concentrations which create a finite probability that a dideoxynucleotide will be incorporated in place of the usual deoxynucleotide at each nucleotide position in the growing chain. Incorporation of a dideoxynucleotide blocks further chain elongation, resulting in a population of truncated fragments of varying length. The identity of the chain-terminating nucleotide at each position can be specified either by running four separate reactions, each of which contains a single dideoxynucleotide (ddATP, ddCTP, ddGTP, ddTTP), or by running one combined reaction but using labels specific for each dideoxynucleotide. The resulting populations of molecules are separated by size on a denaturing high-resolution polyacrylamide gel. The sequence is subsequently specified by correlating the order of the bands on the gel with the dideoxynucleotide used to generate each band.

Label location

In order to detect the picogram quantities of DNA produced in a sequencing reaction, a label that can be readily identified in very small amounts must be introduced into the DNA when it is synthesized (Figure 1). The label can be incorporated into:

i) the oligonucleotide primer used to initiate the reaction
ii) the deoxynucleotides used for chain elongation
iii) the dideoxynucleotides used for chain termination

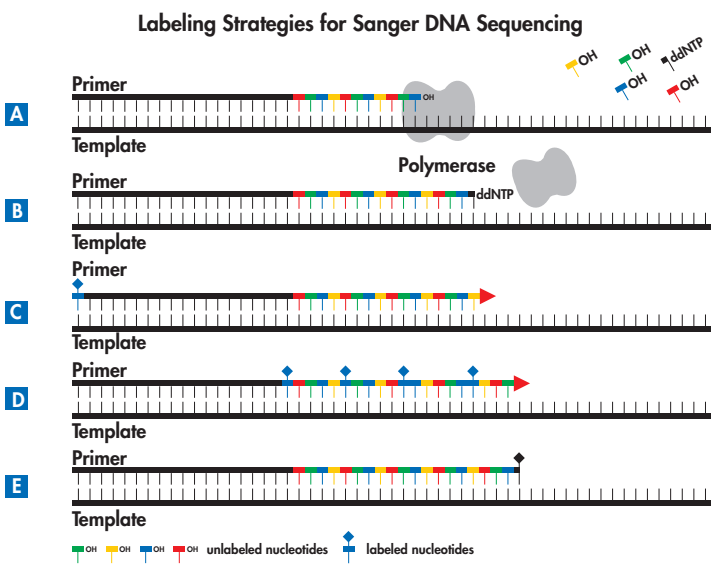

Figure 1. *Labeling strategies for Sanger chain termination sequencing methods.* **A** *a new DNA strand is synthesized as the polymerase moves down the template DNA, away from the priming site.* **B** *nucleotides are added by the polymerase until a dideoxynucleotide is incorporated.* **C**, **D**, *and* **E** *show different label locations.*
C *labeled primer.*
D *labeled deoxynucleotides.*
E *labeled dideoxynucleotide.*

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Radioisotope labels

For manual sequencing, the most common labels are the radioisotopes, ^{32}P, ^{33}P, and ^{35}S (2). The label can be incorporated throughout chain elongation by including a labeled deoxynucleotide in the reaction, or during an initial "labeling reaction" in which the radioactive nucleotide is limiting. Additional unlabeled deoxy- and dideoxynucleotides are then added to complete the reaction. The two-stage labeling procedure significantly increases the specific activity of the label in the newly synthesized DNA relative to that obtained by uniform labeling. Sequencing ladders incorporating radioactive labels are typically visualized using X-ray film.

Chemiluminescent detection sytems

An alternative approach to radioisotope-based manual sequencing uses nucleotides tagged with small molecules as labels to detect bands in a sequence ladder (3,4). The DNA fragments are separated on a standard polyacrylamide sequencing gel and transferred to a nylon membrane, either by blotting or through direct transfer as the gel is running. The molecular tag in the bound DNA is recognized by an enzyme-conjugate that catalyzes the chemiluminescent reaction, and DNA bands can be visualized by exposure to X-ray film. A useful feature of this technology is that the reagents used to detect the bands in one tagged sequence ladder can be removed and additional reactions can be performed to detect ladders with different tags run at the same location (5,6). This approach, called multiplexing, can considerably increase the amount of data obtained from a single gel run.

Fluorescent dyes

In automated sequencing, fluorescent dye labels allow the DNA bands to be detected in the gel during electrophoresis (Figure 2, Refs. 7,8,9). When different dye labels are used to tag each of the four dideoxynucleotides, the reactions can be performed in a single tube and analyzed on a single gel lane, further increasing the capacity and throughput of each sequencing gel. The ability to collect data during electrophoresis is a major contributing

Radioactive Label and Fluorescent Dye Label Sequencing Methods

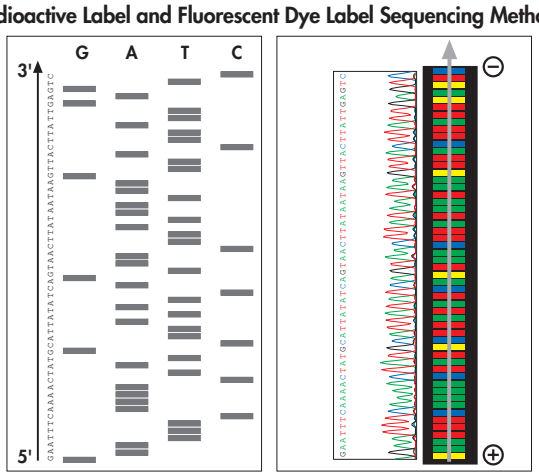

Figure 2. *Comparison of radioactive label and fluorescent dye label sequencing methods.* ***Left panel****: the resulting X-ray film of the conventional radioactive DNA sequencing method. In semiautomated fluorescent DNA sequencing* ***(right panel)****, "lane tracking" (represented by the grey arrow) through the laser-detected fluorescent bands of the electronic gel image is performed by software which measures the signal strength, and extracts an electropherogram to which an automated "base calling" is applied.*

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factor to the greater overall speed of automated sequencing, while the ability to run several samples in a single lane significantly increases the amount of sequence information per gel.

Energy transfer dyes

Recently, there has been a significant increase in new dye system development both for conventional dyes and for an alternative arrangement where two dyes are linked together in an energy transfer system. Energy transfer (ET) dyes were originally developed by Richard Mathies (10), and were commercialized by Amersham as ET primers. The Applied Biosystems Division of Perkin-Elmer then introduced BigDyes™, which can be used with either dye-primer or dye-terminator chemistry (11,12). BigDyes consist of a fluorescein energy donor dye directly linked to an energy acceptor dichlorhodamine dye. Both dyes make up an energy-transfer system that provides significantly greater sensitivity compared to single dyes. BigDyes also exhibit improved color resolution, a property that allows better base calling. The additional sensitivity conferred by energy transfer dyes means that less template is needed for a sequencing reaction, a feature that is valuable for sequencing very large templates and even for direct sequencing of genomic DNA (see Section VIII, page 44).

▼ Tip

The additional sensitivity conferred by energy transfer dyes means that less template is needed for a sequencing reaction, a feature that is valuable for sequencing very large templates and even for direct sequencing of genomic DNA.

Polymerases for DNA sequencing

In the original Sanger protocol, the Klenow Fragment of *E. coli* DNA Polymerase I was used. A significant improvement in the quality of the DNA sequence obtained by the Sanger method occurred with the development of a modified T7 DNA Polymerase with enhanced processivity and nucleotide incorporation capabilities (13,14). Additional modifications to the basic protocol, such as the use of modified nucleotides (e.g. 7-deaza-G) to avoid problems with DNA secondary structure, have resulted in highly reliable manual sequencing methods that work well with most DNA templates (15,16).

In addition, sequencing methods that use the thermostable *Taq* DNA Polymerase and other thermostable polymerases have been developed (17). Unlike the Klenow- and T7 DNA Polymerase-based methods, which involve a single pass of the polymerase along each template, the use of a thermostable polymerase allows the template DNA to be cycled in a series of denaturation/renaturation steps. The input DNA serves as a template many times in a single reaction, reducing the amount of input DNA required by a factor of five or more.

More recently, thermostable DNA polymerases that have a high affinity for dideoxynucleotides have been introduced (13,18–20). These enzymes, AmpliTaq™ FS from ABI and ThermoSequenase™ from Amersham, have a modified active site that accomodates dideoxynucleotides more easily than the unmodified form of the enzyme. The advantage of these enzymes is that much less dye terminator is required and incorporation of the terminators is less affected by sequence context, resulting in more uniform band intensities. The sequence data are therefore more reliable and require less manual editing than data prepared with the original form of the enzyme.

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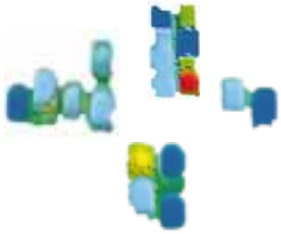

III. Cloning Vectors for DNA Sequencing

Single-stranded phage vectors

The first vectors designed for DNA sequencing were derivatives of the single-stranded bacteriophage M13 (21). Here, the DNA to be sequenced is cloned into the double-stranded replicative form of the phage, transformed into *E. coli*, and harvested from the culture supernatant in the form of phage particles containing single-stranded DNA. Single-stranded template DNA is ideal for sequencing and bypasses problems associated with strand separation. However, single-stranded template DNA can only be sequenced in one direction (without prior PCR amplification) and is of limited use for other molecular biology applications. Larger M13 inserts (>1 kb) also tend to be unstable, leading to difficulties with clone propagation and data reliability. M13 is still used extensively by high-throughput sequencing laboratories, but its use for smaller sequencing efforts has declined dramatically since cycle sequencing methods and thermostable polymerases eliminate the need for a separate template denaturation step.

Double-stranded plasmid vectors

Double-stranded plasmid vectors are suitable for sequencing but require prior separation of the two DNA strands by denaturation. Plasmids to be sequenced with modified T7 DNA Polymerase are typically denatured with sodium hydroxide, then neutralized in a way that maintains the denatured state until a primer can be hybridized to the start site for the sequencing reaction (22,23). Plasmid templates sequenced with thermostable DNA polymerases are subjected to successive cycles of heat denaturation and cooling (primer annealing) and a separate denaturation step is therefore not required (17,24).

▼ Tip

Plasmid templates sequenced with thermostable DNA polymerases are subjected to successive cycles of heat denaturation and cooling (primer annealing) and a separate denaturation step is therefore not required.

Phagemid vectors

Phagemids are hybrid vectors that consist of a double-stranded plasmid carrying an origin of replication from a single-stranded bacteriophage (25,26). When the host strain carrying the plasmid is infected with a helper phage virus, the plasmid is replicated as a single-stranded molecule and packaged into phage particles, from which template DNA can be purified. Mutations in the helper phage limit its replication and ensure that most of the DNA obtained from the mature phage particles is single-stranded phagemid DNA. Many common vectors (pBluescript, pTZ18/19, pGEM18/19) have the ability to function as phagemids but are much more typically used as simple plasmid vectors.

Vectors for large-insert DNA

Vectors capable of replicating large segments of foreign DNA have been developed for use in genome mapping and large sequencing projects. The vectors initially created for this purpose were cosmids (plasmids with lambda phage packaging sites) (27) and YACs (yeast artificial chromosomes) (28). Cosmids have an insert capacity of 35–45 kb, while YAC inserts can reach up to 1 megabase. Since the number of clones that must be analyzed to

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create a physical map depends on the insert size, larger inserts are preferable to smaller inserts. The large inserts of YACs, however, are prone to chimerism and instability and, since they are propagated in yeast, YACs are unfortunately difficult to purify in sufficient quantity from the yeast chromosome background. This limits their utility for subsequent procedures such as large-scale sequencing.

▼ Tip

Substantially higher yields can be obtained from P1 and PAC clones by inducing them prior to purification.

These limitations have been addressed by a new generation of vectors called P1s (29), PACs (phage artificial chromosomes) (30) and BACs (bacterial artificial chromosomes) (31). P1s and PACs are derived from the *E. coli* bacteriophage P1, while BACs are based on the *E. coli* F-plasmid. P1 and PAC insert sizes are typically 90–145 kb, while BAC inserts range in size from 75–350 kb. Insert instability is minimized because the clones are propagated as low-copy plasmids (1–2 copies per cell) in *E. coli*. However, P1 and PAC clones have the added advantage that they can be induced prior to purification for substantially higher yields.

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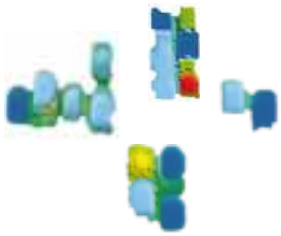

IV. PCR-Amplified Template DNA

The polymerase chain reaction (PCR*) allows a segment of DNA located between two defined priming sites to be amplified (32,33). If equal amounts of the two primers are used, then the amplified DNA will be double-stranded. Alternatively, if one primer is in excess, the final product will contain a proportional amount of single-stranded DNA. Either double-stranded or single-stranded amplified DNA can serve as a template for DNA sequencing (34–36).

PCR has been used to amplify segments of genomic DNA for sequence-based mutational analyses, and to amplify inserts directly from plaques or colonies (37,38). Direct amplification from cells or plaques circumvents the need to grow individual cultures of each clone to obtain enough DNA for sequencing. However, it is essential to ensure that only one fragment is amplified from each clone, and that all PCR components that can interfere with the subsequent sequencing reaction such as primers, nucleotides and salts, are removed.

The reliability of sequence data obtained from PCR-derived templates depends on the number of molecules that served as templates for the original amplification (see Appendix). If the amplification is performed from a small number of template molecules, errors that occur in the early cycles of replication can be propagated through subsequent cycles such that they are detected in the final sequence. These problems can be avoided by sequencing each region at least twice from independent amplification reactions. Problems with PCR fidelity are most often detected when PCR-amplified fragments are cloned, however. This is because fragments with errors that are present at a low level in the amplified DNA will not be detected by direct sequencing but can be selected in the cloning process. No significant differences in error rates were found when data obtained by *Taq* cycle sequencing were compared with data obtained using modified T7 DNA Polymerase (39).

▼ Tip

PCR-amplified fragments containing low-level errors will not be detected by direct sequencing, but can be selected in cloning processes.

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V. Sequencing Applications and Strategies

DNA sequencing applications in molecular biology fall into three broad classes:

- *de novo* sequencing of unknown DNA
- verification of known DNA, such as when a clone junction is sequenced to confirm correct insertion
- diagnostic sequencing to identify variations in an otherwise known sequence (e.g. mutation analysis)

Depending on the application, the DNA to be sequenced is carried on a viral or plasmid cloning vector, or is part of an amplified fragment. The approach used to sequence the target DNA (the sequencing strategy) is designed to obtain the optimum balance between the time required for the project and its cost. The strategy selected is usually determined by the size of the segment to be sequenced, with larger segments requiring additional processing before sequencing can begin.

Primer walking

If the segment of DNA to be sequenced is small, a primer that binds to a known sequence in the flanking DNA can be used to initiate a sequence run across the region of interest. A second sequencing run over the same region from the opposite direction is highly recommended, since factors that affect the sequence quality on one strand are often not present in the opposite strand.

If the length of the DNA to be sequenced exceeds the length of a single sequencing run, sequence data from the far end of the first run can be used to prepare the primer for a second run into the region of interest. This process, called primer walking, can be repeated many times to sequence extensive tracts of DNA (Figure 3). The major advantages of primer walking

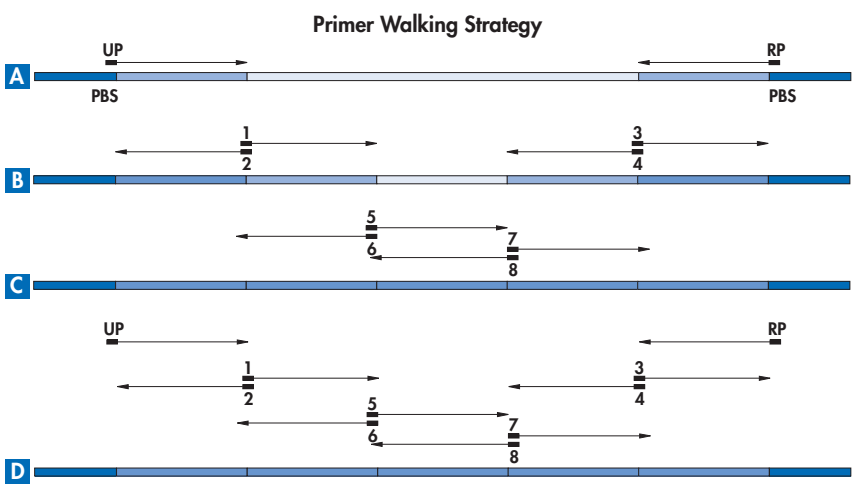

Figure 3. *Primer walking strategy. The bar represents a segment of target DNA (shaded) flanked by vector sequences (solid) containing primer binding sites (PBS).* **A** *sequencing runs are initiated from priming sites in the vector DNA. (UP: –21M13 primer; RP: reverse primer).* **B** *reliable sequence data from the far end of each first run is used to make primers for sequence runs in the forward and reverse directions.* **C** *the priming cycle is repeated to provide complete sequence data for both strands.* **D** *overall sequencing strategy.*

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are that no subcloning is required, and the location and direction of each sequencing run is known. Primer walking minimizes the degree of redundancy needed to obtain the final sequence, and avoids assembly problems caused by repetitive DNA. In theory, this approach can be used to sequence a DNA segment of any size. However, its use is usually limited to projects smaller than 5 kb because the successive cycles of sequencing, primer design, and synthesis for the next walking step are too time-consuming.

The efficiency of primer walking is highly dependent on the length of sequence read from each sequencing run. The priming site for a new sequencing run is selected from the most distant reliable sequence obtained in the previous step. Consequently, the data acquisition phase of a primer walking project with an average accurate read length of 600 bases will be completed in approximately half the time and with half the expense of a project with an average read length of 300 bases.

Nested deletion

Nested deletion strategies work best for DNA segments in the 5–10 kb range and require some prior knowledge of the restriction sites within the target DNA. Nested deletion with exonuclease III requires a unique blunt-end or 5′-overhang restriction site adjacent to the region to be deleted, and a unique 3′-overhang site distal to the deleted region to protect the vector DNA (Figure 4). When these sites are exposed after cleavage, exonuclease III will enter the DNA at the 5′-overhang site and begin to digest one strand in the 3′→5′ direction. The enzyme does not recognize the 3′-overhang and therefore cannot initiate digestion on the opposite strand. At timed intervals, aliquots of the reaction mix are removed, the DNA blunt-ended, and ligated

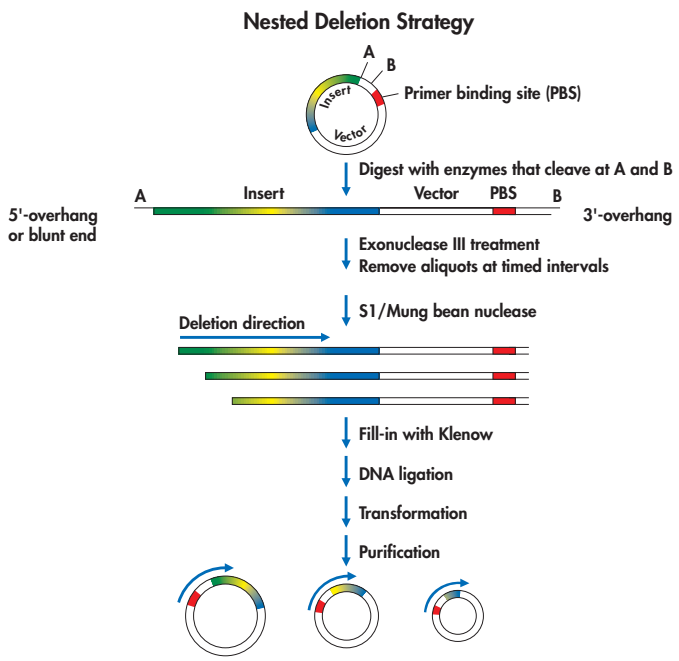

Figure 4. *Generation of clones for nested deletion sequencing.* ***A*** *and* ***B*** *represent restriction endonuclease sites with 5′- and 3′-overhangs, respectively. The arrows on the deleted plasmids at the bottom of the figure represent sequence runs originating from a universal priming site in the flanking DNA of the vector.*

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to recircularize the molecules. Ideally, the time interval between each aliquot corresponds to the amount of time required by the enzyme to digest just under the equivalent of one sequence run (450–500 bases).

This process places a vector priming site next to the 5′-deletion terminus of each molecule — sites that were previously located deep within the insert DNA. The nested deletion approach has the advantage that the sequencing runs are directed, that fewer sequencing runs are required to obtain a complete sequence, and that the sequence data can be obtained from the entire region of interest at one time using standard primers only.

As average read lengths increase, and primer costs decline, this method is being used less frequently. An alternative approach that achieves much of the benefit of the complete procedure is to subclone one or more internal segments of the original clone and sequence these fragments in parallel by primer walking.

Shotgun sequencing

In shotgun sequencing, the target DNA is fragmented by enzymatic digestion or physical shearing to fragment sizes in the range of 0.5–5 kb. Subfractions with a narrower size range (eg. 0.8–1.5 kb) are subcloned into either a plasmid or M13 vector (Figure 5). The subclones can then be sequenced from standard primer binding sites in the flanking vector DNA. Shotgun sequencing requires no prior knowledge of the insert sequence, and puts no limitations on the size of the starting DNA to be sequenced.

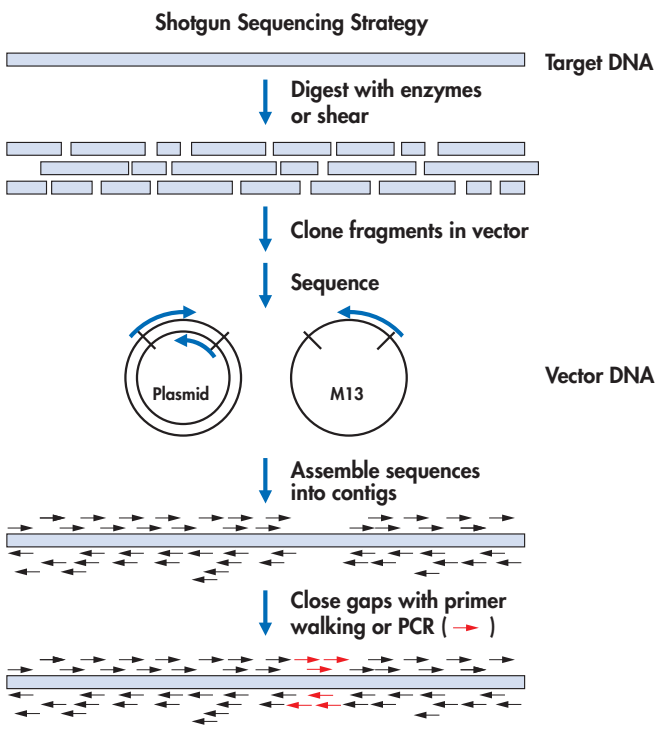

Figure 5. *Shotgun sequencing strategy. Plasmid and M13 vectors are shown as alternatives. Arrows on the vectors indicate sequence runs into subcloned segments of target DNA using standard primers binding in the vector. The arrows above and below the bar (consensus sequence) represent individual sequence runs on different subclones. The red arrows indicate primer walking runs to eventually close the gaps in the consensus sequence.*

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Large projects taken to completion with the shotgun sequencing approach have averaged a seven- to ten-fold level of redundancy. This means that the entire segment has been sequenced the equivalent of seven to ten times to guarantee full double-stranded coverage. 400–600 subclones are required to sequence a cosmid-sized segment of DNA (40 kb) and 1500–1800 may be required to sequence a medium-sized BAC clone (100–120 kb). As with primer walking projects, the number of clones required to complete the project depends on the read lengths obtained. Nested deletion and shotgun sequencing both require a significant initial investment, but once subclones are available, sequence data can be obtained rapidly from the entire target DNA.

In theory it should be possible to complete a sequencing project using just nested deletions or shotgun sequencing. In practice, however, it is often more efficient to fill in gaps in the sequence by primer walking after most of the sequence has been determined (Ref. 40, Figure 5). Recently, projects encompassing entire bacterial genomes have been completed using a whole genome shotgun sequencing approach (41).

Transposon-based strategies

An alternative approach that avoids subcloning uses mobile segments of DNA to introduce priming sites into a large segment of DNA (42,43). A clone containing the target DNA is grown in a bacterial host strain carrying the appropriate element and a gene encoding an enzyme (transposase) that catalyzes transposition of the element into the target DNA (Figure 6).

In addition to sequences required for transposition, the transposable element carries an antibiotic resistance gene or suppressor tRNA gene that can be used as a selectable marker. Clones containing transposable elements are selected by transforming plasmid DNA isolated from the transposase-containing strain into a non-transposase-containing strain, with coselection for markers carried by the plasmid vector and the inserted transposable element. An ordered array of insertions can be obtained by using PCR or restriction sites within the element to identify insertions at regular intervals in

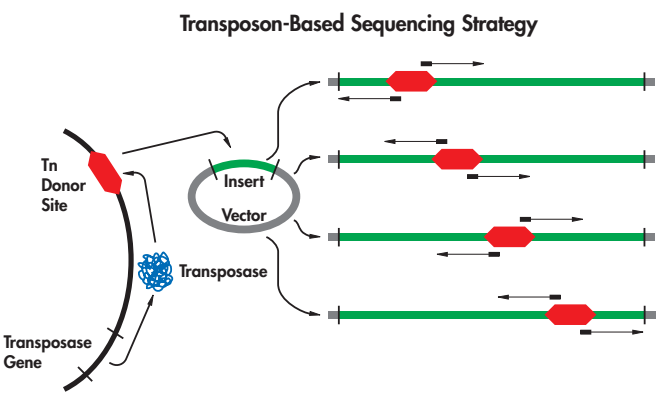

Figure 6. *Transposon-based sequencing strategy. Insertion of primer binding sites.* ***Left****: schematic diagram of a transposition event. The segment on the far left represents a segment of the host cell DNA. The adjacent circle represents the target DNA in a plasmid vector.* ***Right****: transposon insertions in the target DNA of individual plasmid molecules are indicated in red. The arrows represent DNA sequencing runs from priming sites at the ends of each transposon with transposon-specific primers.*

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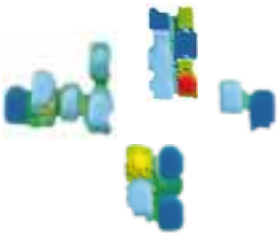

the target DNA. Priming sites at the ends of each element are then used for sequencing runs extending away from the element into the target DNA.

Transposon-based strategies are being used less and less frequently as the prequalification work to identify a good set of clones to be sequenced is relatively high. The alternative strategies described above are faster and easier to perform.

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VI. Automated DNA Sequencing

The development of instruments for automated DNA sequencing has dramatically increased the throughput of individual laboratories. Unlike manual sequencing methods, which generally use a radioactive label and visualize the banding pattern by autoradiography, automated sequencers use a laser to detect DNA fragments labeled with fluorescent dyes (7–9). For many laboratories, the health risks and inconvenience of radioisotope disposal are sufficient incentive to automate much or all of their DNA sequence analysis. Additionally, considerable time and labor savings can be obtained with automated sequencing through increased gel capacity (one lane per reaction instead of four), immediate data acquisition, and automatic base calling and data entry.

Effect of label location

As discussed previously, the label used for detection of the DNA synthesized in a sequencing reaction can be incorporated into the primer, the deoxynucleotides, or the dideoxynucleotides. For manual sequencing, the label is generally supplied in the form of labeled deoxynucleotides, which are incorporated at multiple sites along the newly synthesized DNA. For automated sequencing, the labels are supplied as fluorescent dyes linked to either the primer (dye primer: Ref. 44, Figure 7), the deoxynucleotides (internal labeling: 45) or the dideoxynucleotides (dye terminator: Refs. 46, 47, Figure 7).

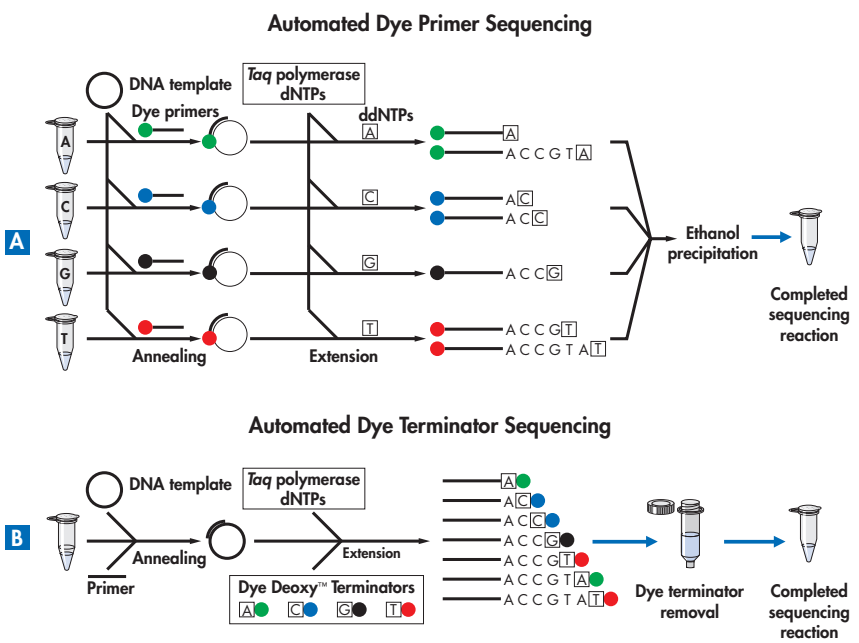

Figure 7. *Automated sequencing chemistries.* **A** *sequencing with dye-labeled primers. Note the correlation between the dye on the labeled primer and the specific dideoxynucleotide in each reaction.* **B** *sequencing with dye-labeled terminators. The colored circles represent different types of fluorescent dye. The extended primers represent the shortest molecules in a population with lengths of up to 1400 bases. (Reproduced by kind permission of Applied Biosystems Division of Perkin-Elmer Corporation.)*

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Labeled primers

Primer labeling with fluorescent dyes is generally limited to commonly used primers. The reason for this is that dye primer synthesis is an expensive process, and not one, but four derivatized primers must be synthesized. The four dye-labeled primers are used in separate sequencing reactions, each of which contains one of the four dideoxynucleotide terminators. Since each of the four dideoxynucleotides is matched with a specific dye primer, the reactions can be pooled before electrophoresis on a sequencing gel (Figure 7A). Sequencing reactions run with dye-labeled primers typically exhibit uniform peak intensities and high signal levels, but are more likely to show higher background levels since nucleotide chains that terminate prematurely will add to the signal.

Sequencing reactions requiring the use of non-standard (custom) primers are usually done with dye-labeled dideoxynucleotides as chain terminators. The reactions are performed in a single tube, and run on a single gel lane (Figure 7B). Prematurely terminated molecules are not detected, since only those molecules that have incorporated a dideoxynucleotide will be labeled. As a result, molecules that contain regions of secondary structure that impede the progress of the polymerase (strong stops) and cause premature termination can be sequenced more readily with dye-labeled terminators than with dye-labeled primers. On the other hand, insertion of modified dideoxynucleotides is more sensitive to context (sequence of adjacent nucleotides) than the incorporation of unmodified nucleotides, so the peak heights of reactions run with dye-labeled terminators are more variable than those prepared with dye-labeled primers (Ref. 20, Figure 8).

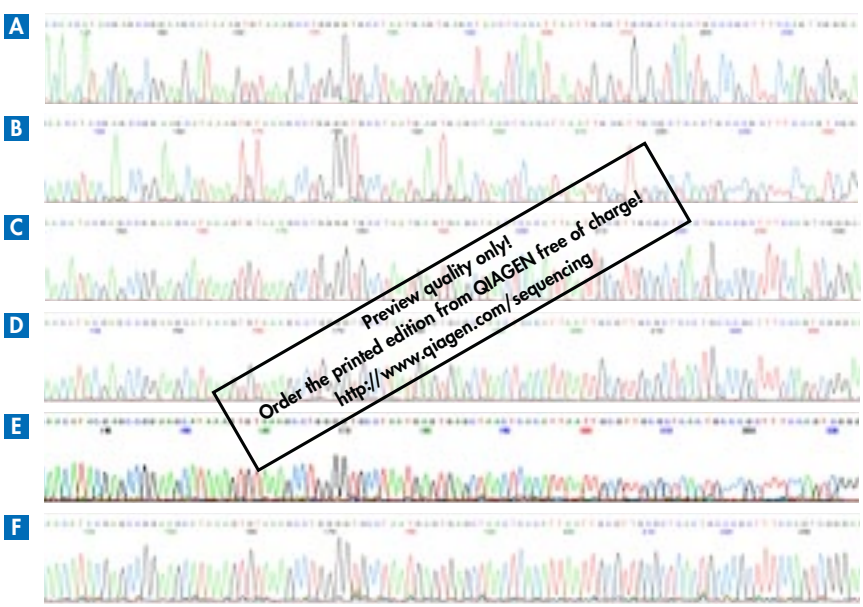

Figure 8. *Sequences of the plasmid pTZ19R with different sequencing chemistries to demonstrate the peak height uniformity obtained. Note the increase in peak height from A to F. Sequencing reaction: 400 ng (**A** and **B**) or 200 ng (**C**, **D**, **E**, and **F**) ultrapure plasmid template were used according to the manufacturer's protocol.* **A** *Taq polymerase and dye terminators.* **B** *Taq polymerase FS and dye terminators.* **C** *Taq polymerase FS dRhodamine dye terminators.* **D** *Taq polymerase FS BigDye terminators.* **E** *Taq polymerase FS dye primers.* **F** *Taq polymerase FS BigDye primers.*

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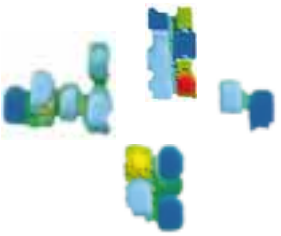

Labeled terminators

In general, modified T7 DNA Polymerase is less sensitive to sequence context than the thermostable *Taq* DNA polymerase. The active sites of the new DNA polymerases, AmpliTaq FS and ThermoSequenase, have been modified to resemble that of T7 DNA Polymerase (13) and consequently exhibit less context sensitivity. The variation that remains is generally predictable, if the one or two bases immediately preceding a particular nucleotide are known (20). This variability can therefore be compensated for by manual editing methods.
A further advance towards improved peak uniformity occurred with the introduction of dichlororhodamine dyes by ABI. These dyes exhibit enhanced spectral resolution and produce larger G peaks after A. Small G peaks after A were a particular problem with the previous dye-terminators (20).

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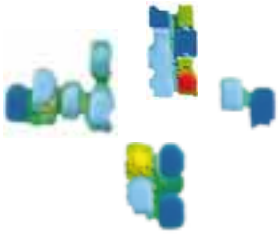

VII. DNA Template Purification

Template purity is an important factor in all types of DNA sequencing, but is most critical for automated sequencing. Data from automated sequencing runs are collected as the gel is running, and samples with low signals cannot be "exposed" for longer periods of time as with radioactive sequencing. Reactions with dye-labeled dideoxynucleotides are particularly susceptible to template contaminants. This is in part because of the additional requirements placed on the polymerase by the bulky fluorescent dye, and because the small peaks that can occur with dye terminator sequencing may be lost if the signal strength is too low. On the other hand, new DNA polymerases such as AmpliTaq FS and ThermoSequenase show improved incorporation of dye terminators and increased tolerance to contaminants relative to the unmodified *Taq* DNA Polymerase enzyme. Therefore, DNA of lower quality that would not have been acceptable before can now produce satisfactory results for some sequencing applications.

QIAGEN has developed a range of product options for template purification to help you achieve the best balance between cost and purity for each DNA sequencing application. These products have been, and are routinely used in the sequencing facility at QIAGEN throughout our participation in the European Yeast Genome Project (48–51), the *Bacillus subtilis* Genome Project (52), the *Arabidopsis thaliana* Genome Project (53,54), the *Schizosaccharomyces pombe* Genome Project (55), and the Full Length Human cDNA Sequencing Project (56). Our products are also used for in-house sequencing demands and contract DNA sequencing services.

▼ Tip

QIAGEN has developed a range of product options for template purification to help you achieve the best balance between cost and purity for each DNA sequencing application.

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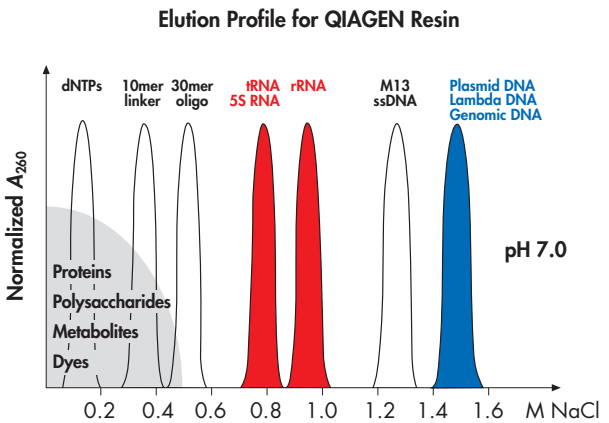

Figure 9. *Efficient separation of nucleic acids from at neutral pH with QIAGEN Anion-Exchange Resin. Elution profile for cellular components in a continuous gradient. QIAGEN Plasmid Kit buffers are designed to purify DNA from other cellular components and not for resolution of individual component peaks.*

QIAGEN Anion-Exchange Resin

For the highest level of template purity, we recommend purification methods based on patented QIAGEN Anion-Exchange Resin. This unique resin contains a high density of positively charged DEAE groups that tightly bind the negatively-charged phosphates of the DNA backbone. The key advantage of this material is that the DNA remains bound over a wide range of salt concentrations (Figure 9). Impurities such as RNA, protein, carbohydrates and small metabolites can be efficiently washed away with low-salt buffers, while the template DNA remains bound until eluted with a high-salt buffer.

QIAGEN-tip columns

The amount of plasmid DNA needed for a DNA sequencing project depends on both the scale of the project and the sequencing strategy selected. To meet these different requirements, QIAGEN Resin is supplied as prepacked columns with capacities ranging from 20 µg to 10 mg of plasmid DNA (Figure 10). QIAGEN Plasmid Kits include all the buffers and components needed for high-yield purification of ultrapure DNA (57–63).

When used together with QIAfilter for efficient lysate filtration, QIAGEN-tip columns provide a fast and effective way to purify ultrapure plasmid DNA from crude lysates.

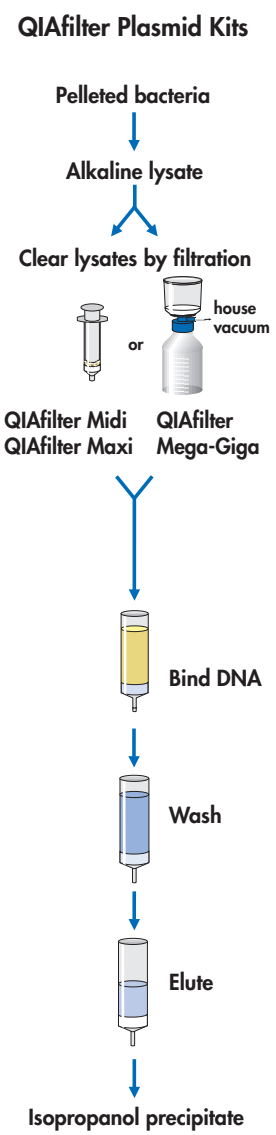

Figure 10. *Schematic representation of ultrapure plasmid DNA purification using QIAGEN-tips.*

QIAfilter is available for Midi, Maxi, Mega, and Giga Kits only.

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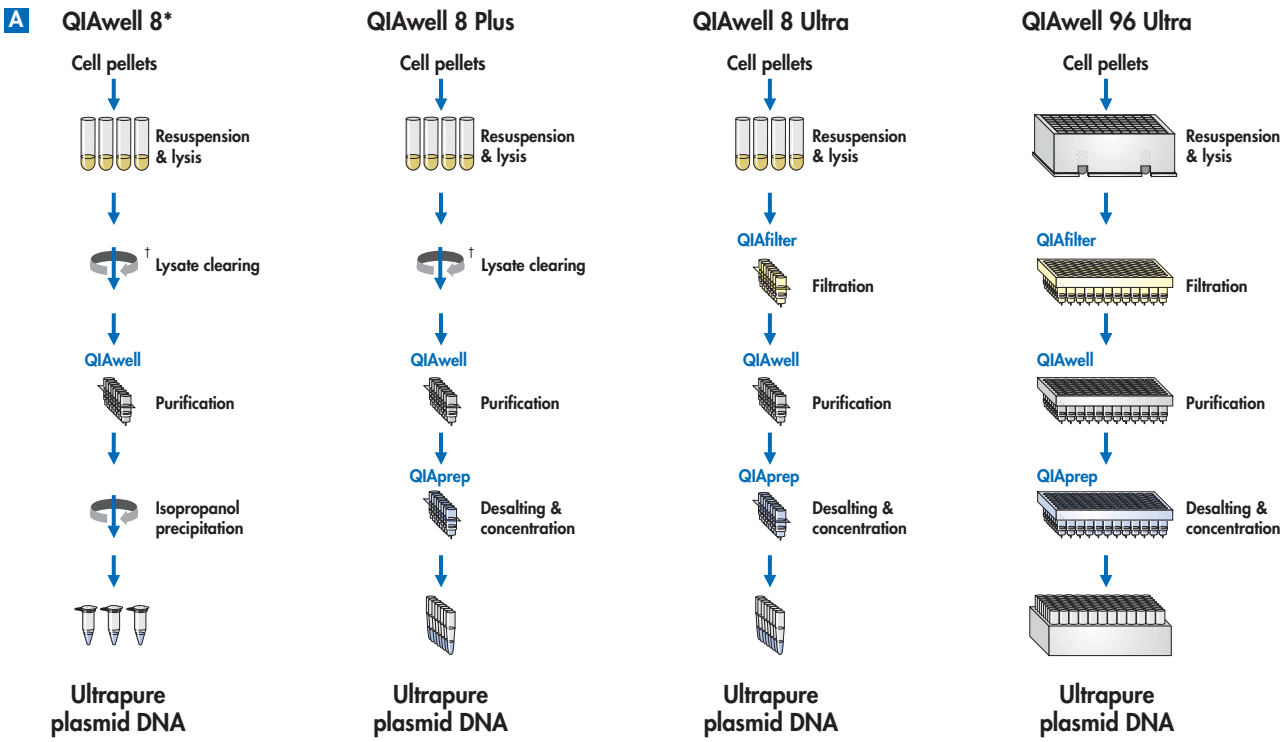

Figure 11. *Schematic representation of plasmid DNA purification from multiple samples in parallel using* **A** *QIAwell with anion-exchange technology for ultrapure DNA,* **B** *QIAprep with specialized silica membrane technology and* **C** *R.E.A.L. with high-efficiency lysate clearing in high-throughput format. All systems are compatible with the BioRobot 96OO (page 25).*

* *Recommended for cosmids and low-copy plasmids*

† *QIAfilter 8 strips are available as an accessory for lysate clearing without centrifugation.*

‡ *Optional, depending on* E.coli *strain.*

§ *Elution of large plasmids (cosmids, BACs, etc) from QIAGEN Resin is not efficient in the buffers used to bind DNA to the silica membrane of the QIAprep module. We therefore recommend QIAGEN columns or QIAwell 8 Plasmid Kit for purifying large plasmids.*

QIAwell Plasmid Purification System: ultrapure plasmid minipreps for all purposes

For projects requiring small amounts of ultrapure DNA from larger numbers of samples, the QIAwell plasmid purification system is available (64,65). QIAwell couples QIAGEN Anion-Exchange Resin chemistry with Empore® membrane technology from 3M. QIAGEN Resin particles are enmeshed in an inert matrix, combining the high capacity and selectivity of anion-exchange chromatography with the handling advantages of a membrane. The core of the QIAwell System is an 8-well or 96-well module containing QIAwell membrane (Figure 11). Each well contains sufficient resin to purify up to 20 µg of plasmid§ DNA per sample . The QIAwell module is designed to be used with additional modules that filter precipitated debris from bacterial alkaline lysates (QIAfilter 8 or 96), and desalt the DNA after QIAwell purification (QIAprep 8 or 96). The purification steps are performed on a vacuum manifold (QIAvac) to facilitate the flow of solutions through the different membranes, and to allow direct transfer of solutions from one unit to the next. QIAvac 6S holds up to six 8-well strips, and QIAvac 96 holds one 96-well plate. The total preparation time for 96 samples of ultrapure DNA is approximately two hours (1.5 min/sample).

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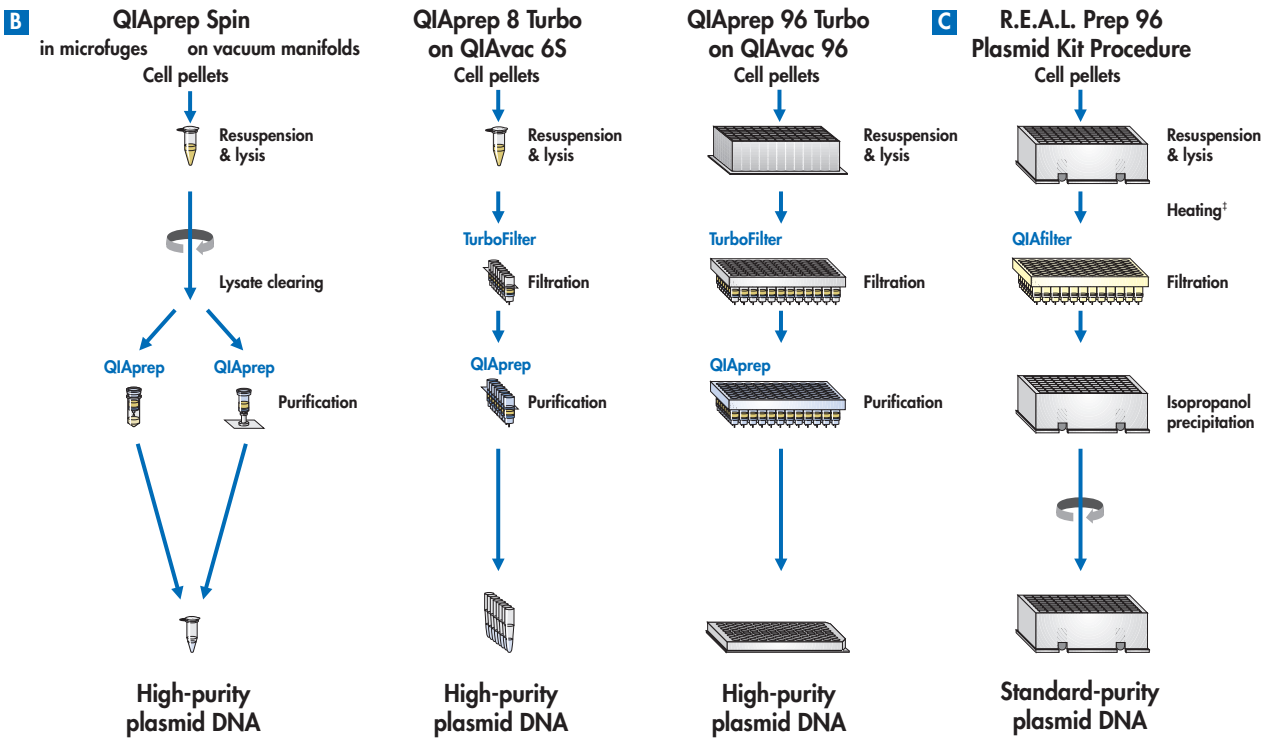

QIAprep Plasmid Kits: high-purity plasmid minipreps for general purpose use

QIAprep Plasmid Kits use a silica gel-based membrane to selectively bind plasmid DNA in the presence of high concentrations of chaotropic salts. Once the DNA is bound, the salt is removed with an 80% ethanol wash, and eluted in a low ionic strength Tris buffer, ready for use in a sequencing reaction (66–68).

Along with the QIAprep 8- or 96-well purification modules, QIAprep Turbo Kits also contain TurboFilter 8 and 96 filter modules for rapid in-line lysate clearing. (Figure 11). Plasmid DNA prepared by this approach is readily digested by restriction endonucleases and performs well in both manual (Figure 12) and automated sequencing reactions. QIAprep Kits (without TurboFilter) are available in a spin-column format for use on either a centrifuge or vacuum manifold (Figure 11), and in 8-well strip format for use with the QIAvac vacuum manifold (up to 48 samples at one time). QIAprep Turbo Kits with TurboFilter are available in both 8-well strip and 96-well plate format for high-throughput sample processing. The total preparation time for 96 samples with QIAprep Turbo 96 is 45 min (<0.5 min/sample).

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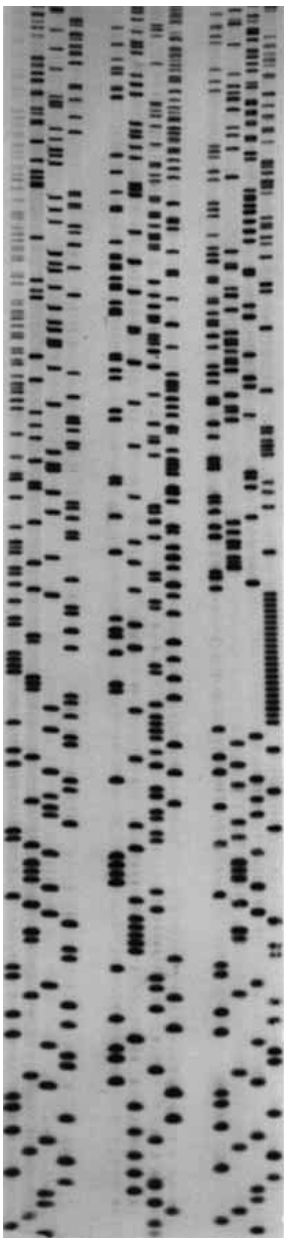

Figure 12. *Radioactive DNA sequencing results of pBluescript clones purified using the QIAprep 8 Miniprep Kit. Double-stranded DNA was sequenced using [^{35}S]dATP and T7 DNA Polymerase.*

R.E.A.L. Prep: standard-purity plasmid minipreps for high-throughput applications

R.E.A.L. (for Rapid Extraction Alkaline Lysis) Prep 96 Kits provide high throughput at low cost for projects involving large numbers of preps (69,70). The R.E.A.L. Prep 96 procedure begins with cell growth in 96-well blocks, followed by a modified alkaline lysis and rapid filtration of the lysates through QIAfilter 96 to remove all precipitated material (Figure 11). The DNA is transferred directly into another 96-well block where it is desalted and concentrated by isopropanol precipitation. Use of a 96-well format throughout avoids individual sample handling, while the ability of QIAfilter to efficiently remove precipitates provides sufficient DNA quality for reliable automated sequencing. With this procedure, a single person can easily process over 1000 preps per day.

QIAprep M13: single-stranded template DNA from phage cultures

QIAprep can also be used for purification of single-stranded viral DNA from culture medium containing phage particles (71). The phage particles are precipitated, collected on the surface of the QIAprep membrane by direct filtration, and treated with a high salt buffer to release the phage DNA and bind it to the membrane (Figure 13). The bound DNA is washed with 80% ethanol to remove the salt, then eluted in water or Tris buffer. QIAprep M13 Kits are available in spin column, 8-well, and 96-well formats.

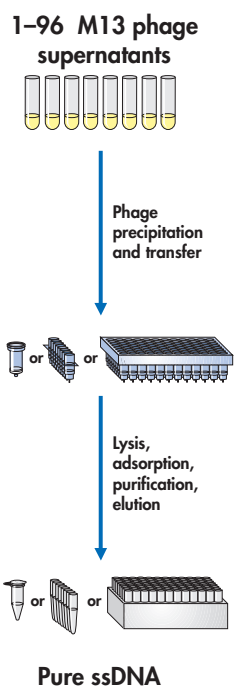

Figure 13. *Schematic representation of single-stranded phage DNA purification using QIAprep M13 Kits.*

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QIAquick: purification of PCR-amplified template DNA

QIAquick PCR Purification Kits and QIAquick Gel Extraction Kits are designed to purify PCR-amplified fragments directly from PCR reaction mixes, agarose gels or enzymatic reactions (Figure 14) (72–74). The simple bind-wash-elute procedure removes proteins, nucleotides and unincorporated primers that can interfere with subsequent reactions. The QIAquick Gel Extraction protocol works equally well with standard or low melting point agarose gels in TBE or TAE buffers. QIAquick PCR Purification and QIAquick Gel Extraction Kits are available in a spin column format, while QIAquick PCR Purification Kits is also available in 8- and 96-well formats.

The BioRobot 9600: automated DNA template purification

To further enhance the convenience of multiwell nucleic acid purification and processing, QIAGEN developed the BioRobot™ 9600 (Figure 15). The BioRobot 9600 is an automated workstation designed to maximize the performance of QIAGEN purification chemistries and to execute related laboratory procedures such as sequencing and PCR reaction setup, restriction digests, and sample pooling (75–79). The versatile QIAsoft™ Operating System includes ready-to-run protocols for plasmid, M13, and PCR fragment purification (plus the laboratory procedures listed above), and features easy-to-use icon-based programming for custom applications. The BioRobot 9600 is equipped with an on-board shaker, vacuum manifold, and multiple pipetting probes. Options include a High-Speed Pipetting System for increased throughput, and a Tip Change system for cross-contamination sensitive applications such as PCR setup. New applications, protocols and kits for the BioRobot 9600 are constantly being developed.

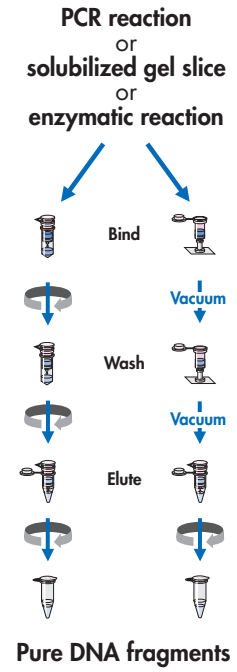

Figure 14. *Schematic representation of PCR product purification and DNA fragment isolation using QIAquick PCR Purification and QIAquick Gel Extraction Kits.*

Figure 15. *The BioRobot 9600 workstation.*

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VIII. Optimizing DNA Sequence Quality

Sequencing methods

A detailed description of the equipment, reagents, and conditions used at QIAGEN for automated sequencing is presented in the Appendix. Many of these procedures were derived through comparative studies, some of which are presented below. Others have been taken from the manufacturers' recommendations without further modification. These recommendations do not imply that other equipment, reagents, and conditions should be considered unsuitable.

The automated sequencing results (electropherograms) in this guide have all been obtained with the Applied Biosystems Model 377 or 377XL DNA Sequencers. The general considerations outlined here for optimizing DNA sequencing quality are likely to apply to other types of sequencers, such as the LI-COR and Pharmacia ALF instruments, and their corresponding sequencing chemistries, although this has not been directly confirmed.

A typical sequencing result obtained with a standard primer and dichlororhodamine dye terminator chemistry using optimum template preps is shown in Figure 16. Read lengths in excess of 700 bases were achieved with >99% unedited accuracy.

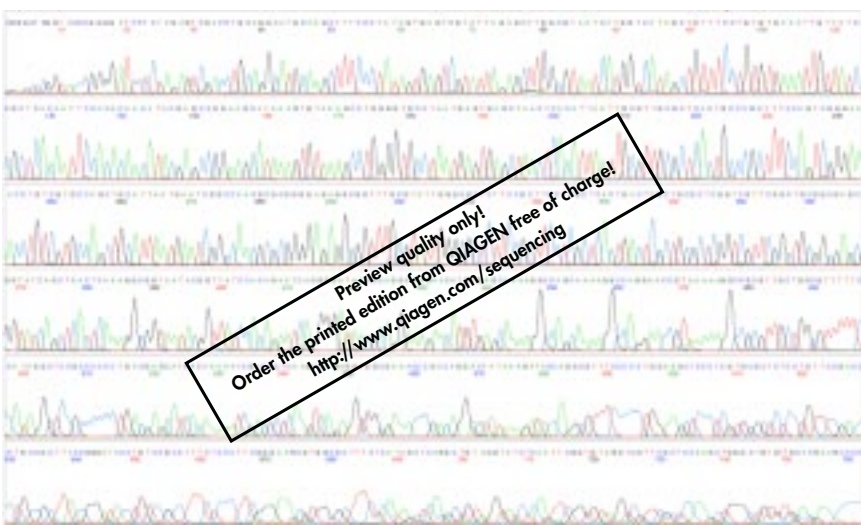
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Figure 16. *Typical sequencing result with a 36 cm well-to-read gel. Plasmid pTZ19R was purified using the QIAGEN Plasmid Midi Kit. Sequencing reaction: 0.2 µg plasmid DNA in a Taq FS dRhodamine dye terminator cycle sequencing reaction under standard conditions with the –21M13 primer. Gel: 5% polyacrylamide, 36 cm well-to-read distance, data collection time 9 hours (see Appendix for conditions).*

The quality of the DNA sequence data can be affected by the quality of any of the components of the sequencing reaction, as well as by suboptimal conditions in any one of the three distinct phases of the sequencing process: the enzymatic reaction, electrophoresis, and data collection. The most common factors which limit sequence quality are impure template DNA, incorrect template or primer concentrations, suboptimal primer selection or annealing, and inefficient removal of unincorporated labeled dideoxynucleotides.

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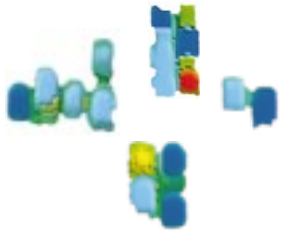

The template-primer-polymerase complex

A key factor in the relative success or failure of a sequencing reaction is the number of template-primer-polymerase complexes that are formed in the course of the reaction. The formation of this complex is necessary (but not sufficient) to produce labeled extension products, and many problems with sequencing reactions can be traced to one or more of these elements.

For example, the ability of an oligonucleotide primer to bind to the template and interact with the polymerase is a major determinant of signal strength. Primers should be selected which have one or more G or C residues at the 3'-end, a base composition of approximately 55% GC and no inverted repeats or homopolymeric regions. These factors affect the stability of the primer–template interaction, which in turn dictates the number of primer–template complexes available to the polymerase under a given set of conditions. It is particularly important to use appropriate annealing temperatures when performing linear amplification (cycle) sequencing with thermostable polymerases, since the remaining steps in the reaction are performed at higher temperatures. Primers with >50% AT content may require a lower annealing temperature than the standard 50°C, while primers with >50% GC content may exhibit nonspecific binding at a low annealing temperature.

Template quantity

The amount of template needs to be within an appropriate range: if the amount is too low, few complexes form and the signal level is too low to be accurately extracted from the background noise. With manual sequencing, a film can be exposed longer to provide a stronger signal. This is not possible with automated sequencing since the data are collected during the run; however, the signal processing software of the automated sequencers appears to perform a similar function by increasing the size of the peaks to fill the space available in each panel. Unfortunately, any background is also amplified and appears as a chaotic pattern underlying the genuine peaks.

To distinguish high background from low signal, check the signal level numbers shown in the header for each sequence. If the average of these numbers is below a certain threshold (below 50 for ABI-type sequencers), ambiguities, inaccuracies, and limited read length due to low signal level may be observed. Low signal levels can arise from any factor that affects the formation of the template-primer-polymerase complex, but is often due to insufficient or low template.

On the other hand, if the template concentration is too high, the nucleotides in the reaction will be distributed over too many growing chains and an overabundance of short fragments will result. In a dye primer reaction, this will be observed as a higher peak intensity towards the beginning of the sequence. In a dye terminator reaction, most of the fragments will result from premature termination. Consequently, no label will be added and the effect will be observed as a loss of signal strength. In addition, a high concentration of DNA may mean a high concentration of contaminant if the template preparation is impure.

▼ Tip

Check the signal levels in each sequence to distinguish high background from low signal. If the average signal level is below a certain threshold, ambiguities, inaccuracies, and limited read lengths may be observed.

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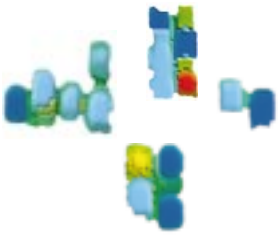

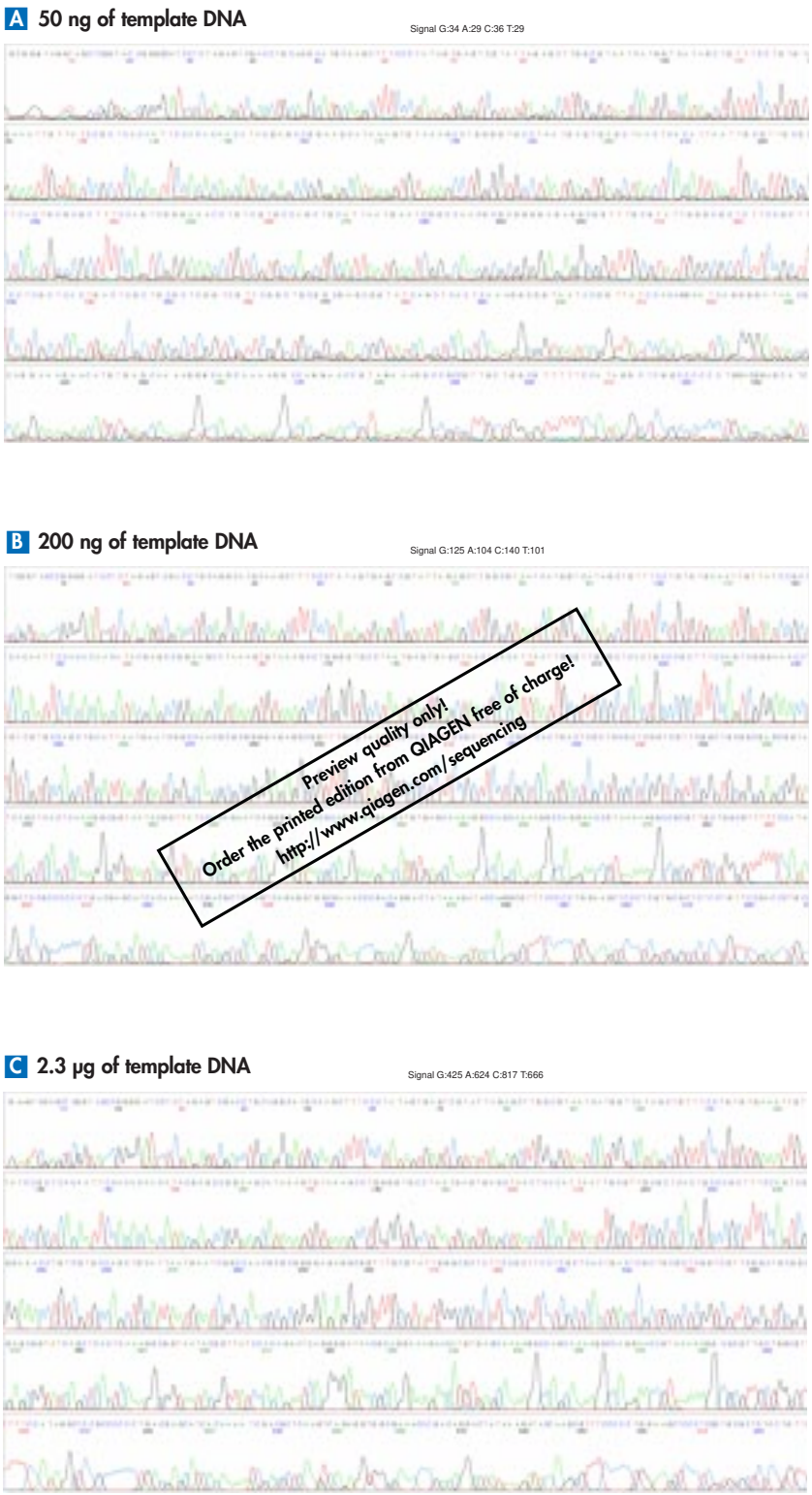

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Figure 17. *Taq FS dRhodamine dye terminator sequencing reactions with different amounts of ultrapure plasmid DNA purified using QIAGEN-tip 100.* **A** *50 ng,* **B** *200 ng,* **C** *2.3 µg plasmid pTZ19R; primer: –21M13 primer. Note the different signal intensities in the header of each electropherogram.*

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These contaminants may also affect the progress of the polymerase, again leading to a predominance of short, prematurely terminated fragments.

In Figures 17–18, the effects of both template amount and general template quality are illustrated. The ability of data analysis software to remove background noise from a low signal scan has improved considerably in the past few years and even a reaction performed with only 50 ng of standard-quality plasmid template is relatively clean. As expected, reactions performed with the recommended quantity of template produce optimal results, while the effect of using too much DNA depends heavily on the quality of DNA used. Ultrapure templates provide excellent results, even when 3 µg of template DNA is used, while a comparable amount of lower quality DNA produces low signal levels and a relatively short read length. This is because the higher contaminant load in the lower quality DNA becomes inhibitory when the concentration of DNA used is excessive.

Depending on the size of the amplified fragment, the amount of PCR-derived template that is required for a successful reaction may be very small. A good guideline to follow is that 5–10 ng of amplified fragment are required for every 100 bp. Thus, only 10–20 ng of a 200 bp fragment are needed for an optimal reaction. The amount required for a 4 kb fragment would be 400 ng, similar to the amount needed for a plasmid template of the same size. The reason less template is required for short fragments is that most, if not all, of the DNA is read and the concentration of actual template is high relative to the same nanogram amount of a larger fragment.

Ideally, template concentrations should be determined using a fluorometer with a DNA-specific fluorescent dye to avoid errors caused by RNA contamination, and confirmed by comparing an aliquot of the template preparation to standards of known concentration on an agarose gel. Table D in the Appendix provides a list of template amounts found to be optimal for the different sequencing chemistries.

▼ Tip

To avoid errors caused by RNA contamination, template concentration should ideally be determined using a fluorometer with a DNA-specific fluorescent dye. It is also recommended to compare an aliquot of the template prep to standards of known concentration on an agarose gel.

Template quality

Culture

An important initial factor in plasmid template purification is the quality of the culture from which the DNA is extracted. Two elements are of particular importance: i) the conditions under which the culture is grown and ii) the host strain selected. Ideally, bacterial cultures for plasmid preparation should be started from fresh colonies grown on selective media. Subculturing directly from glycerol stocks, agar stabs and liquid cultures may lead to uneven culture growth and possible loss of the plasmid. However, use of a preculture (small culture used to inoculate larger culture from which the plasmid is actually purified) may alleviate some of these problems. The effects of culture medium and growth conditions on plasmid DNA quality are discussed in detail in Section H of the Appendix.

Host strain

Plasmids propagated in host strains such as DH1, DH5α, DH10B, C600 and XL1-Blue are highly suitable for template purification. Strain HB101 and its derivatives, including TG1, TG2 and the JM100 series, contain large amounts of carbohydrate that are released during lysis. These strains also have an intact endA locus and produce relatively large amounts of nuclease (16).

Plasmid purification products based on QIAGEN Anion-Exchange Resin efficiently remove carbohydrates and proteins from crude lysates and are recommended for difficult host strains. In QIAprep Plasmid Kits a special wash buffer, Buffer PB, is used to efficiently remove nucleases and other contaminants from suboptimal host strains, while an optional heating step is used to denature contaminating nucleases in the R.E.A.L. Prep 96 protocol. These additional steps will produce satisfactory results with all host strains, even those that typically do not work well with conventional methods.

The general effect of suboptimal template quality is shown in Figure 18. Template DNA was purified by a boiling prep followed by phenol-chloroform extraction and isopropanol precipitation, then sequenced using dye-terminator chemistry. The signal levels were low relative to those obtained with ultrapure DNA (Figure 17), and short fragments predominated. The presence of contaminants in this preparation made the polymerase more likely to dissociate from the template before a labeled terminator could be inserted, so fewer long chains were likely to be produced. These effects were not observed with all templates purified by lower quality preparation methods, but illustrate the greater variability that is encountered relative to results produced with more highly purified templates.

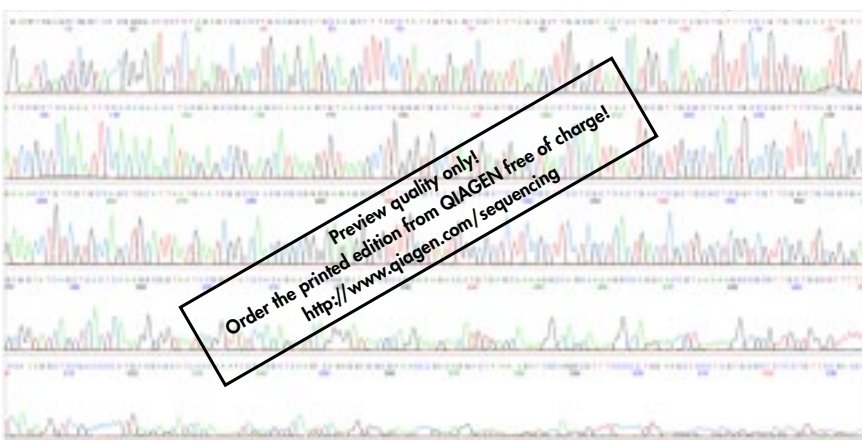
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Figure 18. Taq *FS dRhodamine dye terminator reaction with –21M13 primer using approximately 200 ng pTZ19R plasmid DNA prepared by the boiling preparation method. Note how the quality of the plasmid preparation reduces the signal level and results in short fragments, in comparison to plasmid DNA purified using QIAGEN-tip 100 (Figure 17).*

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Effect of specific contaminants

Nuclease

To demonstrate how nuclease contamination can affect sequence quality, plasmid DNA was contaminated with DNase I at concentrations between 1 ng/ml and 5000 ng/ml, then incubated for 20 min and precipitated prior to dye terminator cycle sequencing. With increasing DNase I concentrations, the quality of the sequencing scans decreased (Figure 19). At 500 ng/ml DNase I, the signal intensity of longer fragments decreased, while at >1000 ng/ml, read length and data accuracy were greatly reduced. These effects were correlated with the appearance of the template on an agarose gel before sequencing (Figure 20). Low amounts of DNase I caused a shift from supercoiled to linear plasmid, while higher amounts further degraded the DNA. The influence of nuclease contamination was even more severe when radioactive sequencing methods were used (Figure 21). An internal radioactive labeling procedure was used, so pre-maturely terminated extension products arising from truncated templates were detected along with those incorporating a ddNTP terminator. In the laboratory, extensive DNA degradation can also occur with lower DNase I concentrations, depending on the sample storage conditions, the length of time the DNA is stored, and the storage temperature.

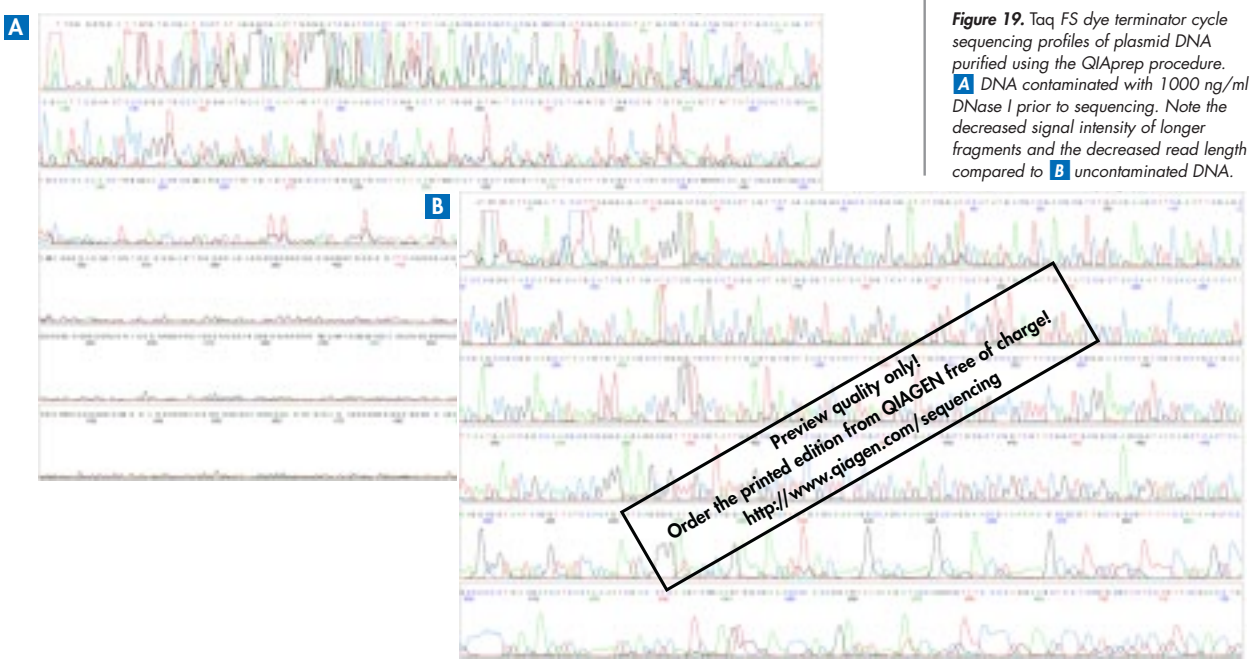

Figure 19. *Taq FS dye terminator cycle sequencing profiles of plasmid DNA purified using the QIAprep procedure.* **A** *DNA contaminated with 1000 ng/ml DNase I prior to sequencing. Note the decreased signal intensity of longer fragments and the decreased read length compared to* **B** *uncontaminated DNA.*

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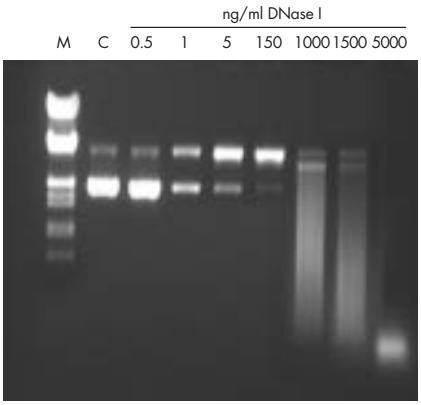

Figure 20. *Agarose gel analysis of DNA samples incubated with the indicated concentrations of DNase I for 20 min.* ***M****: lambda* HindIII-EcoRI *markers.* ***C****: control (untreated purified plasmid DNA). Note that the already low amounts of contaminating DNase I cause a shift from supercoiled to linear plasmid DNA.*

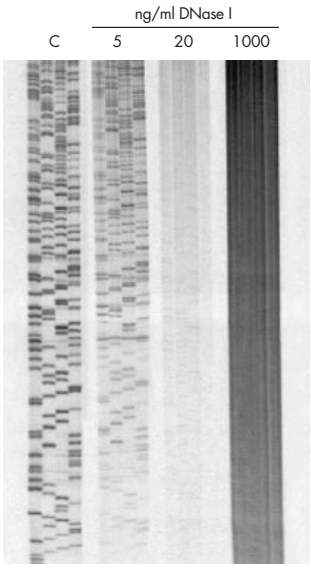

Figure 21. *Effects of nuclease contamination on radioactive sequencing methods. Autoradiogram sequences of plasmid DNA purified with the QIAprep procedure and contaminated with DNase I (at the concentrations indicated) for 20 min prior to sequencing. Double-stranded DNA was sequenced using the Sequenase Version 2.0 DNA Sequencing Kit and [α-^{35}S]dATP.*

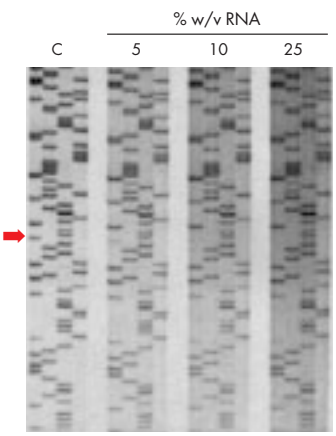

Figure 22. *Effect of RNA contamination on radioactive sequencing. Sequencing profile of plasmid DNA purified using the QIAprep procedure and sequenced in the absence (****C****: control) or the presence of RNA (concentrations indicated) prior to the sequencing reactions. The arrow indicates a low-intensity band which disappears with increasing RNA contamination. Double-stranded DNA was sequenced using the Sequenase Version 2.0 DNA Sequencing Kit and [α-^{35}S] dATP.*

RNA

RNA can be a common contaminant of template preps, particularly in overloading situations where the quantity of cells used exceeds the capacity of the lysis buffers. Figure 22 illustrates the effect of adding 5 to 25% RNA (relative to template) to radioactive sequencing reactions. The RNA-contaminated samples exhibited a high background, which increased with increasing RNA concentration. At high RNA concentrations, some low-intensity bands disappeared, potentially leading to misinterpretation of sequencing data. These effects are due to the contaminating RNA molecules acting as random primers in the sequencing reaction, resulting in a low signal-to-noise ratio. The effect of RNA on automated sequencing results was much less pronounced, most likely because the analysis software effectively removes background noise from the electropherogram (not shown).

Salt

To test the effect of salt contamination on the quality of sequencing data, samples of plasmid DNA were contaminated with 5 to 100 mM sodium acetate and used as templates in fluorescent sequencing dRhodamine dye terminator reactions. The presence of sodium acetate up to a final concentration of 5 mM in the sequencing reactions did not affect the quality of the sequencing data, but higher concentrations of sodium acetate severely inhibited the sequencing reactions (Figure 23). At a final concentration of 20 mM, the signal intensity of the electropherogram as well as the accuracy and the read length were significantly reduced. At concentrations higher than 20 mM, no usable sequencing data could be obtained.

Similar effects were observed if potassium acetate was used in place of sodium acetate. The effects of sodium chloride were less severe: decreases in signal intensity and read length were observed at a concentration greater than 45 mM, with no usable sequence at concentrations above 60 mM (not shown).

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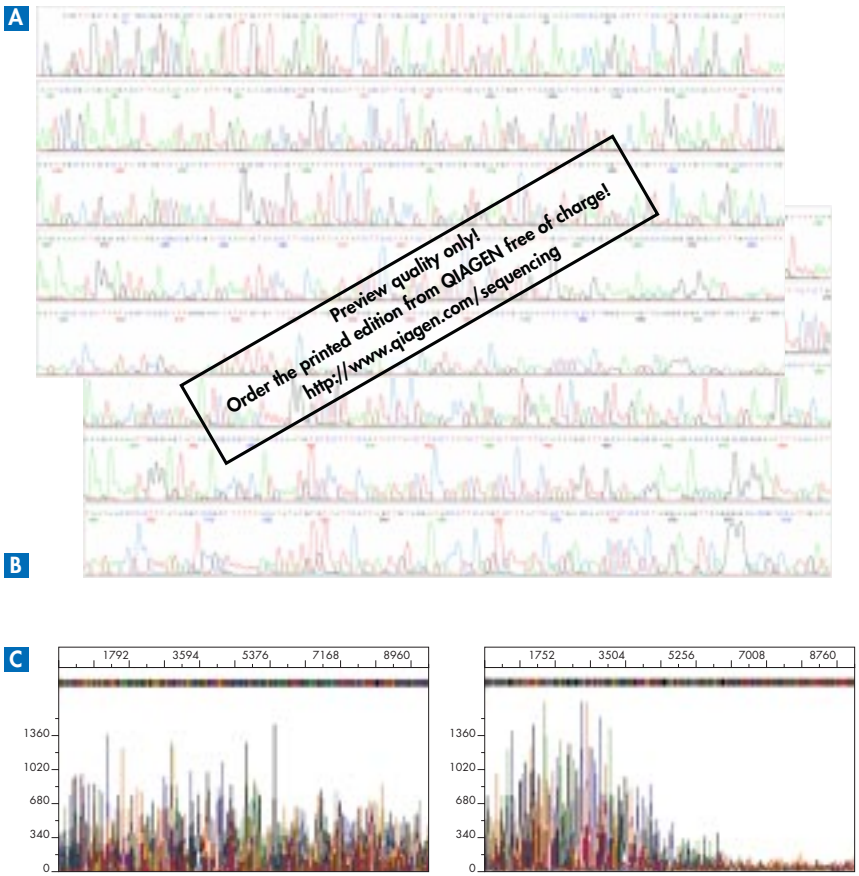

Figure 23. *Effect of salt contamination on Taq FS dye terminator cycle sequencing. The sequencing reactions were performed with 400 ng plasmid pTZ19R using the forward (-20M13) primer and* **A** *20 mM, and* **B** *5 mM sodium actetate in the reaction.* **C** *typical "full view" display of samples that were contaminated with 50 mM NaCl (right panel) compared to a sequence with ultrapure DNA (left panel). Note the decreased signal strength with increasing read length due to salt contamination.*

The observation that sodium acetate has a lower inhibitory concentration than either sodium or potassium chloride indicates that acetate ions inhibit sequencing reactions more than sodium, potassium, or chloride ions.

Salt contamination in template DNA can result from coprecipitation of salt in alcohol precipitations incubated at low temperatures, by insufficient removal of supernatant, or an insufficient 70% wash with ethanol. If traces of salt are suspected, careful precipitation of the template at room temperature, followed by a subsequent room-temperature 70% alcohol wash, can solve the problem.

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Ethanol

The effect of ethanol contamination is shown in Figure 24. The presence of 6% ethanol in a dye terminator reaction significantly reduced peak height in the last three panels, resulting in an accurate read length of only 450 bases. The effect of ethanol is less pronounced in a dye-primer reaction, where usable results were obtained even when 10% ethanol was added to the reaction (data not shown). Contamination with >10% ethanol usually resulted in complete failure of the reaction.

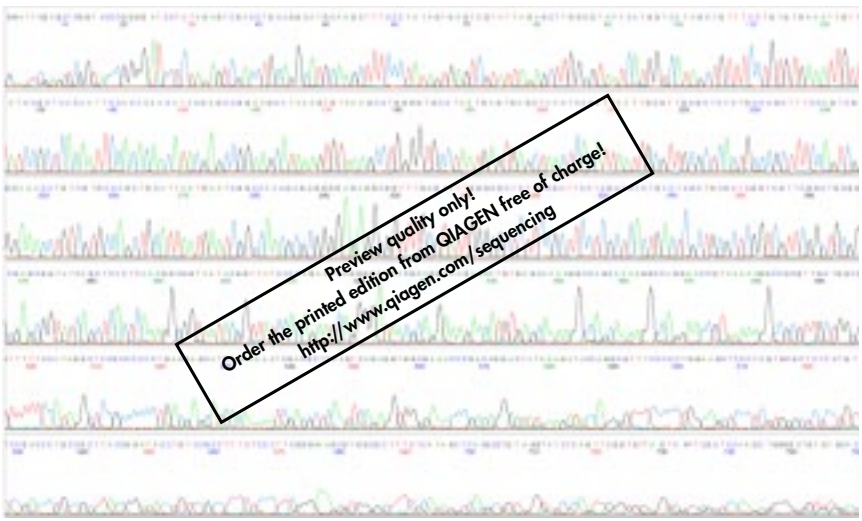
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Figure 24. *Effect of ethanol contamination on Taq FS dRhodamine dye terminator cycle sequencing. Sequence of the plasmid pTZ19R using the –21M13 primer in the presence of 6% ethanol. Note the reduced peak height due to ethanol contamination.*

Alcohol contamination in the template can arise from insufficient drying of a DNA pellet after precipitation, or from incomplete removal of ethanol-containing wash buffer from silica membranes or resin during DNA isolation. If no other contaminants are present, ethanol contamination in a template preparation can be removed by evaporation under vacuum with no loss of template quality.

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Phenol

Some methods of template purification incorporate phenol extraction as a means to remove proteins and other contaminants from cleared lysates. To assess the effect of small amounts of phenol in a sequencing reaction, we tested sequencing reactions containing between 0.1 and 2.25% (w/v) phenol. This corresponds to 0.01–0.25 µl of saturated phenol solution in a 10 µl sequencing reaction. When the phenol concentration was above 0.7% (v/v) the quality of the automated sequencing data decreased with respect to read length, accuracy, and signal intensity (Figure 25). If the phenol concentration exceeded 1.4% (v/v), no usable sequencing data were obtained. Similar results were observed when radioactive sequencing reactions were tested. A clear decline in the quality of the sequence data was observed with 1% phenol, with numerous sites exhibiting bands across all four lanes. (Figure 26). At phenol concentrations greater than 1.5% no sequence data were obtained.

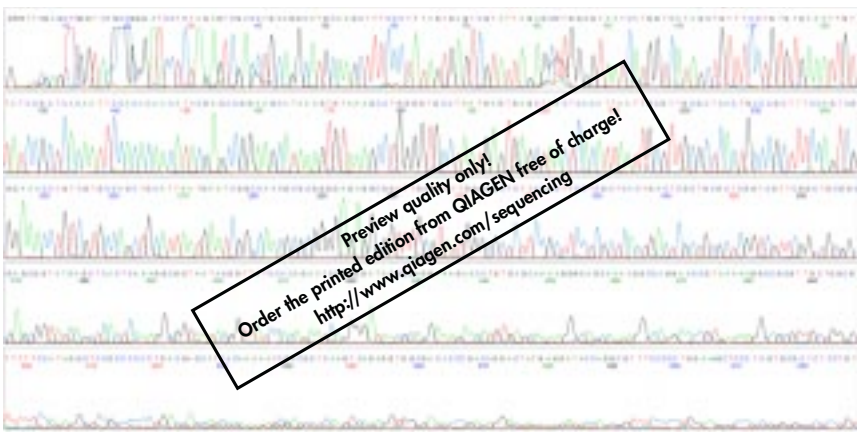
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Figure 25. *Effect of phenol contamination on Taq FS dRhodamine dye terminator cycle sequencing. Sequence of the plasmid pTZ19R using the –21M13 primer in the presence of 0.7% (v/v) phenol. Note the reduced signal intensity, read length and accuracy in the presence of phenol.*

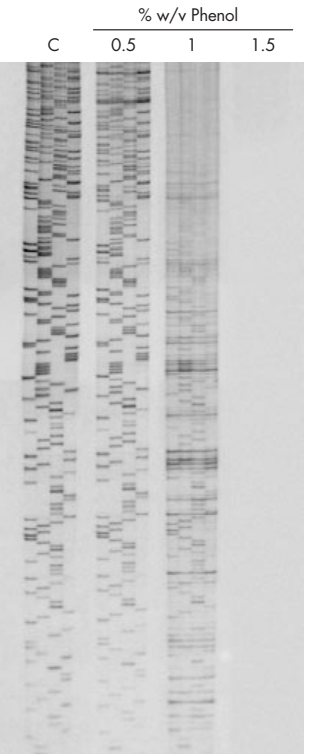

Figure 26. *Effect of phenol contamination on radioactive sequencing. Sequencing profiles of pUC21 plasmid DNA purified using the QIAprep procedure and sequenced in the absence (**C**: control) or presence of phenol (at concentrations indicated) prior to the sequencing reactions. Note that phenol contamination leads to nonspecific chain termination. Double-stranded DNA was sequenced using the Sequenase Version 2.0 DNA Sequencing Kit and [α-^{35}S]dATP.*

Chloroform

Chloroform is often used together with phenol in plasmid preparation methods. However, we observed no effect on the quality of the sequencing profiles when comparable amounts of chloroform were present. Chloroform does not denature proteins as strongly as phenol, and therefore may not affect sequence quality to the same degree.

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Contaminants affecting PCR fragment templates

Primers

To examine the effects of common impurities on the sequencing of PCR-amplified templates, different quantities of contaminants such as nucleotides and primers were added to a purified 1000-bp PCR fragment before sequencing. When the purified fragment was contaminated with the primers used for amplification and sequenced from a nested primer (Figure 27), significantly lower sequence quality was observed with 1 pmol of each amplification primer, while contamination with 2 pmol resulted in unreadable sequence. The excessive background signal obtained arises from sequence products primed by the contaminating primers.

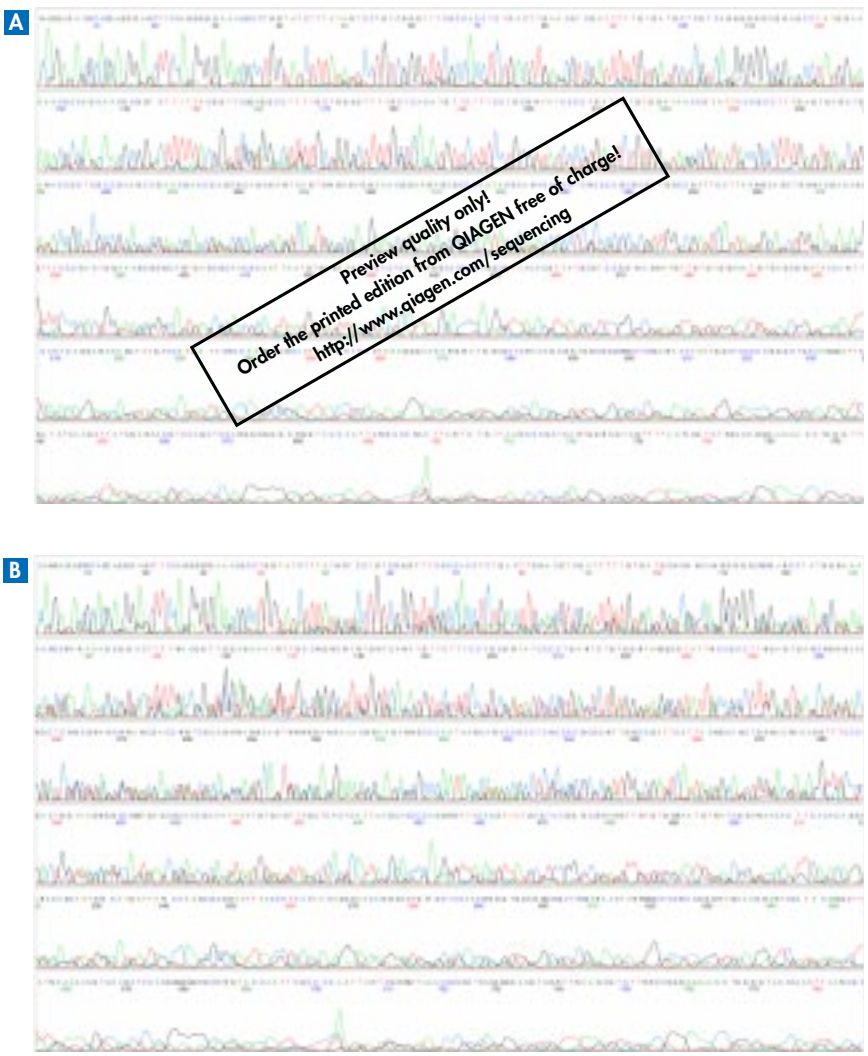

Figure 27. *Effect of primer contamination on fluorescent sequencing. Sequences of 1000-bp PCR fragment contaminated with the amplification primer and sequenced with a nested primer.* **A** *contamination with 1 pmol of each amplification primer. Note the reduced sequence quality.* **B** *contamination with 2 pmol of each amplification primer. This gives an unreadable sequence.*

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dNTPs

In a second set of experiments, nucleotides were added to the purified 1000-bp PCR fragment at concentrations ranging between 2.5 and 100 µM of each dNTP (Figure 28). Contamination with nucleotides at concentrations of 10 µM or more led to a decrease in signal strength and a greater number of ambiguities. An increase in the proportion of long fragments was also observed. At the highest concentration tested, 100 µM, incorporation of dye terminators was inhibited. The presence of contaminating nucleotides alters the ratio of dNTPs to terminators (ddNTPs) in the sequencing reaction, lowering the probability that a ddNTP will be incorporated. This reduces the amount of short extension products that will be produced and may ultimately affect the overall signal level.

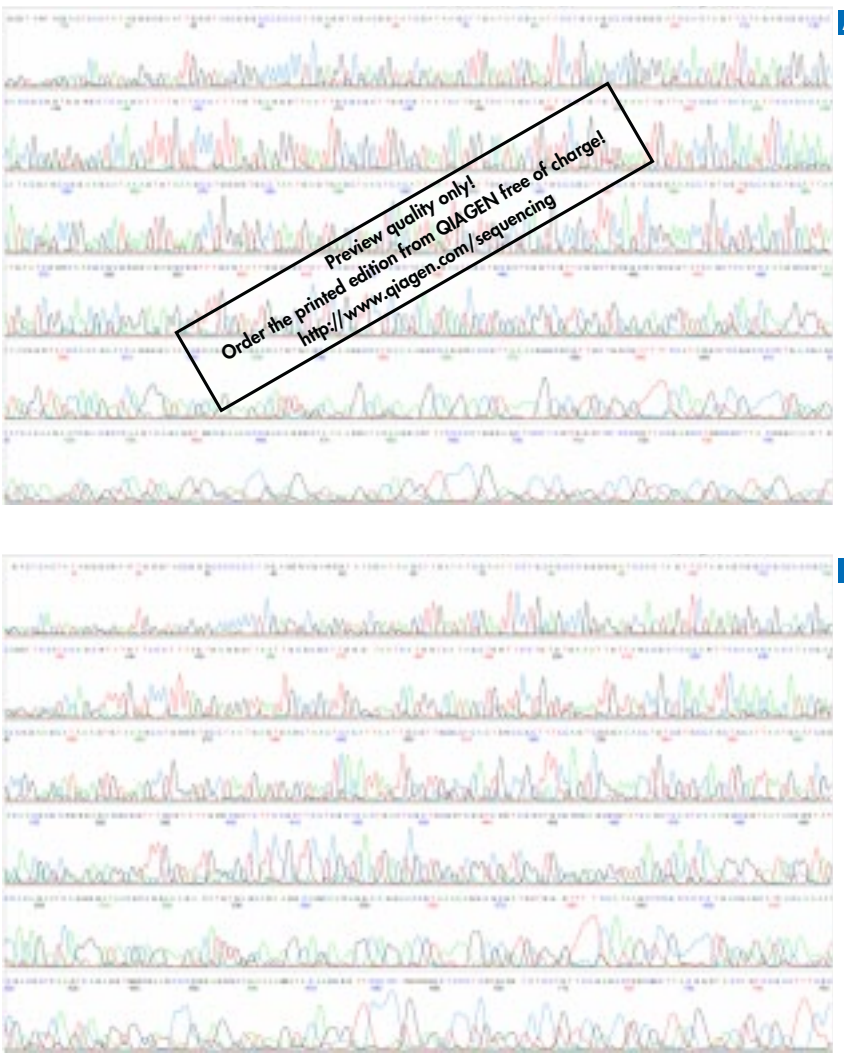

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Figure 28. *Effect of nucleotide contamination on fluorescent sequencing. Sequences of 1000-bp PCR fragment were spiked with nucleotide concentrations ranging between 2.5 and 100 µM of each dNTP.* **A** *contamination with 10 µM of each dNTP.* **B** *contamination with 50 µM of each dNTP. Note the decrease in peak height and greater number of ambiguities at ≥10 µM dNTP.*

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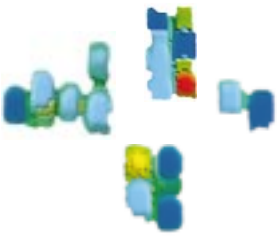

PCR mix

In a typical amplification reaction, the primers and nucleotides used are not completely consumed. Yields of PCR products are typically 0.5–3 µg, from which it can be calculated that, under standard reaction conditions, 11–14 pmol of each primer and 160–190 µM nucleotides remain following PCR. To reproduce the effect of these residual nucleotides and primers in a sequencing reaction with a PCR-derived template, a simulated post-PCR mixture was prepared containing 11 pmol of each primer, 160 µM nucleotides, and 2.5 U *Taq* DNA polymerase. Different amounts of this mixture, corresponding to 0.5–25% of unpurified amplification reaction, were added to 50 ng of purified 1000-bp DNA fragment, which corresponds to 2–10% of the total yield of a typical PCR product. The contaminated DNA fragments were subsequently sequenced with a nested primer (Figure 29). Samples contaminated with 2.5–5% unpurified reaction gave progressively greater suboptimal quality data, while those contaminated with >5% of the simulated unpurified amplification reaction gave unreadable sequence data. These effects would be even more pronounced in PCR systems using >15 pmol primers, or in low efficiency PCR systems which yield <0.5 µg product. At concentrations normally used for PCR amplification, the *Taq* DNA polymerase contained in the reaction did not affect the quality of the sequencing profiles (not shown).

▼ **Tip**

Since remaining nucleotides and primers can significantly decrease sequencing data quality, thorough PCR amplification cleanup is recommended.

Priming artifacts

Mispriming

Annealing of a primer to multiple sites on a template results in superimposed sequence ladders. In most instances, the alternate priming site does not perfectly match the primer, and the spurious signal is less intense than the correct signal from the intended priming site. Molecules of the same length, but different base composition, do not run at exactly the same position. This difference is often sufficient to affect the spacing functions of the base-calling software and leads to artifactual peaks. An example of a multiple priming artifact is shown in Figure 30. Multiple priming can be minimized by prescreening vector and insert DNA for sequences closely matching a proposed priming site, and by selecting temperatures that limit the annealing of mismatched primers (50°C or higher). If closely related primer binding sites are inevitable, as when sequencing through a repeated region by primer walking, selection of a primer that has a 3′-base match only at the desired site should produce acceptable results.

▼ **Tip**

To minimize mispriming, it is advisable to prescreen both vector and insert DNA for sequences closely matching a proposed priming site. An annealing temperature of 50°C or higher should be selected to limit the annealing of mismatched primers.

Priming sites should be selected only from unambiguous sequence regions. Mismatches between the primer and the primer binding site can have serious effects on sequence quality due to a reduction of the stability of primer binding. An example of a sequence obtained using a mismatched primer is shown in Figure 30A. This result was produced with a standard reverse primer (–29Rev dye-labeled reverse M13 primer) on a yeast subclone carried in pTZ18. A comparable reaction using a primer located further

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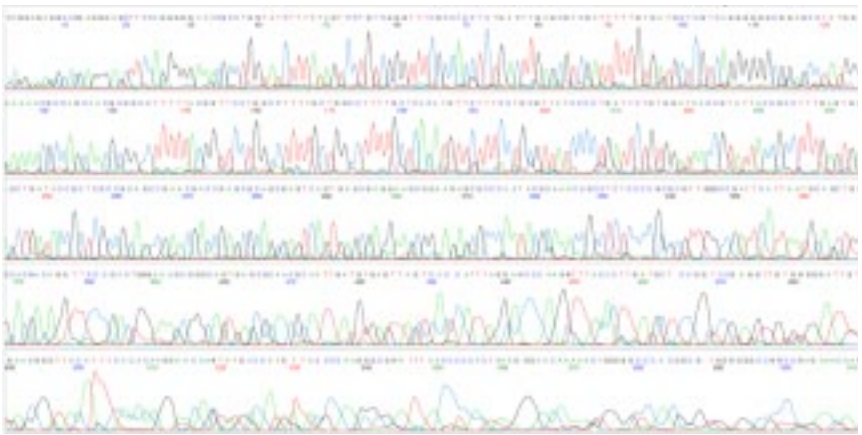
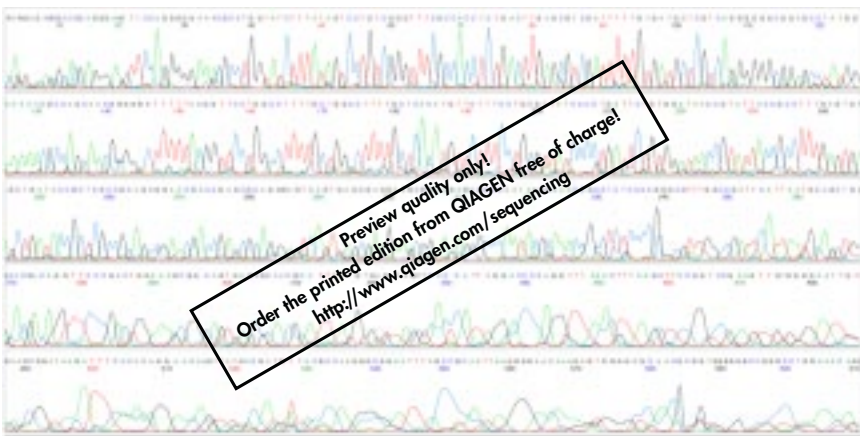
A 2.5% unpurified PCR-amplification reaction



B 5.0% unpurified PCR-amplification reaction



C Control

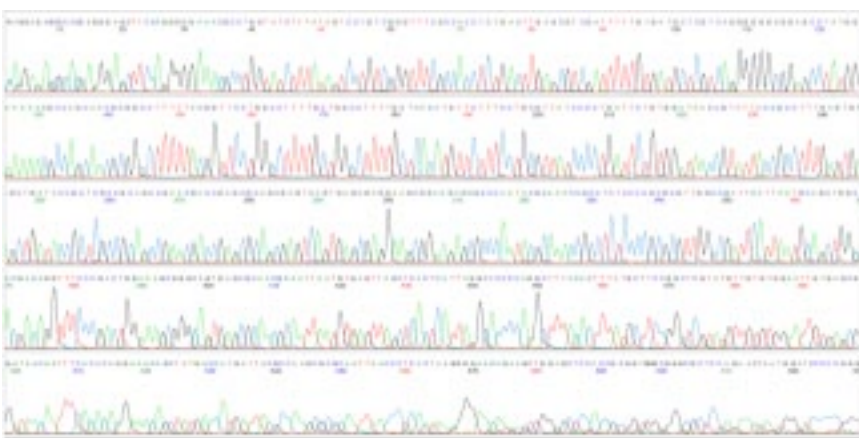


Figure 29. *Simulation of the sequencing of unpurified PCR-amplification reactions. The sequencing reactions of a purified template were spiked with unpurified PCR products.* **A** *contamination with 2.5% unpurified PCR reaction.* **B** *contamination with 5.0% unpurified PCR reaction.* **C** *control.*

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upstream (–48Rev) gave normal results (Figure 30B). Subsequent analysis showed that guanosines at two positions (–19 and –12) were deleted from the primer binding site in the vector (5′-CATGGTCATAGCTGTTTCCTG-3′). Similar deletions were also detected in derivatives of pUC18/19 and pGEM3® from some laboratories. The mutation at position –12 in the second codon of the *lac*Z gene may be a common variant of pUC-like vectors and has been reported before (80). In general, mismatches at or near the 3′-end of the primer affect priming efficiency more strongly than mismatches at the 5′-end, since the polymerase requires an annealed 3′-end to initiate elongation.

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Figure 30. *Priming artifacts — mispriming in the reverse primer binding site. Yeast subclone from chromosome XII in pTZ18 vector was purified using QIAwell 96 Ultra Plasmid Kit.* ***A*** *sequencing reaction with standard reverse (–29Rev) dye primer. Despite the high-purity template and known vector–primer combination, very low signal with high background noise was observed.* ***B*** *same template sequenced with a more distally located primer (–48Rev).*

Poor quality primer

Similar problems can occur when a primer preparation contains a significant amount of n-1 and shorter derivatives. Primers are synthesized in the 3′ to 5′ direction, and a poor-quality synthesis will result in a population of oligonucleotides with a common 3′-end (the end used for priming) but different 5′-ends. Consequently, chains that terminate at the same position may have different lengths and will run at different positions on the gel. An example of this effect is shown in Figure 31. Note how each peak in the electropherogram contains one signal originating from the full-length primer and a shadow peak representing the n-1 signal.

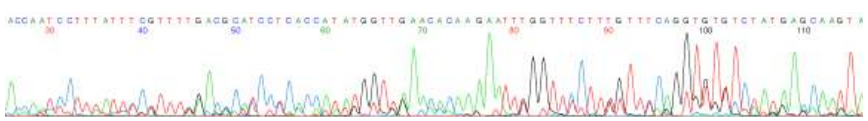

Figure 31. *Primer synthesis artifacts — "n-1" sequences. Primer synthesis artifacts are evident as shadow peaks next to the peak originating from the full-length primer. The formation of shadow peaks from n-1 derivatives is especially noticeable for bases A47, A69, and G91.*

Mixed templates

A variation of the multiple priming site problem occurs when two or more different populations of molecules with a common priming site are present in the template DNA. This happens when more than one recombinant colony or plaque is inoculated into a single culture. When template DNA prepared from such a culture is sequenced into the flanking vector sequences, good vector sequence data will be obtained, but the insert region will show an overlapping band pattern (Figure 32). Such problems can be corrected by retransforming the DNA and inoculating the culture with a single colony or plaque. Similar artifacts will also arise if a plasmid contains more than one vector molecule, or if spontaneous deletions occur during growth.

Figure 32. *Priming artifacts — mixed population bacterial cultures. Shotgun subclones from* Schizosaccharomyces pombe *in pUC18 vector in a* Taq *FS dRhodamine dye terminator reaction using the –21M13 primer. The double sequence starts directly after the* SmaI *restriction site used for subcloning (position 42).*

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A second source of mixed template arises when PCR-amplified templates contain more than one amplified fragment. This can occur when one or more of the primers recognizes more than one site on the template DNA. Such a problem is most difficult to identify when the fragments are amplified from two or more segments of related DNA since the sizes of the amplified fragments may be the same. Amplification of nonspecific PCR products can be avoided by using optimized PCR buffers with a balanced combination of cations that minimize nonspecific annealing (81). Such a system is available with QIAGEN *Taq* DNA Polymerase and the accompanying optimized PCR buffer. Problems with mixed templates may also be circumvented by using a sequencing primer that binds to a site internal to the primers used for PCR.

Template artifacts

The ease with which a polymerase moves along a template can be affected by the overall base composition of the template, and by specific sequences that influence secondary structure.

Unbalanced base composition

Commercially available reaction mixes are formulated for templates with balanced base compositions. When used with templates that have a high GC- or AT-ratio, premature depletion of one or more nucleotides can occur. This results in a higher proportion of prematurely terminated molecules that are detected as increased background in dye primer reactions, and as reduced signal strength in the latter half of the sequence. These effects can be corrected by increasing the concentration of the nucleotides that are in short supply.

GC-rich templates

Problems with secondary structure are often observed with GC-rich template DNA, which may not melt at the temperature of sequencing reactions. This is particularly true for reactions using modified T7 DNA polymerase.

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Figure 33. *Artifacts related to DNA template composition — elongation stop due to secondary structures, and distortion of elongation after a long poly-T stretch.* **A** *sequence with a polymerase elongation stop.* **B** *modified BigDye terminator sequencing protocol that can often resolve elongation stop structures.* **C** *slippage and stuttering effects of polymerase elongation due to a long poly-T stretch. Note that elongation stops can be more easily detected in the "full view" mode of a sequencing file (right panels of* **A** *and* **B** *).*

Effects are seen as one or more strong stops (bands for all four dideoxynucleotides) anywhere along the template. GC-rich regions should always be sequenced by cycle sequencing with a thermostable polymerase, since the elevated temperature will melt hairpins and permit passage of the polymerase. The addition of 5–10% DMSO or formamide to the reaction further reduces the melting temperature of GC-rich regions without otherwise

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reducing sequence quality (Ref. 82, Figure 33, for modified protocol see Appendix). GC-rich templates should also be sequenced using dye-terminator chemistry, since prematurely terminated molecules, which constitute most of the spurious signal in a strong stop in a dye primer reaction, will not be detected when dye terminators are used (Figures 34A and 34B). An example of premature termination created by a highly AT-rich region is shown in Figures 34C and 34D.

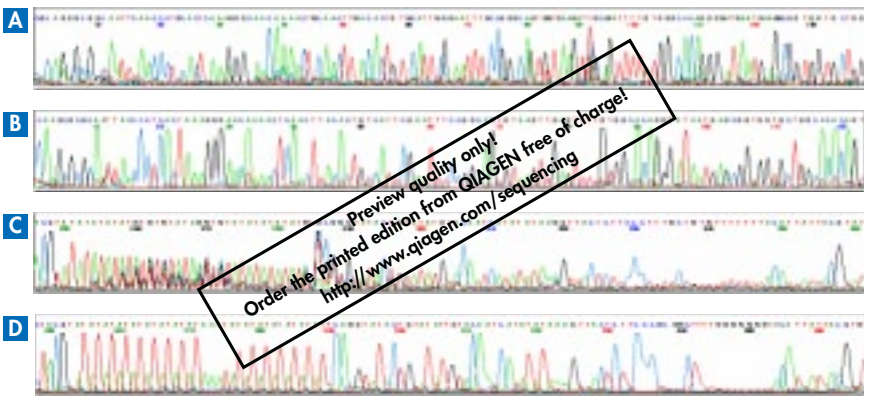

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Figure 34. *Artifacts related to DNA template composition — multiple false terminations. Yeast subclone from chromosome XI in pBluescript II vector purified using QIAwell 8 Plus Plasmid Kit.* ***Reaction****: 0.8 µg of plasmid DNA in Taq dye primer and dye terminator cycle sequencing reactions (see Appendix) with Forward (–20M13) primer.* **A** *dye primer reaction with several false stops, compression, and smeared bands.* **B** *same template used in a dye terminator reaction. The sequence-dependent artifacts from reaction A are all resolved or do not interfere with this chemistry.* **C** *dye primer reaction with high background and false stops within and after an AT stretch.* **D** *same template used in dye-terminator reaction. Most sequence-dependent artifacts from reaction* **C** *are resolved except for several ambiguities related to low signal in the T-rich region.*

Secondary structure can also affect the mobility of molecules in a sequencing gel, resulting in sections of the sequence where the bands cannot be adequately resolved ("compressions"). Most commercial kits for sequencing reactions include nucleotide analogs, such as deoxyinosine-5'-triphosphate or 7-deaza-dGTP, that form fewer hydrogen bonds when paired. These analogs reduce the melting temperature of the template, and alleviate many of the problems associated with GC-rich regions.

Homopolymeric regions

The polymerases used for DNA sequencing often exhibit slipping and stuttering when processing long homopolymeric stretches, such as in the poly-A/poly-T tract of a cDNA (Figure 33C). The best results with these sequences are obtained if *Taq* dye terminator chemistry with higher annealing temperatures and longer denaturation times is used (83), and if the homopolymeric tract occurs in the early part of the sequence, when the nucleotide concentration and enzyme activity are likely to be optimal.

Sequencing large templates

BACs, PACs, and P1s

The sequencing of very large plasmids such as BACs, PACs, and P1s presents particular challenges and is a real test of the sequencer's skill. Part of the challenge arises from the fact that the fraction of the large plasmid template DNA that is actually used for sequencing is much lower than that of a small plasmid template. For example, when 200 ng of a 5-kb plasmid is used in a sequencing reaction, the amount of DNA corresponds to 60 fmol. By comparison, 200 ng of a 150-kb BAC clone corresponds to 2 fmol. To achieve

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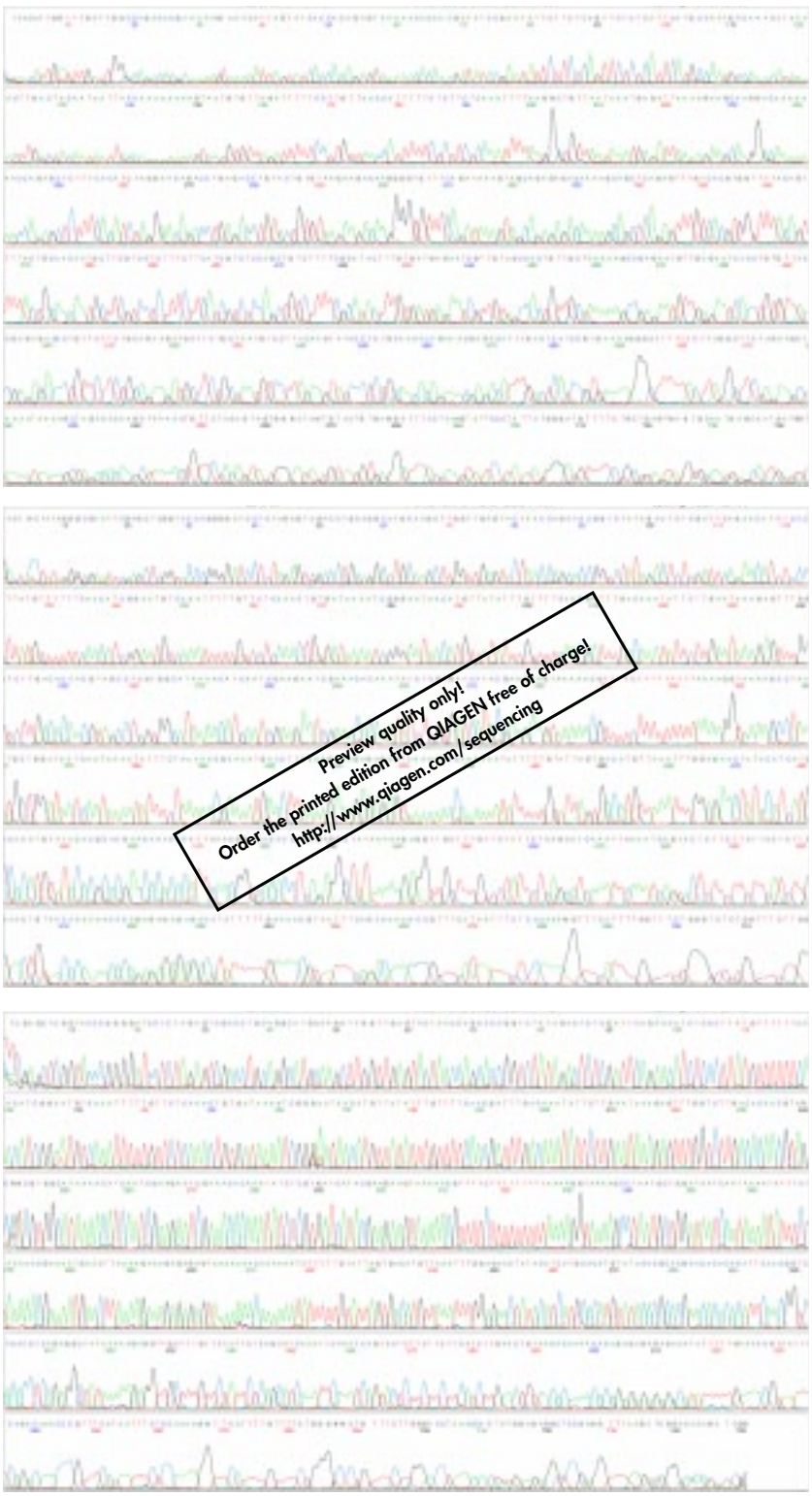

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Figure 35. *BAC end sequences of* Arabidopsis thaliana *BAC clones purified using QIAGEN-tip 500 and sequenced using different sequencing chemistries and template concentrations.* **A** *AmpliTaq FS dRhodamine dye terminator sequence using 2 µg template DNA.* **B** *AmpliTaq FS BigDye dye-terminator sequence using 520 ng template DNA.* **C** *AmpliTaq FS BigDye dye-primer sequence using 132 ng template DNA.*

▼ **Tip**

DNA purified using QIAGEN Anion-Exchange Resin works well for large-plasmid sequencing because contaminants that cause inhibition when concentrated in a low-copy plasmid prep are removed most efficiently by anion exchange.

the same molar concentration of BAC template as plasmid template, 6 µg of BAC DNA would have to be used in the sequencing reaction. Since there is a limit on the amount of DNA possible that can be added to a sequencing reaction, the signal level obtained will be much lower if only 200–500 ng of BAC DNA are used. Most large plasmids exhibit very low-copy number in *E. coli*, and it is often difficult to obtain enough BAC DNA from a small culture (e.g. a culture grown in a 96-well block) for more than one round of sequencing.

The method used to prepare large plasmid template DNA can have a significant effect on the quality of the results. DNA purified using QIAGEN Anion-Exchange Resin works well for large-plasmid sequencing because contaminants that cause inhibition when concentrated in a low-copy plasmid prep are removed most efficiently by anion exchange (60–63). Other methods may be used, however, if the concentration of contaminants in the sequencing reaction can be sufficiently diluted.

Recently, a new series of dyes called BigDyes were introduced that exhibit much higher fluorescence yields than conventional dRhodamine dyes (see Section II, *Energy transfer dyes*). The higher fluorescence yield means that for large plasmid sequencing, less template DNA is needed to obtain acceptable signal levels. A comparison of BAC end sequences obtained with conventional dichlororhodamine-labeled terminators and with BigDye-labeled terminators is shown in Figure 35. In this example, 2 µg of template was required with conventional dRhodamine dye terminator sequencing, 520 ng of template was required with the BigDye terminators, while only 132 ng was required with BigDye primer sequencing. This means that smaller culture volumes can be used to obtain template for sequencing and lower quality templates can be diluted to reduce the concentration of contaminants.

Direct sequencing of genome-size DNA templates

▼ **Tip**

High-quality genomic DNA is essential for successful direct sequencing of genome-size DNA templates, since the amount of template required is high, and significant template contamination would inhibit the sequencing reaction.

The additional sensitivity conferred by energy-transfer dyes like BigDyes raises the possibility of sequencing directly from small genomes such as those of bacteria. This would provide a means of efficiently closing gaps between contigs when using a whole genome shotgun approach (41), and precisely identifying transposon insertion sites and other types of mutations without an intermediate PCR step.

Using protocols from the supplier (84), this was tested with *Bacillus subtilis* (52), which has a genome size of 4.2 Mb, and *E. coli* (85), which has a genome size of 4.7 Mb. The relatively large size of these templates means that the amount of DNA corresponding to the specific region to be sequenced is only one thousandth of a standard 4.2–4.7 kb plasmid template. To overcome the low template concentration, 2.5–3 µg of genomic DNA was used and the number of reaction cycles was increased from 25 to 99. It was observed that the quality of the genomic DNA template is critical for success since, given the amount of template required, a significant contaminant load would inhibit the sequencing reaction. DNA purified on a QIAGEN Genomic-tip 100/G column worked reliably for this purpose (Figures 36 and 37).

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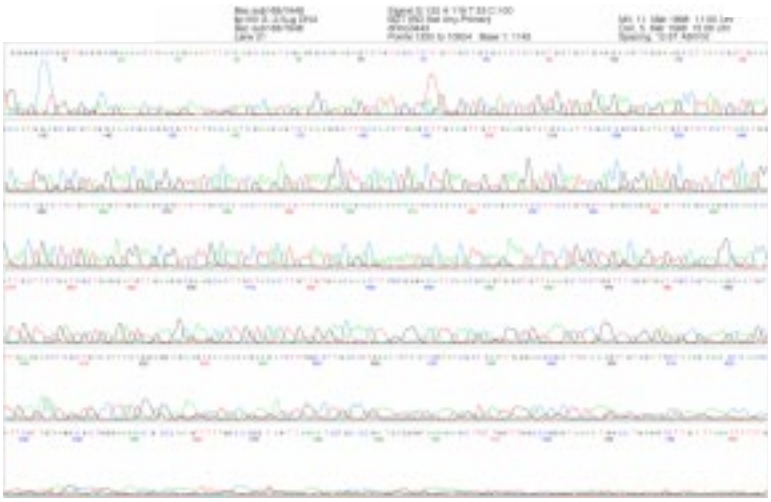

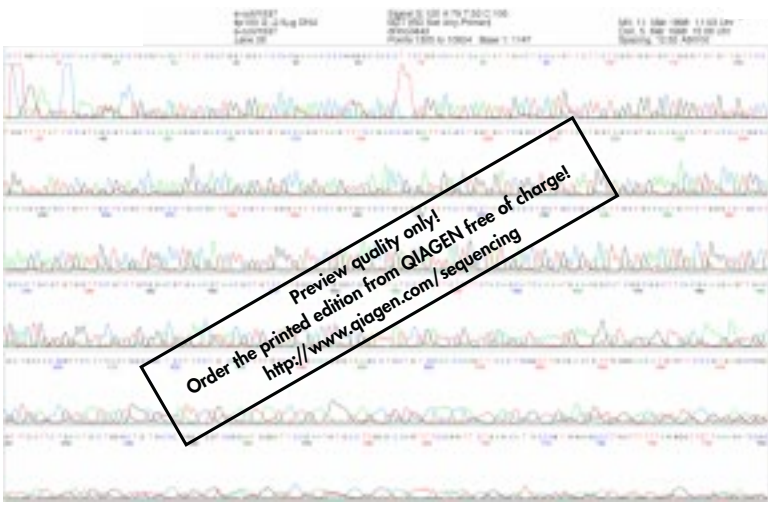

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```
gb|AE000450|ECAE000450 Escherichia coli K-12 MG1655 section 340 of 400 of the complete genome
Length = 11414

Score = 1027 bits (518), Expect = 0.0
Identities = 602/629 (95%), Positives = 602/629 (95%), Gaps = 5/629 (0%)

Query: 17      ctgnacctgcgtacattctcgctggtggatccacnaaaccccnnngccaccttctggaca   76
                ||| ||||||||||||||||||||||||||||||| |||||||  ||||||||||||||
Sbjct: 10444   ctggacctgcgtacattctcgctggtggatccacaaaaccccccagccacccttctggaca  10385

Query: 77      atcaatattgactccatgttcttctcggtggtgctgggtctgttgttcctggttttattc   136
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 10384   atcaatattgactccatgttcttctcggtggtgctgggtctgttgttcctggttttattc  10325

Query: 137     cgtagcgtagccaaaaaggcgaccagcggtgtgccaggtaagtttcagaccgcgattgag   196
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 10324   cgtagcgtagccaaaaaggcgaccagcggtgtgccaggtaagtttcagaccgcgattgag  10265

Query: 197     ctggtgatcggctttgttaatggtagcgtgaaagacatgtaccatggcaaaagcnagctg   256
                |||||||||||||||||||||||||||||||||||||||||||||||||||| |||| |
Sbjct: 10264   ctggtgatcggctttgttaatggtagcgtgaaagacatgtaccatggcaaaagccaagctg  10205

Query: 257     attgctccgctggccctgacgatcttcgtctgggtattcctgatgaacctgatggattta   316
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 10204   attgctccgctggccctgacgatcttcgtctgggtattcctgatgaacctgatggattta  10085

Query: 317     ctgcctatcgacctgctgccgtacattgctgaacatgtactgggtctgcctgcactgcgt   376
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 10144   ctgcctatcgacctgctgccgtacattgctgaacatgtactgggtctgcctgcactgcgt  10085

Query: 377     gtggttccgtctgcggacgtgaacgtaacgctgtctatggcactgggcgtatttatcctg   436
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 10084   gtggttccgtctgcggacgtgaacgtaacgctgtctatggcactgggcgtatttatcctg  10025

Query: 437     attctgttctacagcatcaaaatgaaaggcatcggcggcttcacgaaaagagttgacgctg   496
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 10024   attctgttctacagcatcaaaatgaaaggcatcggcggcttcacgaaaagagttgacgctg  9965

Query: 497     caacccgttcaatcactgggccgtcattcctg-caacttantccttgaaagggtaagcct   555
                | | |||||||||||||||  |||||||| ||||||| |||||||| ||||||||||
Sbjct: 9964    c-agccgttcaatcactgggcgttcattcctgtcaacttaatccttgaaggggtaagcct  9906

Query: 556     gctgtccaaaccaagttcactccggtttgcgactgttnggnaacatgtnttcccgtgagc   615
                |||||||||||||  |||||| ||||||||||||||| || ||||||| | || ||||||
Sbjct: 9905    gctgtccaaaccagtttcact-cggtttgcgactgttcggtaacatgtatgccggtgagc  9847

Query: 616     ctggantttcattctgaatgctggnctgt   644
                ||   ||  |          |
Sbjct: 9846    tgatttccattctgattgctggtctgt   9820
```

Figure 36. *Direct sequencing of* B. subtilis **A** *and* E. coli **B** *genomic DNA purified with the QIAGEN Genomic-tip 100/G protocol. Sequencing reactions: 2.5 µg genomic DNA; primer* B. subtilis*: 25-mer (CTG TAG GCG TGC GTA AAG GGA TGG A) binding inside the groEL gene; primer* E. coli*: 22-mer (CGC CGC AGG ATT ACA TAG GAC A) binding inside the atpB gene. Sequencing was performed with the ABI protocol, see Appendix.*

Figure 37. *BLAST sequence comparison of the unedited* E. coli *genomic sequencing reaction (Figure 36B) with the consensus sequence of* E. coli *(85). Note the very good sequence accuracy for the direct genomic sequencing reaction in the main part of the alignment.*

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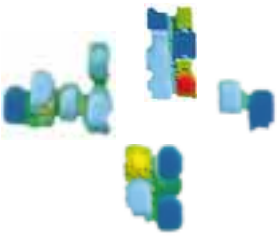

IX. Conclusions

Since the publication of the first edition of *The QIAGEN Guide to Template Purification and DNA Sequencing,* changes in sequencing chemistry and instrumentation have significantly improved both the power, sensitivity, and reliability of automated DNA sequencing. At the same time, the pace of research utilizing DNA sequence data has accelerated, leading to ever higher demands for increased throughput and convenience. QIAGEN will continue to support researchers using DNA sequencing by providing efficient, cost-effective methods for purifying template DNA and automating laboratory procedures. Updates of the methods described in this guide will be provided through an electronic version of the guide located on our website (see below). We welcome your comments on this guide, and look forward to further advances in sequencing and purification methodologies in the years to come.

X. Web Addresses

Sequencing at QIAGEN	http://www.qiagen.com/sequencing
QIAGEN Sequencing Services	http://www.qiagen.com/QSS
QIAGEN homepage	http://www.qiagen.com
QIAGEN Technical Service	http://www.qiagen.com/techservice.html
Useful links to worldwide bioinformatics servers	http://www.qiagen.com/bioinfo/
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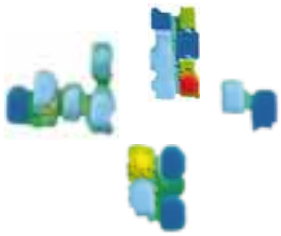

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XII. Appendix: Protocols and Technical Information

A. Sequencing kits and reagents

Kits were used according to the suppliers' (Applied Biosystems) recommendations. All sequencing reactions were analyzed using Applied Biosystems 377 or 377XL Sequencers.

1. PRISM Ready Reaction Dye Primer Cycle Sequencing Kit (–21M13; M13 Reverse)
2. PRISM Ready Reaction Dye Terminator Cycle Sequencing Kit
3. PRISM Ready Reaction BigDye (-21M13) Primer Cycle Sequencing Kit
4. PRISM Ready Reaction BigDye Terminator Cycle Sequencing Kit
5. PRISM Ready Reaction dRhodamine Terminator Cycle Sequencing Kit

B. Standard primers for Dye Terminator cycle sequencing

1. "Forward primer close" (–20M13):
   5'- GTA AAA CGA CGG CCA GT -3'
2. "Forward primer far" (–40M13):
   5'- GTT TTC CCA GTC ACG AC -3'
3. "Reverse primer close" (–24Rev):
   5'- AAC AGC TAT GAC CAT G -3'
4. "Reverse primer far" (–48Rev):
   5'- AGC GGA TAA CAA TTT CAC ACA GGA -3'
5. "T7-19 primer":
   5'- GTA ATA CGA CTC ACT ATA G -3'
6. "T3-19 primer":
   5'- CAA TTA ACC CTC ACT AAA G -3'
7. "KS-18 primer":
   5'- TCG AGG TCG ACG GTA TCG -3'

The forward and reverse M13 primers that are supplied with the Applied Biosystems PRISM Ready Reaction Dye Primer Kits have slightly different compositions and binding sites than the reverse and forward primers listed above:

8. "Forward dye primer" (–21M13):
   5'- TGT AAA ACG ACG GCC AGT -3'
9. "Reverse dye primer" (–29Rev):
   5'- CAG GAA ACA GCT ATG ACC -3'
10. "SP6 primer":
    5'- ATT TAG GTG ACA CTA TAG -3'

C. Cycle sequencing parameters

The following parameters were used for our cycle sequencing reactions. All reactions were carried out using Perkin-Elmer GeneAmp™ PCR Systems 9600 and 9700.

Dye primer AmpliTaq FS cycle sequencing:

- 95°C, 30 sec
- 55°C, 30 sec
- 70°C, 60 sec

for 15 cycles, then

- 95°C, 30 sec
- 70°C, 60 sec

for 15 cycles

Dye terminator AmpliTaq FS cycle sequencing:

- 95°C, 30 sec
- 50°C, 10 sec
- 60°C, 4 min

for 25 cycles

BigDye primer BAC cycle sequencing:

- preheat 5 min at 95°C
- 95°C, 30 sec
- 55°C, 15 sec
- 70°C, 60 sec

for 20 cycles, then

- 95°C, 30 sec
- 70°C, 60 sec

for 15 cycles

BigDye terminator BAC cycle sequencing:

- 95°C, 30 sec
- 50°C, 10 sec
- 60°C, 4 min

for 30 cycles

Dye terminator AmpliTaq FS cycle protocol for resolving stop structures:

- 98°C, 20 sec
- 55°C, 15 sec
- 60°C, 4 min

for 25 cycles

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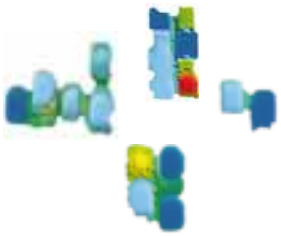

D. Recommended template DNA amounts

The following template amounts were used for all standard sequencing reactions. Optimization of DNA amounts may be necessary for reactions with non-standard custom primers.

	Taq* FS Dye primer	***Taq* FS Dye terminator***	**dRhodamine Dye terminator†**	**BigDye terminator†**	**BigDye primer†**
Plasmids (3–10 kb)	0.8 µg	0.8 µg	0.2 µg	0.1 µg‡§	0.1 µg‡
Plasmids (10–20 kb)	1.2 µg	1 µg	0.4 µg	n.d.	n.d.
Cosmids (30–45 kb)	n.d.	2 µg	0.6 µg	0.2–0.4 µg	0.2–0.4 µg
BACs (80-300 kb)	n.d.	>2 µg	1.5–2 µg	0.25–0.5 µg	0.25 µg
M13 ssDNA	0.3 µg	0.2 µg	0.05–0.1 µg	n.d.	n.d.
PCR products (100–200 bp)	n.d.	0.01–0.02 µg	0.005–0.01 µg	n.d.	n.d.
PCR products (200–500 bp)	n.d.	0.02–0.05 µg	0.01–0.025 µg	n.d.	n.d.
PCR products (500–1000 bp)	n.d.	0.05–0.1 µg	0.025–0.05 µg	n.d.	n.d.
PCR products (1000–2000 bp)	n.d.	0.1–0.2 µg	0.05–0.1 µg	n.d.	n.d.
PCR products (>2000 bp)	n.d.	0.2–0.3 µg	0.1–0.2 µg	n.d.	n.d.

* *"Old" chemistry*
† *Chemistry for 377 and 377XL sequencers only*
‡ *Half volume reaction*
§ ABI recommends 400 ng per full volume reaction
n.d. Not determined

E. Sequencing reaction setup for resolving elongation stop structures*

A*	B*	Setup
400 ng	1 µg	Template DNA
4–10 pmol	10 pmol	Primer
4 µl	16 µl	PRISM Ready Reaction dRhodamine Dye Terminator Premix
2 µl	–	Dimethylsulfoxide (DMSO)
20 µl	40 µl	Reaction volume

F. Direct genomic DNA sequencing protocols

The optimum amount of genomic DNA and the number of cycles are dependent on the genome size and may have to be determined for each bacterial genome.

Protocol		Cycling conditions	
DNA	2–3 µg	Denaturation	95°C, 5 min
Primer (21–25mer)	12–13 pmol	Number of cycles	99
Premix	16 µl	Cycle parameters	95°C, 30 sec
Total reaction volume	40 µl		55°C, 20 sec
			60°C, 4 min

** QIAGEN recommends either of these reaction setups.*

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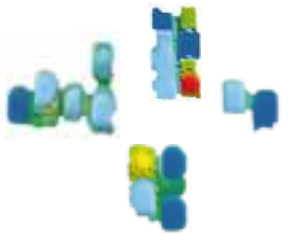

G. Sequencing gel electrophoresis*

2x gel (100 bases/h) – 5% polyacrylamide
Software version ABI PRISM 377XL Collection 2.0

Plates: *36 cm well-to-read*, 0.2 mm spacers and combs
Gel: 5% polyacrylamide, 1x TBE

21 g urea (Life Technologies)
8.4 ml aa-bis solution (30%, 29:1, Roth)
20 ml H_2O

Dissolve (stir and warm, not above 50°C) the acrylamide solution and deionize with Serva Serdolit for 10 min. Meanwhile, filter 6 ml 10x TBE buffer through a 0.2 mm filter. Then pass the dissolved acrylamide solution through the same filter (combines acrylamide solution and 10x TBE), and degas for 1 min under vacuum.

Add 350 µl ammonium persulfate (10%) and 15 µl TEMED for the polymerization (>3h). After 1 hour place a wet paper towel at the bottom of the gel to avoid the occurence of the "red rain" phenomenon.

Electrophoresis: 10 minute prerun with the prerun module PR36A-1200, then 9 h with the run module 36(A/E*)-1200.

Run modules

PR36A-1200	Voltage	1000 V
	Current	35 mA
	Power	50 W constant
	Collection time	1 h
	Gel temperature	51°C
36(A/E†)-1200	Voltage	3000 V
	Current	50 mA
	Power	48 W constant
	Collection time	9 h
	Gel temperature	45°C

† *Depending on the dye set.*

4x gel (200 bases/h) – 4.5% polyacrylamide
Software version ABI PRISM 377XL Collection 2.0

Plates: *36 cm well-to-read*, 0.2 mm spacers and combs
Gel: 4.5% polyacrylamide, 1x TBE

18 g urea (Life Technologies)
7.5 ml aa-bis solution (30%, 29:1, Roth)
22 ml H_2O

Dissolve (stir and warm, not above 50°C) the acrylamide solution and deionize with Serva Serdolit for 10 min. Meanwhile, filter 6 ml 10x TBE buffer through a 0.2 mm filter. Then pass the dissolved acrylamide solution through the same filter (combines acrylamide solution and 10x TBE), and degas for 1 min under vacuum.

Add 350 µl ammonium persulfate (10%) and 15 µl TEMED for the polymerization (>3h). After 1 hour place a wet paper towel at the bottom of the gel to avoid the occurence of the "red rain" phenomenon.

Electrophoresis: 10 minute prerun with the prerun module PR36A-2400, then 4 h with the run module 36(A/E*)-2400

Run modules

PR36A-2400	Voltage	1000 V constant
	Current	35 mA
	Power	50 W
	Collection time	1 h
	Gel temperature	51°C
36(A/E†)-2400	Voltage	3000 V constant
	Current	60 mA
	Power	200 W
	Collection time	4 h
	Gel temperature	45°C

† *Depending on the dye set.*

* *2x gel conditions provide best results for reactions with low signal strength such as BAC and direct genome sequencing.*

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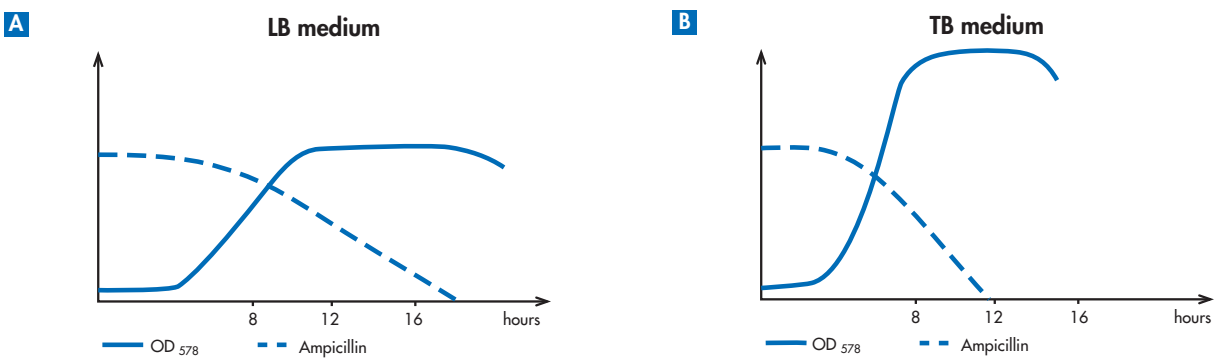
H. Growth of *E. coli* cultures

i. Bacterial culture media

QIAGEN plasmid purification protocols are optimized for use with cultures grown in standard LB medium to a cell density of approximately 1.5–4.0 x 10^9 cells/ml (1.5–4 A_{600} units/ml). A typical growth curve for *E. coli* in LB is shown in panel A. Stationary phase is reached in approximately 10–12 hours and continues for 4–5 hours before cells begin to lyse. With an enriched medium such as TB, stationary phase is reached much faster (8 hours) and ends sooner (panel B). Consequently, an overnight (16 hour) culture grown in LB will contain fewer lysed cells than a comparable TB culture. Lysed cells release degraded genomic and plasmid DNA and yield plasmid DNA of reduced quality. The higher cell densities obtained with enriched medium may also lead to problems with column or membrane overloading.



Medium	Composition[1]		Medium	Composition[1]	
LB Medium [2,3,4,5]	Tryptone	10 g	Super Broth [5]	Tryptone	32 g
	Yeast extract	5 g		Yeast extract	20 g
	NaCl	10 g		NaCl	5 g
	Adjust pH to 7.0			*Adjust pH to 7.0*	
2x YT Medium [3,5]	Tryptone	16 g	Terrific Broth (TB)	Tryptone	12 g
	Yeast extract	10 g		Yeast extract	24 g
	NaCl	5 g		Glycerol	4 ml
	Adjust pH to 7.0			KH_2PO_4	2.31 g
				K_2HPO_4	12.54 g

[1] Per liter. For plates, add 15 g agar per liter. [2] Note that a number of slightly different LB culture broths containing different concentrations of NaCl are in common use. Only the LB composition given here is recommended for plasmid preparation with QIAGEN products. LB containing 5 g NaCl per liter yields ≤30% less plasmid DNA. [3] Recommended for M13 propagation. [4] Recommended for lambda *propagation. [5] Recommended for bacteria carrying QIA*express *pQE expression vectors.*

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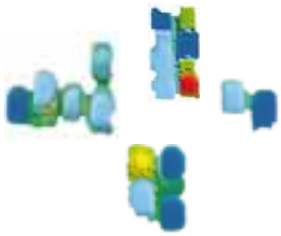

ii. Antibiotic selection

It is important that antibiotic selection is applied throughout culture growth. In the absence of effective selection, cells that do not contain the plasmid will outgrow those that do, leading to poor plasmid yields. The ampicillin depletion curves shown in (i) on the previous page reflect inactivation of ampicillin by plasmid-encoded β-lactamase and thermal breakdown. To ensure effective selection during the critical growth phase of the culture, ampicillin and other antibiotic stocks should be stored frozen in single-use aliquots.

Antibiotic solutions	Stock solution Concentration	Storage	Final concentration (Dilution from stock)
Ampicillin (sodium salt)	50 mg/ml in H_2O	–20°C	100 µg/ml (1:500)
Chloramphenicol	34 mg/ml in ethanol	–20°C	170 µg/ml (1:200)
Kanamycin	10 mg/ml in H_2O	–20°C	50 µg/ml (1:200)
Streptomycin	10 mg/ml in H_2O	–20°C	50 µg/ml (1:200)
Tetracycline·Cl	5 mg/ml in ethanol	–20°C	50 µg/ml (1:100)

Adapted from Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Press, Cold Spring Harbor, NY.

iii. Growth in 96-well blocks

Longer culture times (20–24 h) may be required for 1.3-ml cultures grown in 96-well blocks due to reduced aeration. AirPore™ Tape Sheets may be used to enable gas exchange while guarding against spillover and dust. Maintenance of selection and shaking at 250 rpm or more throughout the culture period are essential.

iv. Origin of replication and copy number of various plasmids and cosmids

DNA construct	Replication origin	Copy number	Classification
Plasmids			
pUC vectors	pMB1*	500–700	High copy
pBluescript vectors	ColE1	300–500	High copy
pGEM vectors	pMB1*	300–400	High copy
pTZ vectors	pMB1*	>1000	High copy
pBR322 and derivatives	pMB1*	15–20	Low copy
pQE vectors	ColE1	~30	Low copy
pREP4	p15A	~30	Low copy
pACYC and derivatives	p15A	10–12	Low copy
pSC101 and derivatives	pSC101	~5	Very low copy
Cosmids			
SuperCos	pMB1*	10–20	Low copy
pWE15	ColE1	10–20	Low copy

* *The pMB1 origin of replication is closely related to that of ColE1 and falls in the same incompatibility group. The high-copy plasmids listed here contain mutated versions of this origin.*

v. Recommended culture volumes for plasmid purification with QIAGEN-tips

Kit	Column size	Culture volume	Volume of lysis buffers†	Expected yield‡	Column capacity
High-copy plasmids (3–5 µg DNA/ml LB culture)* e.g., pBluescript®, pUC, pGEM®, chloramphenicol-amplified low-copy plasmids					
QIAGEN Plasmid Mini Kit	QIAGEN-tip 20	**3 ml**	0.3 ml	9–20 µg	20 µg
QIAGEN Plasmid Midi Kit	QIAGEN-tip 100	**25 ml**	4 ml	75–100 µg	100 µg
QIAfilter Plasmid Midi Kit	QIAGEN-tip 100	**25 ml**	4 ml	75–100 µg	100 µg
QIAGEN Plasmid Maxi Kit	QIAGEN-tip 500	**100 ml**	10 ml	300–500 µg	500 µg
QIAfilter Plasmid Maxi Kit	QIAGEN-tip 500	**100 ml**	10 ml	300–500 µg	500 µg
EndoFree Plasmid Maxi Kit	QIAGEN-tip 500	**100 ml**	10 ml	300–500 µg	500 µg
QIAGEN Plasmid Mega Kit	QIAGEN-tip 2500	**500 ml**	50 ml	1.5–2.5 mg	2.5 mg
QIAfilter Plasmid Mega Kit	QIAGEN-tip 2500	**500 ml**	50 ml	1.5–2.5 mg	2.5 mg
EndoFree Plasmid Mega Kit	QIAGEN-tip 2500	**500 ml**	50 ml	1.5–2.5 mg	2.5 mg
QIAGEN Plasmid Giga Kit	QIAGEN-tip 10000	**2.5 liters**	125 ml	7.5–10 mg	10 mg
QIAfilter Plasmid Giga Kit	QIAGEN-tip 10000	**2.5 liters**	125 ml	7.5–10 mg	10 mg
EndoFree Plasmid Giga Kit	QIAGEN-tip 10000	**2.5 liters**	125 ml	7.5–10 mg	10 mg
Low-copy plasmids (0.2–1 µg DNA/ml LB culture)* e.g., pBR322, most cosmids					
QIAGEN Plasmid Mini Kit	QIAGEN-tip 20	**10 ml**	1 ml	5–15 µg	20 µg
QIAGEN Plasmid Midi Kit	QIAGEN-tip 100	**100 ml**	4 ml	20–100 µg	100 µg
QIAfilter Plasmid Midi Kit	QIAGEN-tip 100	**50 ml**	4 ml	10–50 µg	100 µg
QIAGEN Plasmid Maxi Kit	QIAGEN-tip 500	**500 ml**	10 ml	100–500 µg	500 µg
QIAfilter Plasmid Maxi Kit	QIAGEN-tip 500	**250 ml**	10 ml	50–250 µg	500 µg
EndoFree Plasmid Maxi Kit	QIAGEN-tip 500	**250 ml**	10 ml	50–250 µg	500 µg
QIAGEN Plasmid Mega Kit	QIAGEN-tip 2500	**2.5 liters**	50 ml	0.5–2.5 mg	2.5 mg
QIAfilter Plasmid Mega Kit	QIAGEN-tip 2500	**2.5 liters**	50 ml	0.5–2.5 mg	2.5 mg
EndoFree Plasmid Mega Kit	QIAGEN-tip 2500	**2.5 liters**	50 ml	0.5–2.5 mg	2.5 mg
QIAGEN Plasmid Giga Kit	QIAGEN-tip 10000	**5 liters**	250 ml	1–5 mg	10 mg
QIAfilter Plasmid Giga Kit	Not recommended for low-copy plasmid and cosmid preparations§				
EndoFree Plasmid Giga Kit	Not recommended for low-copy plasmid and cosmid preparations§				
Very low-copy plasmids (0.04–0.2 µg DNA/ml LB culture)* Plasmids and cosmids of <10 copies per cell, e.g. P1 and BAC constructs					
Midi preparation	QIAGEN-tip 100	**500 ml**	20 ml	20–100 µg	100 µg
Maxi preparation	QIAGEN-tip 500	**2.5 liters**	125 ml	100–500 µg	500 µg

** In common host strains, such as XL1-Blue, DH5α™, HB101, and JM109*

† *Buffers P1, P2, and P3.*

‡ *Actual yields depend on plasmid copy number, size of insert, host strain, culture medium, and culture volume.*

§ *Due to the large culture volumes required and the limited capacity of the QIAfilter Mega-Giga Cartridge, QIAfilter/EndoFree Plasmid Mega Kits are a better choice than QIAfilter/EndoFree Plasmid Giga Kits for purification of low-copy plasmids and cosmids.*

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I. QIAGEN sequencing template purification guide

i. Manual sequencing (radioactive or chemiluminescent)

Template	QIAGEN tips or Plasmid Kits	QIAwell Plasmid Kits	QIAprep Kits	R.E.A.L. Prep 96 Kits	QIAprep M13 Kits	QIAquick Kits
ds DNA						
Plasmids	+	+	+	●	·	·
Cosmids	+	●	●	—	·	·
ssDNA						
M13; phagemids	·	·	·	·	+	·
PCR products	·	·	·	·	·	+

+ *Recommended; optimal quality and handling.*
● *Also recommended; optimal quality, but choice depends on sample size, throughput, or handling preference.*
— *Not recommended; other QIAGEN products provide better quality and/or handling advantages.*
· *Not applicable.*

ii. Automated cycle sequencing

Sequencing chemistry Template	QIAGEN tips or Plasmid Kits	QIAwell Plasmid Kits	QIAprep Kits	R.E.A.L. Prep 96 Kits	QIAGEN Genomic tips	QIAprep M13 Kits	QIAquick Kits
dRhodamine Dye Terminator							
ds DNA							
Plasmids	+	+	+	+	·	·	·
Cosmids	+	●	●	●	·	·	·
ssDNA							
M13; phagemids	·	·	·	·	·	+	·
PCR products	·	·	·	·	·	·	+
BigDye Terminator							
ds DNA							
Plasmids	+	+	+	+	·	·	·
Cosmids	+	●	●	●	·	·	·
BACs, PACs	+	●	·	●	·	·	·
Genomic DNA	·	·	·	·	+	·	·
ssDNA							
M13; phagemids	·	·	·	·	·	+	·
PCR products	·	·	·	·	·	·	+
BigDye Primer							
ds DNA							
Plasmids	+	+	+	+	·	·	·
Cosmids	+	●	●	●	·	·	·
BACs, PACs	+	●	·	●	·	·	·
ssDNA							
M13; phagemids	·	·	·	·	·	+	·
PCR products	·	·	·	·	·	·	+

+ *Recommended; optimal quality and handling.*
● *Also recommended; optimal quality, but choice depends on sample size, throughput, or handling preference.*
— *Not recommended; other QIAGEN products provide better quality and/or handling advantages.*
· *Not applicable.*

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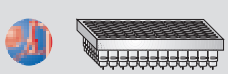
J. Plasmid Miniprep Kit selection guide

DNA purity	Standard-purity DNA for sequencing and screening
Technology	Modified alkaline lysis for highest-throughput, lowest-cost, minipreps

Throughput (number of samples purified in parallel)

No. of samples \ Convenience	Kit	Clearing of lysates	Ready-to-use plasmid DNA
1–24 samples	**Select the perfect miniprep option — simply match your throughput level with your required DNA purity.**		
8–48 samples			
1–4 x 96 samples	**R.E.A.L. Prep 96 Plasmid Kit** 	**Rapid vacuum filtration** (QIAfilter)	Isopropanol precipitation required

Key to formats: Spin column · Gravity-flow column · 8-well strip

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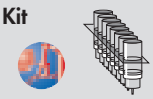
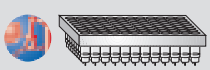
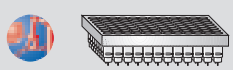
High-purity DNA for all applications			Ultrapure DNA for special applications		
Silica-gel-membrane purification for economical minipreps			Anion-exchange technology for highest-quality minipreps		
Kit	**Clearing of lysates**	**Ready-to-use plasmid DNA**	**Kit**	**Clearing of lysates**	**Ready-to-use plasmid DNA**
QIAprep Spin Miniprep Kit 	Centrifugation	**YES**	**QIAGEN-tip 20**  **QIAGEN Plasmid Mini Kit**	Centrifugation	Isopropanol precipitation required
QIAprep 8 Miniprep Kit 	Centrifugation	**YES**	**QIAwell 8 Plasmid Kit** 	Centrifugation	Isopropanol precipitation required
			QIAwell 8 Plus Plasmid Kit 	Centrifugation	**YES**
QIAprep 8 Turbo Miniprep Kit 	**Rapid vacuum filtration** (TurboFilter)	**YES**	**QIAwell 8 Ultra Plasmid Kit** 	**Rapid vacuum filtration** (QIAfilter)	**YES**
QIAprep 96 Turbo Miniprep Kit 	**Rapid vacuum filtration** (TurboFilter)	**YES**	**QIAwell 96 Ultra Plasmid Kit** 	**Rapid vacuum filtration** (QIAfilter)	**YES**

 **96-well plate**

Special kits are available for automated BioRobot 9600 procedures

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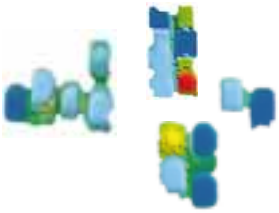

XIII. Ordering Information

Product	Contents	Cat. No.
Plasmid Starter Kits		
QIAGEN Plasmid Starter Kit I	10 QIAGEN-tip 20, 3 QIAGEN-tip100, 1 QIAGEN-tip 500, Reagents, Buffers	12129
Plasmid Mini Kits		
QIAGEN Plasmid Mini Kit (25)	25 QIAGEN-tip 20, Reagents, Buffers	12123
QIAGEN Plasmid Mini Kit (100)	100 QIAGEN-tip 20, Reagents, Buffers	12125
Plasmid Midi Kits*		
QIAGEN Plasmid Midi Kit (25)	25 QIAGEN-tip 100, Reagents, Buffers	12143
QIAfilter Plasmid Midi Kit (25)	25 QIAGEN-tip 100, Reagents, Buffers, 25 QIAfilter Midi Cartridges	12243
Plasmid Maxi Kits*		
QIAGEN Plasmid Maxi Kit (25)	25 QIAGEN-tip 500, Reagents, Buffers	12163
QIAfilter Plasmid Maxi Kit (25)	25 QIAGEN-tip 500, Reagents, Buffers, 25 QIAfilter Maxi Cartridges	12263
Plasmid Mega Kits		
QIAGEN Plasmid Mega Kit (5)	5 QIAGEN-tip 2500, Reagents, Buffers	12181
QIAfilter Plasmid Mega Kit (5)	5 QIAGEN-tip 2500, Reagents, Buffers, 5 QIAfilter Mega-Giga Cartridges	12281
Plasmid Giga Kits		
QIAGEN Plasmid Giga Kit (5)	5 QIAGEN-tip 10000, Reagents, Buffers	12191
QIAfilter Plasmid Giga Kit (5)†	5 QIAGEN-tip 10000, Reagents, Buffers, 5 QIAfilter Mega-Giga Cartridges	12291

Product	Capacity plasmid DNA	Contents	Cat. No.
QIAGEN-tips without buffers			
QIAGEN-tip 20 (25)	20 µg	25 columns	10023
QIAGEN-tip 20 (100)	20 µg	100 columns	10025
QIAGEN-tip 100 (25)	100 µg	100 columns	10043

Product	Contents	Cat. No.
QIAprep Miniprep Kits*	**for high-purity plasmid DNA minipreparation**	
QIAprep Spin Miniprep Kit (50)	For 50 plasmid minipreps: 50 QIAprep Spin Columns, Reagents, Buffers, Collection Tubes (2 ml)	27104
QIAprep Spin Miniprep Kit (250)	For 250 plasmid minipreps: 250 QIAprep Spin Columns, Reagents, Buffers, Collection Tubes (2 ml)	27106
QIAprep 8 Miniprep Kit (50)‡	For 50 x 8 plasmid minipreps: 50 QIAprep 8 Strips, Reagents, Buffers, Collection Microtubes (1.2 ml), Caps	27144
QIAprep 8 Turbo Miniprep Kit (10)‡	For 10 x 8 plasmid minipreps: 10 each: TurboFilter 8 and QIAprep 8 Strips; Reagents, Buffers, Collection Microtubes (1.2 ml), Caps	27152

* *Other kit sizes available*
† *For low-copy plasmid and cosmid preparations, the QIAfilter/EndoFree Plasmid Mega Kit is a more cost-effective option than the QIAfilter Giga Kit.*
‡ *Requires use of QIAvac 6S*

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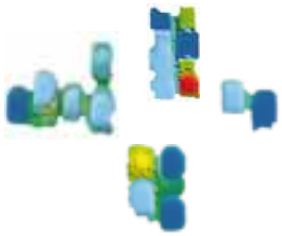

Ordering Information

Product	Contents	Cat. No.
QIAprep 8 Turbo Miniprep Kit (50)*	For 50 x 8 plasmid minipreps: 50 each: TurboFilter 8 and QIAprep 8 Strips; Reagents, Buffers, Collection Microtubes (1.2 ml), Caps	27154
QIAprep 96 Turbo Miniprep Kit (1)†	For 1 x 96 plasmid minipreps: 1 each: TurboFilter 96 and QIAprep 96 Plates; Flat-Bottom Block and Lid, Reagents, Buffers, Collection Microtubes (1.2 ml), Caps	27190
QIAprep 96 Turbo Miniprep Kit (4)*	For 4 x 96 plasmid minipreps: 4 each: TurboFilter 96 and QIAprep 96 Plates; Flat-Bottom Blocks and Lids, Reagents, Buffers, Collection Microtubes (1.2 ml), Caps	27191
R.E.A.L. Prep 96 Kits	**for highest-throughput, lowest-cost plasmid DNA minipreparation**	
R.E.A.L. Prep 96 Plasmid Kit (4)†	For 4 x 96 plasmid minipreps: 4 QIAfilter 96 Plates, Square-Well Blocks, Tape Pad, Reagents, Buffers	26171
R.E.A.L. Prep 96 Plasmid Kit (24)†	For 24 x 96 plasmid minipreps: 24 QIAfilter 96 Plates, Square-Well Blocks, Tape Pads, Reagents, Buffers	26173
R.E.A.L. Prep 96 Accessories		
Tape Pads (5)	Adhesive tape sheets for sealing multiwell plates and blocks: 25 sheets per pad, 5 pads per pack	19570
AirPore Tape Sheets (50)	Microporous tape sheets for covering 96 well-blocks	19571
Square-Well Blocks (8)	96-well blocks with 2.2-ml wells, 8 per case	19572
Square-Well Blocks (24)	96-well blocks with 2.2-ml wells, 24 per case	19573
QIAwell Plasmid Kits‡	**for high-throughput, ultrapure plasmid DNA minipreparation**	
QIAwell 8 Plasmid Kit (50)*	For 50 x 8 plasmid minipreps: 50 QIAwell 8 Strips, Reagents, Buffers, Collection Microtubes (1.2 ml), Caps	17124
QIAwell 8 Plus Plasmid Kit (50)*	For 50 x 8 plasmid minipreps, 50 each: QIAwell 8 and QIAprep 8 Strips; Reagents, Buffers, Collection Microtubes (1.2 ml), Caps	16144
QIAwell 8 Ultra Plasmid Kit (50)*	For 50 x 8 plasmid minipreps, 50 each: QIAfilter 8, QIAwell 8, and QIAprep 8 Strips; Reagents, Buffers, Collection Microtubes (1.2 ml), Caps	16154
QIAwell 96 Ultra Plasmid Kit (4)†	For 4 x 96 plasmid minipreps, 4 each: QIAfilter 96, QIAwell 96, and QIAprep 96 Plates; Reagents, Buffers, Collection Microtubes (1.2 ml), Caps	16191
QIAprep M13 Kits‡		
QIAprep Spin M13 Kit (50)	For 50 ssDNA preparations: 50 QIAprep Spin Columns, Buffers, Collection Tubes (2 ml)	27704
QIAprep 8 M13 Kit (50)*	For 50 x 8 ssDNA preparations: 50 QIAprep 8 Strips, Buffers, Extension Tubes, Collection Microtubes (1.2 ml), Caps	27744
QIAprep 96 M13 Kit (4)†	For 4 x 96 ssDNA preparations: 4 QIAprep 96 Plates, Buffers, Collection Microtubes (1.2 ml), Caps	27781
QIAprep M13 Accessories		
Extension Tubes (100)	For use with QIAGEN 8-well strips, or spin columns on vacuum manifolds: 100/pack	19555

* *Requires use of QIAvac 6S*
† *Requires use of QIAvac 96*
‡ *Other kit sizes available*

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Product	Contents	Cat. No.
QIAGEN Genomic-tips	**for small- to large-scale purification of high-molecular-weight DNA**	
QIAGEN Genomic-tip 20/G	25 columns	10223
QIAGEN Genomic-tip 100/G	25 columns	10243
Accessories		
Genomic DNA Buffer Set	Buffers, including specific lysis buffers for yeast, bacteria, cells, blood, and tissue: Y1, B1, B2, C1, G2, QBT, QC, QF; for 75 mini-, 25 midi-, or 10 maxipreps	19060
QIAquick PCR Purification Kits*	**for direct purification of PCR fragments from amplification reactions**	
QIAquick PCR Purification Kit (50)	50 QIAquick Spin Columns, Buffers, Collection Tubes (2 ml)	28104
QIAquick Nucleotide Removal Kits*	**for cleanup of oligonucleotides and DNA fragments from enzymatic reactions**	
QIAquick Nucleotide Removal Kit (50)	50 QIAquick Spin Columns, Buffers, Collection Tubes (2 ml)	28304
QIAquick Gel Extraction Kits*	**for purification of DNA fragments (70 bp – 10 kb) from agarose gels**	
QIAquick Gel Extraction Kit (50)	50 QIAquick Spin Columns, Buffers, Collection Tubes (2 ml)	28704
QIAquick Multiwell PCR Purification Kits*	**for high-throughput purification of PCR fragments from amplification reactions**	
QIAquick 8 PCR Purification Kit (50)†	For purification of 50 x 8 PCR reactions: 50 QIAquick 8 Strips, Buffers, Collection Microtubes (1.2 ml), Caps	28144
QIAquick 96 PCR Purification Kit (4)‡	For purification of 4 x 96 PCR reactions: 4 QIAquick 96 Plates, Buffers, Collection Microtubes (1.2 ml), Caps	28181
BioRobot 9600§	System includes: robotic workstation with microprocessor-controlled shaker and vacuum pump; vacuum manifold; QIAsoft Operating System; computer, cables; installation and training; 1 year warranty on parts and labor	900200
BioRobot 9600 Accessories§		
Accessory Pipetting System	Dilutor unit with ceramic-coated, stainless steel pipetting probe for 1–500 µl volumes	900510
High-Speed Pipetting System	Specially designed microprocessor-controlled peristaltic pump with solenoid valves, Reagent Delivery Module for high-throughput applications, bottles and tubing	900520
Tip-Change System	Multifunctional system for error-free use of conducting tips, Tip-Disposal Station for the release and the disposal of used tips; Sensing of tip uptake and disposal using the Tip-Check Station, cooling block for reaction mix; racks for disposable tips	900530
Disposable Tips 300 µl (960)	Conducting disposable tips for use on the BioRobot 9600, pack of 960 tips	990032
Disposable Tips 300 µl (18 x 960)	Conducting disposable tips for use on the BioRobot 9600, pack of 18 x 960 tips	990036

* *Other kit sizes available*
† *Requires use of QIAvac 6S*
‡ *Requires use of QIAvac 96*
§ *The BioRobot 9600 is not available in all countries. Please inquire.*

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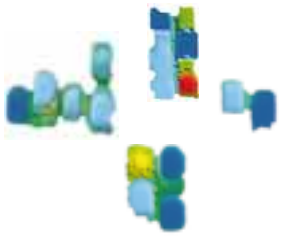

Ordering Information

Product	Contents	Cat. No.
BioRobot Kits[§]		
QIAwell 8 Ultra BioRobot Kit (48)	For 48 x 8 plasmid minipreps, 48 each: QIAfilter 8, QIAwell 8, and QIAprep 8 Strips; Reagents, Buffers, Collection Microtubes (1.2 ml), Caps, 96-Well Microplates and Lids	960134
QIAwell 96 Ultra BioRobot Kit (4)	For 4 x 96 plasmid minipreps, 4 each: QIAfilter 96, QIAwell 96, and QIAprep 96 Plates; Flat-Bottom Blocks and Lids, Reagents, Buffers, Collection Microtubes (1.2 ml), Caps, 96-Well Microplates and Lids, Tape Pads	960141
QIAprep 8 Turbo BioRobot Kit (48)	For 48 x 8 plasmid minipreps, 48 each: TurboFilter 8 and QIAprep 8 Strips; Reagents, Buffers, Collection Microtubes (1.2 ml), Caps, 96-Well Microplates and Lids	962134
QIAprep 96 Turbo BioRobot Kit (4)	For 4 x 96 plasmid minipreps, 4 each: TurboFilter 96, and QIAprep 96 Plates; Flat-Bottom Blocks and Lids, Reagents, Buffers, Collection Microtubes (1.2 ml), Caps, 96-Well Microplates and Lids, Tape Pads	962141
R.E.A.L. Prep 96 BioRobot Kit (4)	For 4 x 96 rapid extraction alkaline lysis minipreps: 4 QIAfilter 96 Plates; Flat-Bottom Blocks, Square-Well Blocks, Reagents, Buffers, Tape Pads	961141
QIAquick 8 PCR BioRobot Kit (48)	For purification of 48 x 8 PCR products: 48 QIAquick 8 Strips; Buffers, Collection Microtubes (1.2 ml), Caps, 96-Well Microplates and Lids	963134
QIAquick 96 PCR BioRobot Kit (4)	For purification of 4 x 96 PCR products: 4 QIAquick 96 Plates; Buffers, Collection Microtubes (1.2 ml), Caps, 96-Well Microplates and Lids	963141
QIAprep 8 M13 BioRobot Kit (48)	For 48 x 8 ssDNA preparations: 48 QIAprep 8 Strips; Reagents, Buffers; Extension Tubes, Collection Microtubes (1.2 ml), Caps, 96-Well Microplates and Lids	962534
QIAprep 96 M13 BioRobot Kit (4)	For 4 x 96 ssDNA preparations: 4 QIAprep 96 Plates; Square-Well Blocks, Reagents, Buffers, Collection Microtubes (1.2 ml), Caps, 96-Well Microplates and Lids, Tape Pads	962541
QIAGEN 96-Well Centrifugation System		
Plate Rotor 2 x 96*	Rotor for 2 QIAGEN 96 plates for use with Centrifuge 4-15*	81031
Centrifuge 4-15 (230 V)[†]	Universal laboratory centrifuge with brushless motor (230 V/50 Hz)	81020
Centrifuge 4-15 (120 V)[†]	Universal laboratory centrifuge with brushless motor (120 V/60 Hz)	81010
QIAvac Vacuum Manifolds		
QIAvac 6S	Vacuum manifold for processing 1–6 QIAGEN 8-well strips: includes QIAvac 6S Top Plate with flip-up lid, Base, Waste Tray, Blanks, Strip Holder	19503
QIAvac 96	Vacuum manifold for processing QIAGEN 96-well plates: includes QIAvac 96 Top Plate, Base, Waste Tray, Plate Holder	19504
QIAvac Manifold Components and Replacement Parts		
Vacuum Regulator	For use with QIAvac Manifolds	19530

* *The Plate Rotor 2 x 96 is available exclusively from QIAGEN and its distributors. Under the current liability and warranty conditions, the rotor may only be used in Centrifuge 4-15 from QIAGEN or the freely programmable models of centrifuges 4-15, 4K15, 6-10, and 6K10 from Sigma Laborzentrifugen GmbH.*

† *Not available in all countries. Please contact your local QIAGEN company or distributor.*

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Ordering Information

Product	Contents	Cat. No.
QIAGEN Products for PCR*		
***Taq* DNA Polymerase**		
Taq DNA Polymerase (250)	250 units *Taq* DNA Polymerase, 10x PCR Buffer†, 5x Q-Solution, 25 mM $MgCl_2$	201203
***Taq* PCR Core Kit**		
Taq PCR Core Kit (250)	250 units *Taq* DNA Polymerase, 10x PCR Buffer†, 5x Q-Solution, 25 mM $MgCl_2$, dNTP Mix‡	201223
***Taq* PCR Master Mix Kit**		
Taq PCR Master Mix Kit (250)	3 x 1.7 ml *Taq* PCR Master Mix§ containing 250 units *Taq* DNA Polymerase total, 3 x 1.7 ml distilled water	201443

Notice to Purchasers of QIAGEN Taq DNA Polymerase: Limited License

Purchase of *Taq* DNA Polymerase or *Taq* PCR Core Kit is accompanied by a limited license to use them in the Polymerase Chain Reaction (PCR) process for research and development activities in conjunction with a thermal cycler whose use in the automated performance of the PCR process is covered by the up-front license fee, either by payment to Perkin-Elmer or as purchased, i.e. an authorized thermal cycler.

* *Larger kit sizes available.*
† *Contains 15 mM $MgCl_2$.*
‡ *Contains 10 mM each dNTP.*
§ *Provides a final concentration of 1.5 mM $MgCl_2$ and 200 µM each dNTP*

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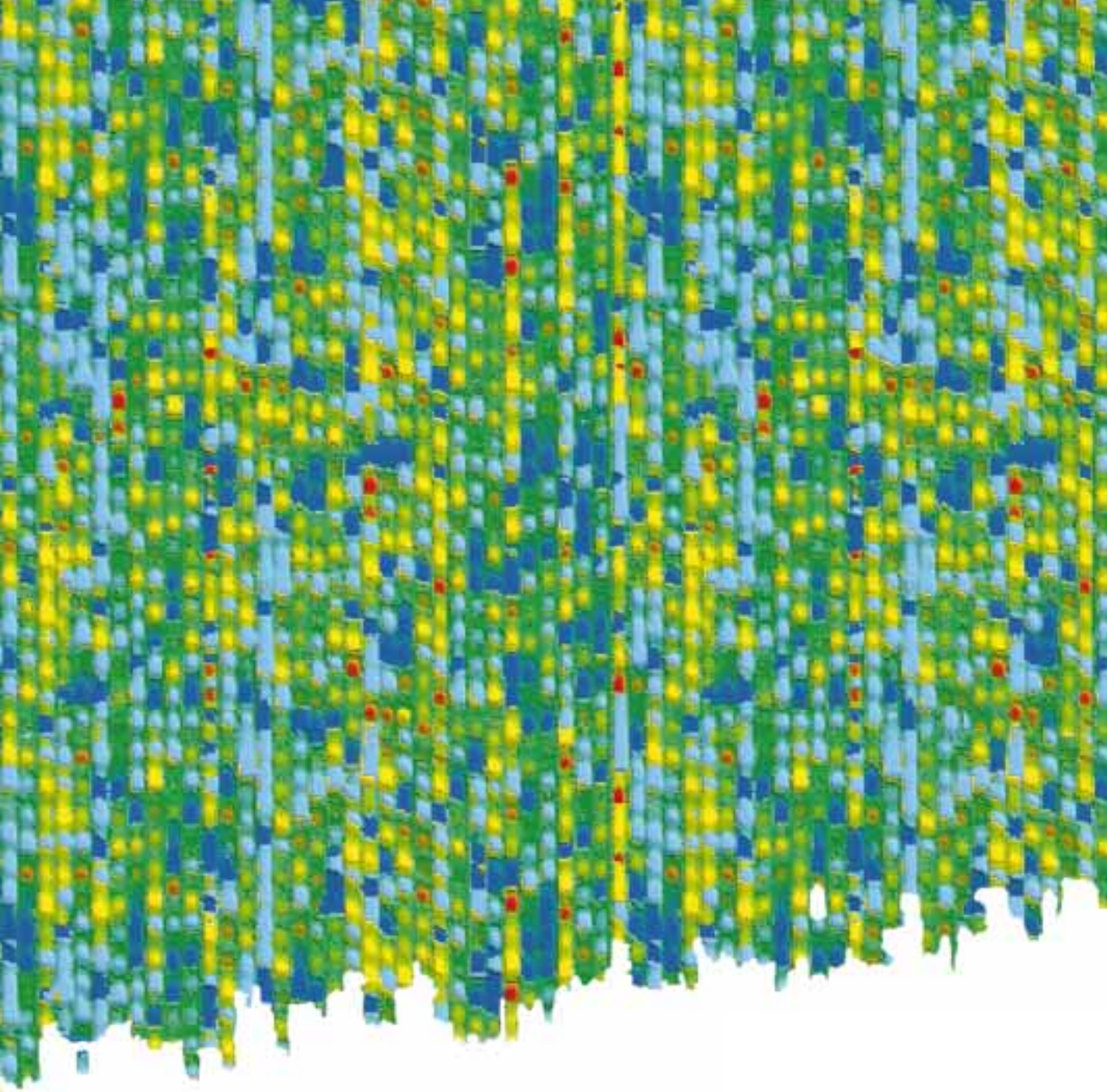

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