GE Healthcare

Thermo Sequenase Cy5 Dye terminator Cycle Sequencing Kit

Product Booklet

Code: 27-2682-01

Page finder

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Thermo Sequenase DNA Polymerase – this product and/or its method of use is covered by one or more of the following patent numbers: US 4,962,020, US 5,173,411, US 5,409,811, US 5,498,523 and US 5,614,365 in the name of Harvard and their equivalents in other countries.

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Contact your GE Healthcare representative for the most current information and a copy of the terms and conditions.

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2. Handling

2.1. Safety warnings and precautions

Warning: ***For research use only.*** Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.

Warning: This protocol requires the use of ethanol and formamide. Gel reagents may contain acrylamide, a neurotoxin and suspected carcinogen. Please follow the manufacturer's Material Safety Data Sheet regarding safe handling and use of these materials.

2.2. Storage

Store at -15°C to -30°C.

2.3. Quality control

All kit components are functionally tested as described in this protocol. Release specifications are based upon sequence length, band intensity, and sequence quality.

3. Materials not supplied

Reagents

- **Ammonium acetate** - 7.5 M aqueous solution.
- **Water** - Only deionized, distilled water should be used for the sequencing reactions.
- **Sequencing primers** - For most applications, use 1–2.5 pmol of primer for each set of four sequencing reactions. Always determine the concentration of the primer stock by reading the optical density at 260 nm (OD_{260}).

  For primers containing N bases (measured in a cuvette with a 1 cm path length), the approximate concentration in pmol/µl is given by the formula:

  Concentration (pmol/µl) = OD_{260} /(0.01 x N) where N is the number of bases.
- **Ethanol (100% and 70% ice cold)** - Required only for ethanol purification protocol.
- **AutoSeq™ G-50 Columns** - Required only for spin column purification protocol (GE Healthcare product code: 27-5340).
- **Mineral oil** - If required for the thermal cycler being used.

Equipment

- **Liquid handling supplies** - Vials, pipettes, and microcentrifuge. All sequencing reactions should be run in plastic microcentrifuge tubes (typically 0.2 ml or 0.5 ml) suitable for thermal cycling.
- **Thermal cycler** - For thermally cycled incubations between 40°C and 95°C (1–100 cycles).
- **Sequencing instrument** - Fluorescent sequencing instrument with 675 nm laser.

4. Components of the kit

The Thermo Sequenase™ Cy™5 Dye Terminator Kit is designed for use with the ALFexpress™ DNA Sequencer from GE Healthcare. The kit contains sufficient reagents for 100 sets of dideoxy cycle sequencing reactions using Thermo Sequenase I DNA polymerase. The kit also includes a modified Universal Primer for sequencing pUC-derived templates.

The solutions included in the Thermo Sequenase Cy5 Dye Terminator Cycle Sequencing Kit have been carefully prepared to yield optimal sequencing results. Each reagent has been tested extensively and its concentration adjusted to meet the standards of GE Healthcare.

It is strongly recommended that the reagents supplied in the kit be used exactly as described in this protocol.

The following components are included in this product:

dNTP Mix:	1.1 mM each dATP, dCTP, dGTP, and dTTP
Cy5-ddATP:	8.8 µM Cy5-labelled ddATP in isocitrate
Cy5-ddCTP:	7.0 µM Cy5-labelled ddCTP in isocitrate.
Cy5-ddGTP:	17.6 µM Cy5-labelled ddGTP in isocitrate.
Cy5-ddTTP:	14.3 µM Cy5 labelled ddTTP in isocitrate.
Thermo Sequenase I DNA Polymerase:	10 units/µl in 20 mM Tris-HCl (pH 9.5), 1 mM DTT, 0.1 mM EDTA, 0.5% Tween™-20, 0.5% Nonidet™P-40, 50% glycerol with pyrophosphatase.
Reaction buffer	150 mM Tris-HCl (pH 9.5), 67 mM $MgCl_2$
Control Primer:	Modified M13 Universal Primer in aqueous solution; 2.0 pmol/µl.
Control Template:	Double-stranded pUC19; 0.5 µg/µl.
Glycogen Solution:	10 mg/ml, aqueous solution

Formamide loading dye	Deionized formamide containing a proprietary dye.

The kit components should be stored at -15°C to -30°C (NOT in a frost-free freezer). All reagents should be kept on ice when removed from storage for use. For convenience, the kit can be stored at 2–4°C for up to three months with no loss of performance; however, this should be avoided if the reagents will not be completely consumed within this period of time.

Also please be aware that repeated freeze-thawing of the Cy-labelled terminators is not recommended. Aliquot Cy5-labelled terminators into smaller volumes if repeated freezing and thawing is necessary.

5. Introduction

ALFexpress DNA Sequencing instruments, in conjunction with fluorescent chemistry, offer convenient, automated analysis of either single- or double-stranded DNA templates. When used with ALFexpress DNA Sequencers, the Thermo Sequenase Cy5 Dye Terminator Kit enables DNA sequencing using a nonradioactive Cy5 label and thermal cycling (cycle sequencing).

Cycle Sequencing

In cycle sequencing the template is combined with a single unlabelled primer, a thermostable polymerase, Cy5-labelled ddNTPs and dNTPs. This mixture is then subjected to repeated cycles of thermal denaturation and polymerization to generate products that terminate with specific Cy5 dye-labelled dideoxynucleotides. The thermal cycling procedure increases the amount of chain terminated product in a linear fashion with each round of denaturation and polymerization.

The Thermo Sequenase Cy5 Dye Terminator Kit uses Thermo Sequenase I DNA polymerase for cycle sequencing. Thermo Sequenase I DNA polymerase is stable at 90°C for at least one h, and retains 50% of its activity when incubated at 95°C for 60 minutes. The enzyme is exonuclease-free and, like T7 DNA polymerase, generates very uniform signal peaks.

Dye Terminators

For each template being sequenced, four separate Cy5 dideoxy reactions (A, C, G and T) are required. One advantage of this labelled dye-terminator method is that prematurely terminated chains are not labelled. This reduces artificial stops and background noise. For analysis, reactions are loaded into four adjacent lanes on an ALFexpress sequencing gel and electrophoresed. As the DNA fragments in each lane migrate through the gel, they pass a fixed laser beam, generating fluorescent signals that are detected and stored automatically for subsequent sequence determination.

6. Protocols

6.1. Preliminary preparations and general handling instructions

For information concerning preparation of template DNA, primer selection, and cycling parameters, see Appendixes 1–3 on pages 17–21.

Pre-program a thermal cycler as described on page 13 (step 9.). Centrifuge all reagent tubes before opening them. Whenever possible, keep the tubes capped and on ice to minimize evaporation of the small volumes of the reagents used. Dispense all reagents using disposable-tip micropipettes, and apply great care to avoid contamination of stock solutions. Thoroughly mix all reaction mixtures after each addition, typically by "pumping" the solution two or three times with a micropipettor without creating air bubbles. Centrifuge tubes briefly to collect the reaction mixtures at the bottoms of the tubes.

6.2. Sequencing reactions

6.2.1. Sequencing reactions using Cy5 dye-labelled ddNTP terminators

1. Label four tubes A, C, G, and T for the respective termination mixes.

2. To each tube, add the components indicated below to prepare the dNTP/Cy5 ddNTP termination mixes. The volumes are sufficient for 10 sequencing reactions.

	A Mix	C Mix	G Mix	T Mix
1.1 mM dNTP	4 µl	4 µl	4 µl	4 µl
Cy5-ddNTP	2 µl	2 µl	2 µl	2 µl
deionized water	16 µl	16 µl	16 µl	16 µl
Total volume	22 µl	22 µl	22 µl	22 µl

Note: Prepare only enough of each dNTP/Cy5-ddNTP mix for the day's sequencing activities.

3. Vortex the mixes and centrifuge briefly to collect the contents at the bottom of each tube.

4. Store the dNTP/Cy5-ddNTP mixes on ice and in the dark until needed.

5. For each template to be sequenced, label four new tubes "A", "C", "G" and "T" and place on ice.

6. Dispense 2 µl of the "A" mix from step 2. into each tube labelled "A". Repeat this process for the "C", "G", and "T" tubes. Cap each tube to prevent evaporation.

7. For each template to be sequenced, prepare the following master mix in a microcentrifuge tube.

DNA template	1.0–20.5 µl
Primer (4 pmol total)	2 µl
Reaction Buffer	3.5 µl
Thermo Sequenase I	1 µl
DNA polymerase (10 U/µl) Distilled water	(to final volume of 27 µl)
Total volume	27 µl

8. Vortex the tube gently, then centrifuge it briefly. Aliquot 6 µl of the Master Mix into each of the tubes containing A Mix, C Mix, G Mix, and T Mix (prepared in step 6.).

9. Mix each sequencing reaction thoroughly and centrifuge the tubes if necessary. Overlay each reaction with 10–20 µl of mineral oil (or one drop from a 200 µl micropipette tip) unless the thermal cycler being used does not require it (e.g. a thermal cycler with a heated lid). Cap each tube and place into the thermal cycler.

10. Start the cycling program.
95°C for 30 seconds
60°C for 30 seconds
72°C for 80 seconds
(typically 30 cycles taking 2–3 hours)
End the program with a file to hold the completed reactions at 4°C.

11. After completion of the cycling program, centrifuge the tubes briefly to collect any condensation and place them on ice.

Purification of sequencing reactions

Unincorporated dye terminators can be removed from the sequencing reactions by either ethanol precipitation or with spin columns.

6.2.2. Removal of unincorporated dye terminators using ethanol precipitation

1. To each tube add:
2 µl of 7.5 M Ammonium acetate
2 µl Glycogen solution
30 µl (~ 3 times the reaction volume) of chilled 100% ethanol
Mix thoroughly by vortexing and place the tubes on ice for 20 minutes or incubate overnight at -20°C to precipitate the DNA.

2. Centrifuge the tubes in a microcentrifuge at full speed (10 000–16 000 x g) for 20–30 minutes at either room temperature or 4°C.

3. Carefully remove each supernatant and wash the pellets with 200 µl of chilled 70% ethanol.

4. Centrifuge for 5 minutes. Remove the supernatants and dry the pellets for 2–3 minutes in a vacuum centrifuge. Do not overdry.

Note: If pellets are overdried, they may be difficult to resuspend.

5. Resuspend each pellet in 6 µl of Formamide loading dye and vortex vigorously (10–20 seconds) to ensure complete resuspension. Briefly centrifuge to collect the samples at the bottoms of the tubes.

Note: The DNA pellets must be completely resuspended at this step for optimal sequencing results.

6. Just prior to loading the samples onto the gel, heat each sample at 70°C for 2–3 minutes to denature, then cool immediately on ice.
7. Load the entire volume of each sample into separate lanes of the sequencing gel.

6.2.3. Purification of sequencing reactions using microspin columns

This purification protocol has been verified using AutoSeq G-50 Columns from GE Healthcare. Results obtained using size-exclusion spin columns from other manufacturers cannot be guaranteed.

1. Resuspend the resin in each column by gentle vortexing. Prepare four columns for each set of sequencing reactions performed.
2. Loosen the cap on the column one-fourth turn and snap off the bottom closure.
3. Place each column in a 1.5 ml microcentrifuge tube for support (or cut the cap off a flip-top tube).
4. Prespin the columns at 2 000 x g for 1 minute.

Note: Do not use the pulse feature because this will override the variable speed setting on the instrument.

5. Remove the top caps and place each column in a clean support tube.
6. Add distilled water to each sample to bring the final volume to 20–25 µl. Mix thoroughly and then slowly apply each sample to the center of the angled surface of the compacted resin bed.

Note: Do not disturb the resin or allow any sample to flow around the sides of the bed during sample application.

7. Centrifuge the columns at 2 000 x g for 1 minute. Collect the purified sample in the bottom of the support tube.
8. Add 6 µl of Formamide loading dye to each tube and mix thoroughly.
9. Partially dry the purified samples for 15–20 minutes under vacuum without the use of heat. Don't dry the samples to completion; each volume should be reduced to 6 µl.
10. Just prior to loading the samples onto the gel, heat each sample at 70–72°C for 2–3 minutes and then immediately quench on ice.
11. Load the entire volume of each sample into separate lanes of the sequencing gel.

7. Appendix 1:

7.1. Preparation of template DNA - general considerations

Preparation of single-stranded plasmid DNA

Several published methods describe the preparation of single-stranded DNA from clones in M13 vectors and hybrid plasmid-phage (phagemid) vectors (4–6).

Note: It has been observed that DNA prepared from plate lysates contains an inhibitor of DNA polymerase.

Preparation of double-stranded plasmid DNA

Sequencing double-stranded templates with the Thermo Sequenase Cy5 Dye Terminator Cycle Sequencing Kit requires no change in the protocol. Alkaline denaturation is not required for these templates. For optimal results, use purified plasmid DNA. Cesium chloride gradients, polyethylene glycol (PEG) precipitation, adsorption to glass, columns, and other common DNA purification methods all produce DNA suitable for use in this system. However, since such small quantities of DNA are used in the reactions, even impure DNA samples can yield acceptable sequence data. Although there are many popular protocols for purifying plasmid DNA from 2 ml to 10 ml cultures, scientists at GE Healthcare consistently achieve success using boiling (7) and alkaline lysis (8) mini-prep methods.

Enzymatic pre-treatment of PCR product

This procedure involves treating Polymerase Chain Reaction (PCR) products with a combination of Exonuclease I (Product Number E700737) and Shrimp Alkaline Phosphatase (E70092Y). These enzymes are also available in kit form (USB 78200—ExoSAP-IT™). Both of these enzymes are active in the buffer used for PCR, so no change in buffer is required. Exonuclease I removes residual

single-stranded primers and any extraneous single-stranded DNA produced by PCR. Shrimp alkaline phosphatase removes any remaining dNTPs from the PCR mixture, which will interfere with the labelling step of the sequencing process. Both enzymes are easily inactivated by heating for 15 minutes at 80°C.

1. Mix the PCR amplification mixture and enzymes as shown below:

PCR amplification mixture	5 µl
Exonuclease I (10.0 U/µl)	1 µl
Shrimp alkaline phosphatase (2.0 U/µl)	1 µl
Total volume	7 µl

2. Incubate the mixture for 15 minutes at 37°C. It is convenient to do this in the thermal cycler.

Note: When treating more than 5 µl of PCR product, increase the amount of exonuclease and phosphatase proportionally.

3. Inactivate the exonuclease and phosphatase by heating for 15 minutes at 80°C. It is convenient to do this in the thermal cycler. The DNA is now ready for direct sequencing with the Thermo Sequenase Cy5 Dye Terminator Cycle Sequencing Kit following the standard protocol. Use the equivalent of 30–100 fmol of PCR product (often 1 µl or less) per sequencing reaction with 20–30 cycles (see below).

8. Appendix 2

8.1. Primer selection

Since the popular multiple cloning sites are all derived from similar sequences, the control -40 M13 forward primer can be used to sequence insert DNA within the most common cloning vectors, including M13mp8, M13mp9, M13mp12, M13mp13, M13mp18, M13mp19, pUC18, pUC19, and virtually any vector featuring blue/white screening with β-galactosidase activity.

Designing a new sequencing primer

The length and sequence of a primer will determine its melting temperature and specificity. For cycling temperatures normally used, the primer should be 18–25 bases in length. The sequence of the primer should be checked for potential self-annealing (dimer formation could result) and hairpin formation, especially at its 3′ end. Possible sites of false priming in the vector or other known sequences should also be identified, again stressing matches which include the 3′ end of the primer.

9. Appendix 3

9.1. Cycling parameters

Cycling temperatures

It is important to consider the melting temperature of the primer when choosing cycling temperatures. The control -40 M13 forward primer included in the kit is a moderately long 23-mer with 50% G/C content and a melting temperature of ~ 73°C under sequencing reaction conditions. For this primer, cycling between 60°C and 95°C is effective. The duration of the steps does not seem to be critical, and even brief pauses at these temperatures seem to be effective. If in doubt, select a wide temperature range with brief pauses at the temperature extremes.

Always use a denaturation temperature of 95–98°C for the termination reaction cycles; however, avoid extended steps at 98°C because Thermo Sequenase I DNA polymerase has a half-life of less than 1 hour at this temperature. Since the optimum temperature for polymerization is approximately 70–75°C, 72°C is a good choice for the termination.

Number of cycles and quantity of template

Good sequence data can be obtained with this product using 0.1–0.2 µg of M13 DNA, 0.2–0.5 µg of plasmid DNA, or 50–200 fmol of PCR product. When smaller amounts of template are used, signal intensity is strongly influenced by the number of cycles. If the number of cycles is increased from 30 (recommended in the protocol) to 60, for example, a significant increase in signal intensity is noted for template amounts less than ~ 50 fmol. However, as the numbers of cycles are increased, the amount of thermal breakdown products from the dye terminators also increases. It is possible to increase the number of cycles to the point that the purification procedures will not remove all of the breakdown products, leading

to some loss of data. When using smaller amounts of template, the number of cycles should first be increased to 45 and the results assessed. If the signal intensities remain low after 45 cycles, only then should 60 cycles be attempted. Weak signal intensities encountered with low template amounts can also be improved by adding two- to five-fold more primer to the reaction. This may improve signal strengths to the point that only a minimum of extra cycles is required.

10. Appendix 4

10.1. Processing sequence data

Process the data using the **Extended Shift** function available in ALFwin™ Sequence Analyser 2.10. ALFwin Sequence Analyser software can be upgraded by downloading version 2.10 from the internet site http://phenix.amershambiosciences.com/ADAProducts/Download/download.html or http://autodna.amershambiosciences.com.

If version ALFwin Sequence Analyser 2.10 is not available, use the following instructions to shift the sequence for processing:

When sequencing the Control Template using the Modified Universal Primer on a 5.5% Acrylamide gel comprised of 1 X TBE (running buffer 0.5 X TBE) the shift values are as follows:

(A: -20) (C: 10) (G: -5) (T: 15)

If using the ALFwin software, apply shift values after the run has completed. DO NOT HAVE THE SOFTWARE PROCESS YOUR DATA AS A POST-RUN ACTION!

Open the Evaluation program and go to **Analyze Process**. In this window enter a dot in the circle next to **Use these shifts**. In the space under each base enter your shift values. Click the **Process** or **Process All** button.

It might be necessary to determine shift values for your gel conditions. Begin with unprocessed raw data for clone 4, 5 or 6. If your data was processed with the automatic shift or the above-mentioned values, it is best to reprocess the data with shift values of 0, to return to raw data. Move to approximately 400 minutes into the run. Apply the shift, which should not change from raw data. Look for groups of multimer peaks; these are the easiest to align. Manually move each base. In every system the A and G must be

moved left relative to the C and T. Once the alignment is satisfactory, process that clone.

To find your shift values go to the **Casebook**, **Evaluation Log**. Scroll down to the last action to see the value of the shift applied for each base in that clone. Process the remaining clones (following the above-mentioned procedure) using the new shift values. **The Evaluation Log** lists the relative shift of each clone in comparison to the previous shift values of 0 (raw data). Prior to beginning this process, the values in the evaluation log are not absolute values.

Each clone must be manually shifted if ALF™ Manager software is in use. DO NOT HAVE THE SOFTWARE PROCESS YOUR DATA AS A POST RUN ACTION! Once the run is complete, open each clone. Begin with either clones 4, 5 or 6. Move to approximately 400 minutes into the run. Apply the shift, which should not change from the raw data. Move each base to the appropriate number of ticks, either negative (i.e. to the left) or positive (i.e. to the right). In every system the A and G will need to be moved relative to the C and T. Record the number of ticks moved and in which direction for future reference. Enter **Control**, **Process Raw Data** and click the **Process** button. Repeat for each clone.

11. Troubleshooting

Problem	Possible causes/solutions
1. Sequencing signals are weak in all four lanes.	**1.1.** The template concentration was low. Either increase the number of cycles or the amount of template and/or primer used in the reaction.
	1.2. The cycling parameters were not optimal. Check the parameters used.
2. Sequencing signals are weak in one or more lanes.	**2.1.** Poor technique was used when loading the sequencing gel. Minimize the time between application of samples and the start of electrophoresis.
	2.2. Sample resuspension was inadequate. Decrease the amount of time used to dry the precipitated DNA pellets under vacuum. Vortex the samples for a longer period after adding the loading solution.
	2.3. The ethanol precipitation was inadequate. Use extra care when resuspending the DNA pellets after precipitation. Perform ethanol precipitation on dry ice or at -20°C. Repeat the reactions.

Problem	Possible causes/solutions
3. Secondary sequence or "ghost" bands are present.	**3.1.** The primer annealed nonspecifically to the template. Repeat the reactions using a higher annealing temperature (no higher than 70°C). **3.2.** The reaction resulted in the generation of nonspecific PCR products from the template. Repeat the reactions using a higher annealing temperature or use a "nested" primer for the sequencing reactions. **3.3.** The clone used for sequencing was impure. Repurify the clone and then reisolate the template DNA.

If problems persist, please contact GE Healthcare Technical Service for assistance.

12. References

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13. Companion products available from GE Healthcare

Product	Pack size	Product number
Thermo Sequenase Cy5.5 Dye Terminator Sequencing Kit	50 rxns	US79840
Thermo Sequenase Primer Cycle Sequencing Kit	100 rxns	25-2438-01
ALFexpress AutoRead Sequencing Kit	100 rxns	27-2690-02
AutoRead Sequencing Kit	100 rxns	27-1690-04
AutoRead 200 Sequencing Kit	200 rxns	27-1791-12
AutoLoad Solid Phase Sequencing Kit	1 kit	17-1198-10
Cy5 Amidite	100 mg	27-1801-02
Cy5.5 Amidite	100 mg	27-1799-01
FluorePrime Fluorescein Amidite	82 mg	27-1796-01
AutoSeq G-50 Columns	250 cols	27-5340-02
AutoSeq G-50 Columns	1000 cols	27-5340-03

A full range of sequencing kits and reagents can be found in the GE Healthcare catalog.

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