

STRANDASE™ KIT

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Product Information
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Product Description

Strandase™ Kit is designed for the convenient production of single-stranded DNA template suitable for sequencing. The kit takes advantage of the characteristic of I exonuclease to digest one strand of duplex DNA from a 5' phosphorylated end, releasing 5'-phosphomononucleotides¹. Suitable DNA templates can be easily generated during PCR[†] amplification in which one primer contains a 5'-terminal phosphate. Templates containing one phosphorylated primer and one non-phosphorylated primer allow for the strand containing the phosphorylated primer to be selectively degraded. Sequencing is then carried out using a primer that anneals to the strand containing the non-phosphorylated 5' end.

In practice, PCR products are first precipitated with sodium acetate and isopropanol to concentrate the DNA and remove primers and other reaction components. The DNA is then digested with Strandase, heated to inactivate the enzyme, and added directly to a sequencing reaction. Unlike other approaches for PCR product sequencing, the Strandase method produces a concentrated, purified ssDNA that can be sequenced by conventional primer extension techniques that do not require cycling.

Amplification Strategy

When planning to sequence a PCR product using ssDNA prepared with Strandase, it is important to account for both the sense and size of the template being produced. PCR reactions are carried out using one phosphorylated primer and one non-phosphorylated primer. Strandase will degrade the strand containing the phosphorylated primer; therefore, sequencing will be performed using the same phosphorylated primer or another primer of the same sense. Approximately 0.8 pmol of ssDNA is recommended for sequencing; therefore, more DNA mass is required for larger amplification products than for smaller fragments.

Another factor to consider is the extent of phosphorylation of the primer targeted for digestion. More template will be required as the extent of phosphorylation decreases, since non-phosphorylated DNA is very inefficiently digested. The chart below indicates

the amount DNA required to produce 0.8 pmol of single-stranded DNA for templates of various sizes. Substantially more starting dsDNA is required to produce an equivalent amount of ssDNA when a lower percentage of the primer is phosphorylated.

Amount of DNA required for sequencing

DNA length (bp)	0.8 pmol ssDNA (µg)	µg dsDNA needed to produce 0.8 pmol ssDNA when primer is:	
		90% phosphorylated	70% phosphorylated
300	0.079	0.176	0.226
750	0.197	0.438	0.563
1500	0.395	0.878	1.129
3000	0.789	1.753	2.254

See page 4 for a discussion of primer phosphorylation strategies

Reagents

Sufficient components are provided to generate 50 ssDNA templates for sequencing.

- Strandase I Exonuclease, Product No. S8554, 5 units/µl 250 units
- 10X Strandase Buffer, Product No. S8679 50 µl
100 mM Tris-HCl, pH 8.8 at 25°C,
500 mM KCl, 15 mM MgCl₂, 1% Triton X-100
- Glycogen, 10 mg/ml in water 250 µl
Product No. G3788
- 3 M Sodium Acetate, pH 5.2, Product No. S1555 1 ml
- Water, Nuclease-Free, Product No. W3885 1.5 ml

Reagents Required but Not Provided

(Sigma product numbers have been given where appropriate)

- dNTP mix (10 mM each dATP, dCTP, dGTP, TTP), Product No. D7295
- Phosphorylated primer
- Non-phosphorylated primer
- *Taq* Polymerase, Product No. D1806, shipped with 10X PCR buffer, Product No. P2192
- Mineral oil, PCR Reagent, Product No. M8662
- Isopropanol, Product No. I9516
- T7 DNA polymerase, Product No. D0410
- T4 Polynucleotide kinase, Product No. P4390

Precautions and Disclaimer

Sigma's Strandase Kit is for laboratory use only; not for drug, household or other uses.

Storage and Stability

Store all kit components at -20°C. This kit should be stable for 6 months.

Procedure

A. PCR Amplification

A 100 µl PCR reaction typically generates 1-5 µg of DNA. Conditions that can be used for direct colony PCR are given below. The same reaction conditions can be used to amplify DNA from purified plasmids; simply omit steps 1-5 and use a 20 µl sample containing 0.5-1 ng plasmid in sterile water as the template.

1. Pick a colony from an agar plate by touching a 200 µl pipet tip or sterile toothpick to the colony. Choose colonies that are at least 1 mm in diameter and try to get as many cells as possible. If a "copy" of the colony is desired, touch the pipet tip to a master plate before transferring the bulk of the colony to the tube in the next step.
 2. Transfer the bacteria to a 1.5 ml tube containing 50 µl of sterile water. Vortex to disperse the pellet.
 3. Place the tubes in boiling water or a heat block set at 99°C for 5 minutes to lyse the cells and denature DNases.
 4. Centrifuge at 12,000 x g for 1 minute to remove cell debris.
 5. Place the tubes on ice until ready to use (up to 1 hour). Samples can be stored for longer periods by transferring the supernatant to fresh tubes and placing at -20°C.
 6. Make a master reaction mix as follows (assemble on ice just prior to use):

Per reaction:

63.5 µl	Molecular biology grade water
2.0 µl	dNTP mix
2.0 µl	Phosphorylated primer, ~5 pmol/µl
2.0 µl	Non-phosphorylated primer, ~5 pmol/µl
10 µl	10X PCR buffer
0.5 µl	2.5 units DNA <i>Taq</i> polymerase
- Mix together the above components in a single tube using amounts corresponding to the number of reactions desired. It is convenient to multiply the amounts by (X+0.5) µl, where X is the number of reactions, in order to account for pipetting losses.
7. Add 20 µl of each DNA sample to a 0.5 ml PCR tube. Perform a "hot start" by preheating the tubes at 80°C in the thermocycler for about 0.5-1 minute prior to adding 80 µl aliquots of the master mix to each tube. Add 3 drops (~65 µl) of mineral oil to each tube if not using a thermocycler with a heated lid, cap the tubes and process for 35 cycles of 1 minute at 94°C, 1 minute at 55°C and 2 minutes at 72°C, with a final extension at 72°C for 5 minutes. These conditions are suitable for 20-mer primers having approximately 50% G:C content.
 8. To analyze the reaction products, remove a 5 µl sample from beneath the oil overlay and add to an appropriate loading buffer. Load and run a 1% agarose gel containing 0.5 µg/ml ethidium bromide and visualize the bands under UV illumination. BlueView™ may be used for the electrophoresis running buffer, eliminating the need for ethidium bromide staining. The PCR product should appear as a single intense band. The approximate yield of the PCR product can be estimated from comparing the target band intensity with known amounts of standards run on the same gel. If insufficient DNA is produced from a 100 µl PCR, several reactions can be performed and combined prior to precipitation to produce enough material for sequencing. When estimating the amount of product required, be sure to account for both the size and extent of phosphorylation of the primer as

discussed above. If the PCR produces multiple bands instead of a single species, either purifying the desired band from a gel or repeating the PCR using more stringent conditions (e.g. higher annealing temperature) to eliminate the spurious products is recommended.

B. Post-PCR Precipitation

Following the PCR reaction, a precipitation step is required to remove unreacted primers from the desired amplification products. With the following protocol, DNA greater than 150 bp is efficiently precipitated, leaving products less than 50 bp in solution.

1. Carefully transfer the PCR reaction mix (approximately 90 μ l) to a 1.5 ml tube leaving the oil layer behind. Add the following reagents equilibrated at room temperature: 1 μ l (10 μ g) glycogen solution, 9 μ l 3 M sodium acetate, pH 5.2, and 60 μ l isopropanol.
2. Vortex and incubate at room temperature for 5 minutes.
3. Centrifuge for 10 minutes at 12,000 x g at room temperature. Remove the supernatant.
4. Add 0.25 ml 70% ethanol to rinse the DNA pellet. Centrifuge as above for 2 minutes. Carefully remove the supernatant with a pipet.
5. Repeat the pellet wash in step 4 with 100% ethanol. Carefully remove the supernatant. The pellet will be very loose.
6. Allow the DNA to air dry (usually 5-10 minutes) and resuspend it in 10 μ l sterile water. Measure the amount of DNA. Read the absorbance of a 1-2 μ l sample diluted into 0.3 ml water at 260 nm (A_{260} of 1 = 50 μ g/ml). Alternatively, the DNA can be analyzed by agarose gel electrophoresis and the amount estimated based on band intensity vs. known markers (see Step 8 under PCR Amplification).

C. Strandase Exonuclease Reaction

1. Assemble the following components in a 0.5 ml tube on ice:

8.0 μ l	Amplified, primer-free DNA from step 6, Post-PCR Precipitation
1.0 μ l	10X Strandase buffer
1.0 μ l	Strandase I exonuclease (5 units/ μ l)
2. Incubate at 37°C for 20 minutes. Stop the reaction by heating at 75°C for 10 minutes.
3. Centrifuge briefly to collect the contents at the bottom of the tube. Run a 1 μ l sample of the reaction along side an aliquot of undigested DNA on an agarose gel. Single-stranded DNA migrates faster than the double-stranded counterpart; approximately 90% of the DNA should be converted to single-stranded form using Novagen's phosphorylated primers for PCR. Since single-stranded DNA binds ethidium bromide poorly, it will appear much more faint than the equivalent amount of double-stranded material. Staining gels following electrophoresis with 0.5 μ g/ml ethidium bromide in water for 20 minutes generates more visible single-stranded bands than are seen by including ethidium bromide in the gel.

Notes:

- Five units (1 μ l) of enzyme are sufficient to digest up to 2 μ g of DNA under these conditions. Up to 4 μ g of DNA can be digested in 10 μ l; use 10 units (2 μ l) of Strandase for 2-4 μ g DNA.
- The observed fraction of dsDNA resistant to digestion presumably represents material derived from unphosphorylated primers. Increasing the digestion period or amount of enzyme has little effect on the remaining double-stranded DNA. However, as long as there is a sufficient amount of ssDNA produced, normal sequencing can be performed. As noted above, estimating the amount of ssDNA produced by comparing gel band intensities of dsDNA and ssDNA can be deceiving due to the large difference in ethidium binding. The most critical factor in producing reliable sequence is ensuring that sufficient quality and quantity of starting PCR product is used for the Strandase reaction. This is achieved by (1) compensating for the extent of primer phosphorylation and (2) checking the PCR reaction products on a gel and correctly estimating the amount of material produced.

- The use of two phosphorylated primers under these reaction conditions results in complete degradation of the dsDNA, rather than the accumulation of molecules representing half of each strand.

D. Sequencing Conditions

The single-stranded products resulting from Strandase treatment can be sequenced by a variety of conventional primer extension methods. Reaction components are compatible with commercial sequencing reagents and enzymes, such that up to 5 μ l of heat-inactivated Strandase reactions can be used with commercial kits based on modified T7 DNA polymerase or *Taq* DNA polymerase. Typical conditions for annealing a sequencing primer with ssDNA prepared with Strandase are given below. A 1:1 primer:template ratio is recommended for optimal sequencing results.

1. Assemble in a 0.5 ml or 1.5 ml tube:

X μ l	Strandase reaction containing approximately 0.8 pmol DNA
1.0 μ l	Sequencing primer (diluted to 0.8 pmol/ μ l)
2.0 μ l	5X Sequencing buffer
Y μ l	<u>Molecular biology grade water</u>
10 μ l	Total Volume

2. Place 200 ml water in a 500 ml beaker and heat to 65°C. Place annealing reactions in the water and allow to cool slowly to 23-30°C on the bench top. Centrifuge briefly to collect the liquid at the bottom of the tubes. The annealed primer/template is ready for the sequencing reaction using standard protocols.

E. Primer Phosphorylation

As described above, the amount of DNA required for sequencing is dependent upon the extent of phosphorylation of the target primer. Higher phosphorylation will result in greater efficiencies; however, efficient PCR reactions will usually produce enough DNA to offset the effect of under-phosphorylated primers.

F. Chemical Phosphorylation

The most highly phosphorylated primers are obtained via chemical phosphorylation during synthesis followed by purification, preferably by HPLC. This process yields primers that are full-length and approximately 90-95% phosphorylated. Novagen offers

phosphorylated primers prepared by this method that are compatible with Novagen vectors.

Oligonucleotides can be phosphorylated either during synthesis ("chemically") or by treatment with polynucleotide kinase. Unpurified primers that are chemically phosphorylated vary significantly in the extent of phosphorylation in the final product; as little as 40% of an unpurified 20-mer or 33% of a 35-mer will be full-length and phosphorylated. This low yield arises from the generation of non-full length species during synthesis (they are built 3'→5') and loss of the phosphate group during the deprotection and deblocking steps. Therefore, using chemically phosphorylated purified primers is recommended.

G. Phosphorylation Using T4 Polynucleotide Kinase

A convenient method for phosphorylating previously synthesized oligonucleotides is treatment with T4 polynucleotide kinase. Typically, 60-80% of a purified primer is phosphorylated under conditions described below. With unpurified primers, the extent of phosphorylation also depends on length due to the presence of truncated products that arise through incomplete addition of residues to the 5' end during each synthesis cycle. Since only full-length oligonucleotides have a 5'-hydroxyl group, incomplete chains do not serve as substrates for kinase. Assuming that each cycle is 99% efficient, an unpurified 20-mer would be 80% full-length, whereas an unpurified 35-mer would be 65% full-length. Therefore, given the efficiency of the kinase reaction, the 20-mer would be ~50-65% phosphorylated and the 35-mer would be ~40-50% phosphorylated by polynucleotide kinase. These considerations should be applied when estimating the amount of PCR product needed to ensure that enough ssDNA will be produced for sequencing a given target.

It is also important to use oligonucleotides that are free of ammonium ions, which are a potent inhibitor of polynucleotide kinase. Most, but not all, oligonucleotide synthesis companies desalt the final product (which contains ammonium ions) prior to lyophilization.

1. Polynucleotide Kinase reaction conditions:

1.5 μ l	10X Kinase buffer (330 mM Tris acetate, pH 7.8, 660 mM potassium acetate, 100 mM magnesium acetate, 5 mM DTT)
1.0 μ l	10 mM ATP
1-5 μ g	Primer
5 units	T4 Polynucleotide kinase
<u>X</u> μ l	<u>Molecular biology grade water</u>
15 μ l	Total Volume

2. Incubate at 37°C for 10 minutes. Heat at 70°C for 10 minutes to inactivate the enzyme. Store at -20°C.

To determine the extent of phosphorylation, a reaction containing a tracer amount of [³²P] γ -ATP can be carried out in parallel. Use conditions identical to those described above, except add 1 μ l (1-2 μ Ci) labeled ATP to the tube (i.e. in addition to the cold ATP). Following the 10 minute incubation, spot duplicate 1 μ l samples on Whatman DE81 filters. Wash one filter by immersing in 20-50 ml of 0.5 M sodium phosphate, pH 7.0, for 5 minutes. Repeat the wash step with fresh buffer 4 more times. Dry both filters under a heat lamp, add appropriate scintillant and count. Determine the percent incorporation by dividing the cpm observed for the washed filter by the total cpm observed for the unwashed filter and multiplying by 100%.

Results

Calculations:

The 15 μ l reaction with 1 mM ATP contains 15 nmol ATP (the amount added by the ³²P-ATP is negligible). If 4% incorporation was observed, then 15 nmol x 0.04 = 0.6 nmol of ATP was incorporated.

The extent of phosphorylation of the primer is then calculated by dividing the nmol ATP incorporated by the nmol oligonucleotide in the reaction and multiplying by 100%.

The amount of oligonucleotide in the reaction is determined from its absorbance at 260 nm, where a reading of 1 is equivalent to a concentration of 30 μ g/ml. Divide the mass of oligonucleotide by its molecular weight to obtain the number of nmoles in the reaction.

For example, 5 μ g of a 20-mer with a molecular weight of 6600 was phosphorylated as above, giving 4% incorporation in the labeling reaction. Therefore, 5 μ g \div 6.6 μ g/nmol = 0.76 nmol oligonucleotide was present in the reaction. Since 0.6 nmol ATP was incorporated, this represents 0.6/0.76 x 100% = 79%.

Related Products

Blot Stain Blue, Product No. B1177
 BlueView TBE Buffer, Product No. T9060
 BlueView TAE Buffer, Product No. T8935
 Ethidium bromide, Product No. E7637
 Whatman DE81 Filters, Product Nos. Z,28,659-1 or Z28,660-5
 Sodium phosphate, dibasic, Product No. S3264
 Sodium Phosphate, monobasic, Product No. S3139

References

1. J.W. Little, I.R. Lehman and A.D. Kaiser, J. Biol. Chem., **242**, 672 (1967)

† The PCR process is covered by patents owned by Hoffman-LaRoche, Inc. Purchase of this product does not convey a license under these patents.

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