GE Healthcare

Thermo Sequenase fluorescent labelled primer cycle sequencing kit

Product Booklet

Codes: RPN2436
RPN2536

Page finder

1. Legal

http//www.gehealthcare.com/lifesciences

GE Healthcare UK Limited.
Amersham Place, Little Chalfont,
Buckinghamshire, HP7 9NA UK

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.

Warning: The formamide loading dye contains 95%(v/v) formamide.

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.

Note that the protocol requires the use of acrylamide/bis acrylamide.

Warning: Acrylamide and bisacrylamide are neurotoxins.

Please follow the manufacturer's safety data sheets relating to the safe handling and use of these materials.

2.2. Storage

Store at -15°C to -30°C.

3. Thermo Sequenase fluorescent labelled primer cycle sequencing kits

RPN 2436	**Thermo Sequenase fluorescent labelled primer cycle sequencing kit**	
	Sufficient for 100 templates	
	A reagent	1 tube
	C reagent	1 tube
	G reagent	1 tube
	T reagent	1 tube
	Formamide loading dye (for fluorescent samples)	2x1 ml
RPN 2536	**Thermo Sequenase fluorescent labelled primer cycle sequencing kit**	
	Sufficient for 100 templates	
	A reagent	5 tubes
	C reagent	5 tubes
	G reagent	5 tubes
	T reagent	5 tubes
	Formamide loading dye (for fluorescent samples)	1x10 ml

4. Components of the system

Each pack contains the following components:

Component	Contents	Cap colour
A reagent	Tris-HCl (pH 9.5), magnesium chloride, Tween™20, Nonidet™ P-40, 2-mercaptoethanol, dATP, dCTP, dGTP, dTTP ddATP, thermostable pyrophosphatase and Thermo Sequenase DNA polymerase	green
C reagent	Tris-HCl (pH 9.5), magnesium chloride, Tween 20, Nonidet P-40, 2-mercapto-ethanol, dATP, dCTP, dGTP, dTTP, ddCTP, thermostable pyrophosphatase and Thermo Sequenase DNA polymerase	blue
G reagent	Tris-HCl (pH 9.5), magnesium chloride, Tween 20, Nonidet P-40, 2-mercapto-ethanol, dATP, dCTP, dGTP, dTTP, ddGTP, thermostable pyrophosphatase and Thermo Sequenase DNA polymerase	yellow
T reagent	Tris-HCl (pH 9.5), magnesium chloride, Tween 20, Nonidet P-40, 2-mercapto-ethanol, dATP, dCTP, dGTP, dTTP, ddTTP, thermostable pyrophosphatase and Thermo Sequenase DNA polymerase	red
Formamide loading dye (for fluorescent samples)	Formamide, EDTA and fuchsin	clear

5. Description

The Thermo Sequenase™ fluorescent labelled primer cycle sequencing kit is recommended for fluorescent dye primer sequencing of single stranded or double stranded DNA templates.

5.1. Quality control

The Thermo Sequenase fluorescent labelled primer cycle sequencing kit is tested in fluorescent sequencing as follows:

5.2. DNA templates

XL-1 Blue/NZY propagated M13 mp8 ssDNA

TOP 10F'/TSB propagated pBluescript miniprep dsDNA, prepared by a modification of the alkaline lysis method

M13 mp8 ssDNA from CsCl template preparation

pUC 18 dsDNA from CsCl template preparation.

5.3. Assay method

ABI 373 34 cm well-to-read and 4 dye-primer cycle sequencing with Thermo Sequenase DNA polymerase.

5.4. Quality

98.5% accurate from 20 bases beyond the 3'-end of the primer to 500 bases beyond the 3'-end of the primer.

5.5. Storage

The components in this kit may be stored at either 2–8°C or -15°C to -30°C with no difference in stability over 3 months. For longer term storage, we recommend -15°C to -30°C.

6. Additional reagents and materials required

Reagents and materials that may be required but which are not supplied with the kit are detailed below. Many of these items are available from GE Healthcare.

Please refer to the section on **related products available from GE Healthcare** on page 30 of this booklet.

Fluorescent dye labelled primers	for fluorescent sequencing. A range of dye labelled primers can be obtained for use with commercially available sequencing instruments.
Acrylamide gel electrophoresis materials	for separation of the sequencing reaction products. See the **supplementary protocols** section on page 22 of this booklet.
Light mineral oil	for overlaying the sequencing reactions. Use Sigma light mineral oil (catalogue code M-3516).

7. Thermo Sequenase fluorescent labelled primer cycle sequencing

7.1. Preparation of template DNA

High quality template DNA is essential to obtain the best results from the Thermo Sequenase fluorescent labelled primer cycle sequencing kit. Protocols and reagent kits are available from GE Healthcare for the preparation of M13 and plasmid sequencing templates.

7.2. Thermo Sequenase cycle sequencing with fluorescent 4 dye primer labelling

The reagents in the Thermo Sequenase fluorescent labelled primer cycle sequencing kit are suitable for use with fluorescently labelled primers such as those used with the Applied Biosystems 373 and 377 Fluorescent Sequencers.

1. Remove the following components from the kit and allow to thaw (if stored frozen): A reagent (green cap), C reagent (blue cap), G reagent (yellow cap), T reagent (red cap) and formamide loading dye (for fluorescent samples) (clear cap). Store on ice until required. All of the tubes should be well mixed before use by briefly vortexing the tube.

2. Prepare any other materials that may be required. The following quantities of template are recommended: 0.5–2 µg of single stranded DNA or 0.5–5 µg of double stranded DNA. The DNA should be in a total volume of at least 30 µl. If the template is the product of a polymerase chain reaction* (PCR), please refer to the section on **sequencing the products of a polymerase chain reaction** on page 21 of this booklet.

* See licensing information in legal section, page 3.

3. Label four tubes or microplate wells as follows: A reaction, C reaction, G reaction and T reaction. Reactions should be prepared as follows:

A and C reactions

	For each sample:
Primer (JOE for A terminations, FAM for C terminations)	1 µl
Template DNA	5 µl
A or C reagent	2 µl

Reagents and primers may be conveniently premixed before addition to the template DNA. The following table gives the volumes of primers and reagents needed for some commonly used numbers of samples.

Number of samples	Primer JOE FAM	Reagent A C	Total volume
4	4 µl	8 µl	12 µl
8	8 µl	16 µl	24 µl
12	12 µl	24 µl	36 µl
16	16 µl	32 µl	48 µl
24	24 µl	48 µl	72 µl

Mix thoroughly after the addition of each reagent by pipetting up and down two or three times. The premixes can be freshly prepared for each set of sequencing reactions or they can be prepared in bulk and stored at 2–8ºC. Premixes should be kept on ice until used.

G and T reactions

For each sample:	
Primer (TAMRA for G terminations, ROX for T terminations)	2 µl
Template DNA	10 µl
G or T reagent	4 µl

Again, reagents and primers may be conveniently premixed before addition to the template DNA. The following table gives the volumes of primers and reagents needed for some commonly used numbers of samples.

Number of samples	Primer	Reagent	Total volume
	TAMRA ROX	G T	
4	8 µl	16 µl	24 µl
8	16 µl	32 µl	48 µl
12	24 µl	48 µl	72 µl
16	32 µl	64 µl	96 µl
24	48 µl	96 µl	144 µl

Mix thoroughly after the addition of each reagent by pipetting up and down two or three times. Again, the premixes can be freshly prepared for each set of sequencing reactions or they can be prepared in bulk and stored at 2–8°C. Premixes should be kept on ice until used.

4. When all of the reagents have been prepared; overlay with light mineral oil, or add a wax barrier pellet, or cap the tube with an evaporation-preventing lid as appropriate for your thermocycler. We recommend an overlay with 50 µl of light mineral oil for reactions in standard 96 well microplates and Eppendorf type tubes.

5. Thermocycle the reactions. The exact thermocycling conditions will depend upon the primer set used, the degree of amplification required, the type of template (ssDNA or dsDNA) and the amount of template. The best thermocycling conditions can only be found by experiment. Some recommended starting conditions for the optimization of the thermocycling conditions are given below.

Note that the conditions given are only guidelines in order to start this optimization process.

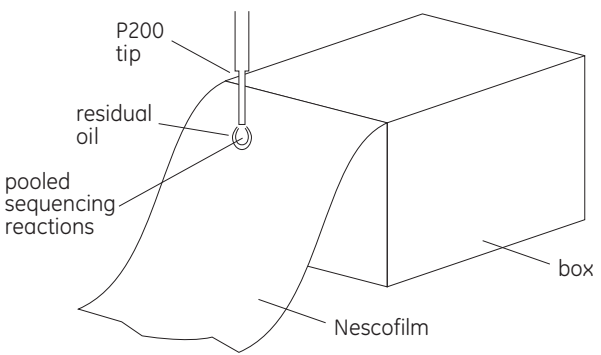
Prime set	Template	Amount	Annealing	Denaturation	Number of cycles
GE Healthcare -					
21M13	plasmid dsDNA	>1 µg	60ºC, 30sec	95ºC, 30sec	20
RPN 2540	plasmid dsDNA	<1 µg	60ºC, 30sec	95ºC, 30sec	25
(100 templates)	M13 ssDNA	>1 µg	60ºC, 30sec	95ºC, 30sec	15
RPN 2542	M13 ssDNA	<1 µg	60ºC, 30sec	95ºC, 30sec	20
(500 templates)					
GE Healthcare					
reverse	plasmid dsDNA	>1 µg	55ºC, 30sec	95ºC, 30sec	20
RPN 2541	plasmid dsDNA	<1 µg	55ºC, 30sec	95ºC, 30sec	25
(100 templates)	M13 ssDNA	>1 µg	55ºC, 30sec	95ºC, 30sec	15
RPN 2543	M13 ssDNA	<1 µg	55ºC, 30sec	95ºC, 30sec	20
(500 templates)					
DYEnamic ET	plasmid dsDNA	>1 µg	50ºC, 30sec	95ºC, 30sec	7-8*
primers	plasmid dsDNA	<1 µg	50ºC, 30sec	95ºC, 30sec	10
-40 M13 forward	M13 ssDNA	>1 µg	50ºC, 30sec	95ºC, 30sec	3-5
US 79339	M13 ssDNA	<1 µg	50ºC, 30sec	95ºC, 30sec	5
(500 templates)					
-28 M13 reverse					
US 79357					
(500 templates)					
ABI T7	plasmid dsDNA	>1 µg	50ºC, 30sec	95ºC, 30sec	25
(#401127)	plasmid dsDNA	<1 µg	50ºC, 30sec	95ºC, 30sec	30
	M13 ssDNA	>1 µg	50ºC, 30sec	95ºC, 30sec	20
	M13 ssDNA	<1 µg	50ºC, 30sec	95ºC, 30sec	25
ABI T3	plasmid dsDNA	>1 µg	55ºC, 30sec	95ºC, 30sec	20
(#401128)	plasmid dsDNA	<1 µg	55ºC, 30sec	95ºC, 30sec	25
	M13 ssDNA	>1 µg	55ºC, 30sec	95ºC, 30sec	15
	M13 ssDNA	<1 µg	55ºC, 30sec	95ºC, 30sec	20
ABI SP6	plasmid dsDNA	>1 µg	55ºC, 30sec	95ºC, 30sec	20
(#401129)	plasmid dsDNA	<1 µg	55ºC, 30sec	95ºC, 30sec	25
	M13 ssDNA	>1 µg	55ºC, 30sec	95ºC, 30sec	15
	M13 ssDNA	<1 µg	55ºC, 30sec	95ºC, 30sec	20

*See Dyenamic™ ET primer protocol book for detailed reaction conditions

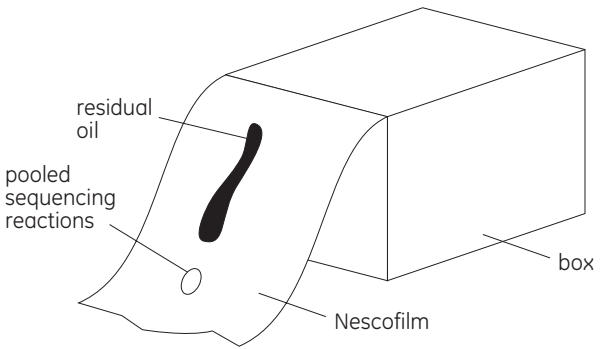
For very GC-rich samples, we recommend a denaturation temperature of 98ºC. In addition, a pre-denaturation for 5 minutes at 95ºC may be beneficial for plasmid cycle sequencing. For full details of related products see pages 30–31.

6. After the thermocycling has been comlpeted, the A, C, G and T reactions should be pooled together. If an oil overlay has been used in order to prevent evaporation during the thermocycling, it is imperative that traces of oil are removed before ethanol precipitation is carried out. Residual oil droplets actually sink in ethanol and are immediately centrifuged to the bottom of the tube. The sequencing reaction may therefore be centrifuged on to this residual oil rather than the plastic surface of the tube. Removal of the ethanol-containing supernatant after centrifugation can easily result in loss of most or all of the sequenced DNA. We find the following technique for removal of residual oil both simple and completely effective.

Pool the sequencing reactions together using a P200 tip. Try to minimize the amount of oil taken but be sure to collect all of the sequencing reactions. Set up a 'slope' of Nescofilm™ or Parafilm™ as illustrated below. Pipette the pooled sequencing reactions and residual oil on to the top of the slope as shown:



The pooled sequencing reactions will run down the slope, leaving a 'trail' of residual oil behind on the Nescofilm or Parafilm.



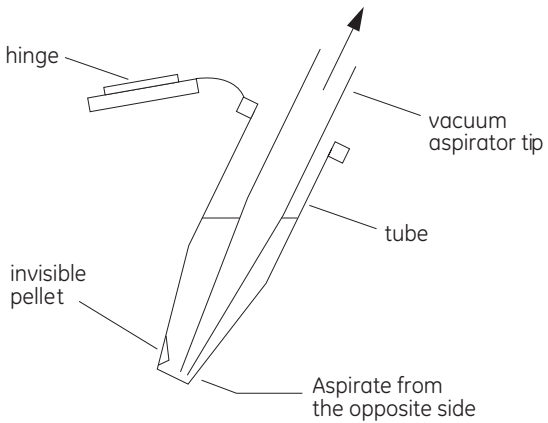
Use a **fresh** P200 tip to transfer the pooled sequencing reactions (now free of any residual oil) for ethanol precipitation.

7. When residual oil has been removed, the pooled sequencing reactions can be ethanol precipitated. We recommend the use of a hinged Eppendorf type tube and the following protocol:

Add~1/10th volume 3 M NaOAc (pH 5.2) (5 µl)
Add 2.5 volumes of ethanol and mix well (132 µl)
Leave for 5 minutes at room temperature.

Spin for 10 minutes in a microcentrifuge with the hinge facing **outwards**.

Remove tube and use a vacuum aspirator plus a fresh P200 tip to remove the supernatant. Place the P200 tip **opposite** the hinge for supernatant aspiration as shown below:



Gently add 500 µl of 80% ethanol with a P1000 tip.

Use a vacuum aspirator plus a fresh P200 tip to remove the supernatant. There is no need to vortex the tube and re-spin (this only increases the chances of detaching the pellet from the tube wall). Again, place the P200 tip **opposite** the hing for supernatant aspiration.

Add 2–5 µl of formamide loading dye (for fluorescent samples) and vortex well or pipette up and down to resuspend. The exact volume of formamide loading dye (for fluorescent samples) used for resuspension will depend on the type of comb used for loading the sequencer.

8. Denature the samples for 2 minutes at 90°C prior to gel electrophoresis.

7.3. Thermo Sequenase cycle sequencing with fluorescent 1 dye primer labelling

1. Remove the following components from the kit and allow to thaw (if stored frozen): A reagent (green cap), C reagent (blue cap), G reagent (yellow cap), T reagent (red cap) and formamide loading dye (for fluorescent samples) (clear cap). Store on ice until required. All of the tubes should be well mixed before use by briefly vortexing the tube.

2. Prepare any other materials that may be required. The following quantities of template are recommended: 0.5–2 µg of single stranded DNA or 0.5–5 µg of double stranded DNA. The DNA should be in a total volume of at least 20 µl. If the template is the product of a polymerase chain reaction (PCR), please refer to the section on sequencing the products of a polymerase chain reaction on page 21 of this booklet.

3. Label four tubes or microplate wells as follows: A reaction, C reaction, G reaction and T reaction. Reactions should be prepared as follows:

	For each sample:
Fluorescent primer (1–2 pmol/µl)	1 µl
Template DNA	5 µl
A, C, G or T reagent	2 µl

Reagents and primers may be conveniently premixed before addition to the template DNA. The following table gives the volumes of primers and reagents needed for some commonly used numbers of samples.

Number of samples	Primer	Reagent A, C, G or T	Total volume
4	4 µl	8 µl	12 µl
8	8 µl	16 µl	24 µl
12	12 µl	24 µl	36 µl
16	16 µl	32 µl	48 µl
24	24 µl	48 µl	72 µl

Mix thoroughly after the addition of each reagent by pipetting up and down two or three times. The premixes can be freshly prepared for each set of sequencing reactions or they can be prepared in bulk and stored at 2-8°C. Premixes should be kept on ice until used.

4. When all of the reactions have been prepared; overlay with light mineral oil, or add a wax barrier pellet, or cap the tube with an evaporation-preventing lid as appropriate for your thermocycler. We recommend an overlay with 50 µl of light mineral oil for reactions in standard 96 well microplates and Eppendorf type tubes.

5. Thermocycle the reactions. The exact thermocycling conditions will depend upon the primer set used, the degree of amplification required, the type of template (ssDNA or dsDNA) and the amount of template. The best thermocycling conditions can only be found by experiment. Some recommended starting conditions for the optimization of the thermocycling conditions are given below. Note that the conditions given are only guidelines in order to start this optimization process.

Primer set	Template	Amount	Annealing	Denaturation	Number of cycles
M13 universal	plasmid dsDNA	>1 µg	60°C, 30sec	95°C, 30sec	20
	plasmid dsDNA	<1 µg	60°C, 30sec	95°C, 30sec	25
	M13 ssDNA	>1 µg	60°C, 30sec	95°C, 30sec	15
	M13 ssDNA	<1 µg	60°C, 30sec	95°C, 30sec	20
M13 reverse	plasmid dsDNA	>1 µg	60°C, 30sec	95°C, 30sec	20
	plasmid dsDNA	<1 µg	60°C, 30sec	95°C, 30sec	25
	M13 ssDNA	>1 µg	60°C, 30sec	95°C, 30sec	15
	M13 ssDNA	<1 µg	60°C, 30sec	95°C, 30sec	20

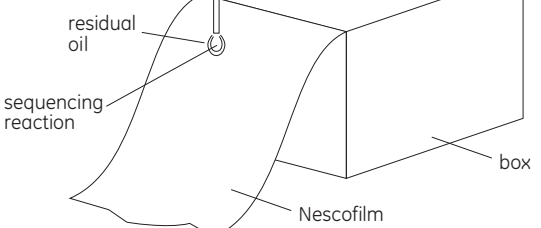
For very GC-rich samples, we recommend a denaturation temperature of 98°C.

In addition, a pre-denaturation for 5 minutes at 95°C may be beneficial for plasmid cycle sequencing.

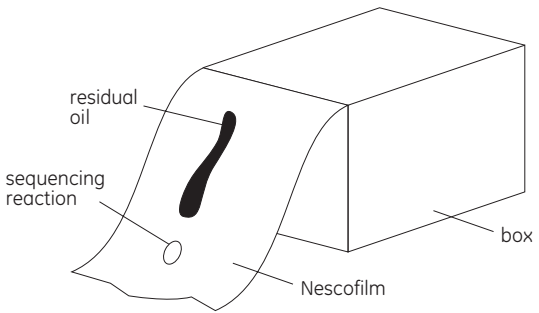
6. After the thermocycling has been completed, the A, C, G and T reactions should each be ethanol precipitated. This result in the best possible gel resolution, however it is possible to omit this step and still obtain acceptable data quality. Users wishing to do this should follow the recommendations of the manufacturer of the particular fluorescent sequencer being used. If an oil overlay has been used in order to prevent evaporation during the thermocycling, it is imperative that traces of oil are removed before ethanol precipitation is carried out. Residual oil droplets actually sink in ethanol and are immediately centrifuged to the bottom of the tube. The sequencing reaction may therefore be centrifuged on to this residual oil rather than the plastic surface of the tube. Removal of the ethanol-containing supernatant after centrifugation can easily result in loss of most or all of the sequenced DNA.

We find the following technique for removal of residual oil both simple and completely effective

Recover each of the sequencing reactions using a fresh P200 tip for each one. Try to minimize the amount of oil taken but be sure to collect all of each sequencing reaction. Set up a 'slope' of Nescofilm or Parafilm as illustrated below. Pipette each of the sequencing reactions and residual oil on to the top of the slope as shown:



The sequencing reactions will run down the slope, leaving a 'trail' of residual oil behind on the Nescofilm or Parafilm.



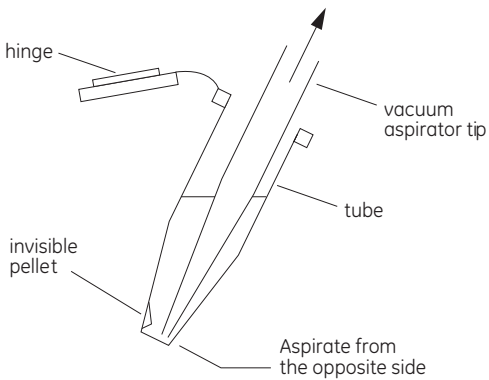
Use a **fresh** P200 tip to transfer the sequencing reactions (now free of any residual oil) for ethanol precipitation.

7. When residual oil has been removed, the sequencing reactions can be ethanol precipitated. We recommend the use of a hinged Eppendorf type tube and the following protocol:

Add ~1/10th volume 3 M NaOAc (pH 5.2) (1 µl)
Add 2.5 volumes of ethanol and mix well (23 µl)
Leave for 5 minutes at room temperature.

Spin for 10 minutes in a microcentrifuge with the hinge facing **outwards**.

Remove tube and use a vacuum aspirator plus a fresh P200 tip to remove the supernatant. Place the P200 tip **opposite** the hinge for supernatant aspiration as shown below:



Gently add 500 µl of 80% ethanol with a P1000 tip.

Use a vacuum aspirator plus a fresh P200 tip to remove the supernatant. There is no need to vortex the tube and re-spin (this only increases the chances of detaching the pellet from the tube wall). Again, place the P200 tip **opposite** the hinge for supernatant aspiration.

Add 2–5 µl of formamide loading dye (for fluorescent samples) and vortex well or pipette up and down to resuspend. The exact volume of formamide loading dye (for fluorescent samples) used for resuspension will depend of the type of comb used for loading the sequencer.

8. Denature the samples for 2 minutes at 90ºC prior to gel electrophoresis.

8. Additional information

8.1. Sequencing the products of a polymerase chain reaction

DNA generated by the polymerase chain reaction (PCR) (4) may also be used as a template for sequencing. The pre-treatment of the PCR product with the GE Healthcare reagent pack for PCR product sequencing (US 70995) is recommended.

It is also possible to successfully sequence unpurified PCR products using a simple dilution protocol. The following section contains guidelines about how to use this approach.

- The dilution method relies on the PCR product being as homogeneous as possible. Care should be taken in the design of the PCR to ensure that truncated and misprimed fragments are minimized. PCR products should be analyzed on an agarose gel to check the homogeneity of the product.
- In general, it is better to use a different primer for the sequencing reaction than was used for the PCR. We recommend that the 5'-end of the sequencing primer is positioned at least 20 bases from the 3'-end of the PCR primer.
- The dilution of the PCR product that will be required will vary according to the size and concentration. We recommend that a series is carried out to find the optimum dilution for each PCR product. In general, we have found that dilutions in the range 1:10 to 1:100 are suitable for most cases.
- If possible, sequence the PCR product with both forward and reverse sequencing primers.

8.2. Supplementary protocols

8.2.1. Preparation of a 6% acrylamide gel mix

Warning: Acrylamide and NN'-methylenebisacrylamide (bis) are neurotoxins. Avoid inhalation and skin contact. Wear gloves when handling these materials and when pouring a gel. Pour the gel in a tray lined with absorbent material. Polymerized acrylamide is generally regarded as being safe but safe handling precautions should nevertheless be observed.

The preparation of a high quality gel mix is important in obtaining the maximum amount of data from the separated sequencing reactions. This is particularly critical when using fluorescent detection.

Premixed acrylamide stocks are available from a number of manufacturers. These are usually supplied as preweighed powders containing a 19:1 acrylamide/bis-acrylamide mixture that can be used to make a 40% solution. Once the 40% solution has been prepared, it should be stored at 2–8°C in the dark. Acrylamide solutions should be stored for no longer than two weeks.

The 6% acrylamide gel mix is prepared as follows. The quantities below produce 500 ml of gel mix. This can be used for up to a week.

1. Weigh out 250 g of urea (electrophoresis grade).

2. Add 75 ml of 40% acrylamide stock solution.

3. Add 187.5 ml of deionized water.

4. Add 3–5 g of mixed bed resin.

5. The urea can be dissolved by gently heating the solution while stirring. Be careful not to overheat the solution - a stream of water from a hot water tap on the outside of the beaker should be sufficient to dissolve the urea.

Warning: Never microwave solutions containing acrylamide.

6. Filter 50 ml of 10 x TBE through a 0.2 µm cellulose acetate filter. Filter the acrylamide solution through the same filter into the same container. Degas for 5 minutes.

7. Store the gel mix at 2–8ºC in the dark.

   The gel plates should be cleaned and assembled using standard procedures. For fluorescent sequencing, follow the recommendations from the manufacturer of the sequencer.

   Immediately before pouring the gel add 1/1000 volume of NNN'N'-tetramethylethylene diamine (TEMED) and 1/200 volume of 10% ammonium persulphate (AMPS). For example, for 80 ml of gel mix, add 80 µl of TEMED and 0.40 ml of AMPS. Solutions of AMPS should always be freshly prepared.

   Mix well and pour the gel. Allow 1–2 hours for polymerization before using the gel.

8.3. Troubleshooting

8.3.1. Sequencing chemistry problems

Problem: Faint or no sequence on all samples.

Possible cause	Action
1. Insufficient template DNA.	**1.** Check an aliquot of the template preparation on an agarose gel to assess the yield. Ensure that at least 0.5–2 µg ssDNA or 0.5–5 µg dsDNA was used for fluorescent sequencing and 0.1–1 µg ssDNA.
2. Missing reaction component.	**2.** Check that all of the premixes were prepared correctly.
3. Inactive enzyme.	**3.** When stored under the recommended conditions Thermo Sequenase DNA polymerase should be stable for greater than 6 months. Check that the enzyme has been correctly stored and that the kit is within the stated expiry date.
4. No priming.	**4.** Check that the primer is suitable for the template to be sequenced.

Problem: High background on sequence.

Possible cause	Action
1. Poor quality DNA preparation.	**1.** Check the DNA preparation on an agarose gel.
2. Plasmid propagated in an *endA1*+ strain.	**2.** Some common *endA1*+ strains such as TG1 can produce plasmids which give a high background on sequencing.

Problem: High background on sequence *Continued***.**

Possible cause	Action
3. Secondary priming sites.	**3.** Check the sequencing primer for homology to other sequences in the vector, particularly within the 8–10 bases at the 3'-end. If there is a problem with secondary binding, the background is usually seen as specific bands rather than generalized background. Try using a different primer.

Problem: Bands smeared.

Possible cause	Action
1. Poor quality gel materials.	**1.** Follow the recommendations in the section on **preparation of a 6% acrylamide gel mix** on page 22 of this booklet.
2. Gel run too hot or too cold.	**2.** Sequencing gels should be run at 50–55°C.
3. Samples not denatured.	**3.** Ensure that samples are heated for at least 2 minutes at 90°C immediately before loading.

Problem: Bands across all four tracks.

Possible cause	Action
1. Poor quality DNA.	**1.** Check the DNA preparation on an agarose gel.
2. Strong secondary structure.	**2.** The enzyme may pause at sites of high secondary structure. If possible, try sequencing the other strand.

Problem: Bands across two or three tracks.

Possible cause	Action
1. Compressions during electrophoresis.	**1.** Some DNA sequences, usually G-C rich do not remain fully denatured during electrophoresis. Including formamide in the gel mix may help eliminate the problem.

Problem: Very short length of read in fluorescent sequencing

Possible cause	Action
1. Plasmid propagated in an *endA1* + strain.	**1.** Some common *endA1*+ strains such as TGI can produce plasmids which give a high background and short read length in fluorescent sequencing.

If problems persist, please contact GE Healthcare's Technical Services for assistance. Please be prepared to give as much information as possible about the exact circumstances under which the problem occurred and always quote the batch number of any reagents that you have used.

8.4. Background and references

8.4.1. Thermo Sequenase

Thermo Sequenase is a new thermostable DNA polymerase specifically engineered for DNA sequencing. GE Healthcare has used a recent discovery of Tabor and Richardson (1) to construct this exonuclease-free thermostable DNA polymerase. Like Sequenase™ T7 DNA polymerase, Thermo Sequenase generates uniform (and therefore easy to read) sequence band patterns. However, the thermostability of this enzyme also makes it suitable for cycle sequencing. Thermo Sequenase therefore combines accuracy comparable with Sequenase T7 DNA polymerase with the sensitivity of cycle sequencing.

8.4.2. Cycle sequencing

DNA sequencing relies on the synthesis of a new strand of DNA starting at a specific priming site and ending with the incorporation of a chain terminating nucleotide such as a dideoxynucleoside triphosphate (2). In fluorescent dye-primer sequencing and radioactive primer sequencing, the label is incorporated on the 5′-end of the primer. The relative concentrations of dNTPs and ddNTPs are balanced so that, on average, at each position within the desired length of sequence, there is an equal chance of a ddNTP being incorporated.

When these fragments are separated on a suitable gel matrix, sequence information can be obtained.

Cycle sequencing (3) uses repeated cycles of thermal denaturation, annealing and extension/termination to increase signal levels and therefore decrease the amount of template required. For each cycle, the amount of product DNA will be equivalent to the amount of primed template. However, if a thermostable enzyme such as Thermo Sequenase DNA polymerase is used, many cycles can be performed without the need to add fresh enzyme.

For example, after ten cycles there could be up to ten times as much product as template (in practice, each cycle is not 100% efficient and the amplification is somewhat lower than this).

Cycle sequencing is therefore ideally suited for applications where the amount of template may be limiting or where the sensitivity of the detection system is not high. For these reasons, we recommend using the Thermo Sequenase fluorescent labelled primer cycle sequencing kit for fluorescent sequencing using dye labelled primers.

1. Tabor, S. and Richardson, C., *Proc. Nat. Acad. Sci. (USA).*

2. Sanger, F., Niklen, S. and Coulson, A.R., *Proc. Nat. Acad. Sci. (USA),* **74**, pp.5463-5467, 1977.

3. Murray, V. *et al., Nucleic Acids Research*, **17**, p.8889, 1989.

4. Saiki, R.K. *et al., Science,* **230**, pp.1350-1354, 1985.

8.5. Related products available from GE Healthcare

8.5.1. Sequencing reagents

Product	Application	Pack size	Product number
Acrylamide, Ultrapure	Gel electrophoresis	1k g 500 g	US 75820-1KG US 75820-500G
Boric acid	Electrophoresis buffer	1 kg	US 76324-1KG
DNA sequencing gel mix, 6%, Ultrapure	Supplied with TBE running buffer. Each bottle makes 125 ml of gel solution	5 bottles	US 72990-5BTL
DNA sequencing gel mix, 8%, Ultrapure	Supplied with TBE running buffer. Each bottle makes 125 ml of gel solution	5 bottles	US 70090-5BTL
N,N'-methylene-bis acrylamide, Ultrapure	Gel electrophoresis	1 kg 500 g	US 75821-1KG US 75821-500G
Urea, Ultrapure	Gel electrophoresis	1 kg 500 g	US 75826-1KG US 75826-500G

8.5.2. Dye-labelled primers

Standard primers for 4 dye labelled primer sequencing

Primer	Pack size	Product code
-21 M13 forward primer 5'-TGTAAAACGACGGCCAGT-3'	100 templates 500 templates	RPN 2540 RPN 2542
M13 reverse primer 5'-CAGGAAACAGCTATGACC-3'	100 templates 500 templates	RPN 2541 RPN 2543

8.5.3. DYEnamic ET primers

DYEnamic ET primers for 4 dye labelled primer sequencing

Primer	Pack size	Product code
-40 M13 forward primer	100 templates (Start up kit)	US 79345
5'-GTTTTCCCAGTCACGACG-3'	500 templates 10 000 templates	US 79339 US 79340
-28 M13 reverse primer 5'-AGGAAACAGCTATGACCAT-3'	500 templates 10 000 templates	US 79355 US 79357

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