GE Healthcare
Life Sciences

illustra™ TempliPhi 100 Amplification Kit TempliPhi 500 Amplification Kit

Product Booklet

Codes: 25-6400-10
25-6400-50

Page finder

1. Legal

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http//www.gelifesciences.com/illustra

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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.

2.2. Storage

The product can be stored for 2 months or less at -20°C.

For storage longer than 2 months, store at -70°C.

Thaw components on ice and maintain at 0–4°C during handling.

DO NOT warm above 4°C prior to amplification.

3. Components of the kits

TempliPhi™100 and TempliPhi 500 contain sufficient reagents to perform 100 and 500 sequencing template amplification reactions respectively.

Kit component	**25-6400-10**	**25-6400-50**	**Storage**
Sample buffer (Red cap)	1 × 0.5 ml	5 × 0.5 ml	-70°C or -20°C
Reaction buffer (blue cap)	1 × 0.5 ml	5 × 0.5 ml	-70°C or -20°C
Enzyme mix (yellow cap)	1 × 20 µl	5 × 20 µl	-70°C
Positive control DNA (pUC19, 2 ng/µl)	1 × 50 µl	1 × 50 µl	-70°C or -20°C

4. Quality control

The control reaction must generate a minimum of 0.5 µg of DNA within 4 hours.

5. Materials not supplied

Reagents

- **RNase A-TEG buffer—**(25 mM Tris-HCl, pH 8, 10 mM EDTA, 50 mM glucose, 100 µg/ml RNase A)
- **Lysis solution—**(0.2 N NaOH, 1% SDS)
- **3 M KOAc—**(294 g KOAc, 114 ml acetic acid; bring to 1 liter with water; store at 4°C).
- **Ethanol—**95%
- **TE buffer—**10 mM Tris, 1 mM EDTA, pH 8.1

Equipment

- **Water bath**
- **Vortex mixer**
- **Microcentrifuge**
- **Thermal cycler**

6. Introduction

TempliPhi DNA amplification kits are novel products developed specifically to prepare templates for DNA sequencing. As illustrated in Figure 1, the TempliPhi method utilizes bacteriophage ø29 DNA polymerase to exponentially amplify single- or double-stranded circular DNA templates by rolling circle amplification (RCA) (1, 2). This isothermal amplification method produces microgram quantities of DNA from picogram amounts of starting material in a few hours. Amplification *in vitro* of very small amounts of template DNA eliminates the need for overnight cell culture and conventional plasmid or M13 DNA purification. The proofreading activity of ø29 DNA polymerase ensures high fidelity DNA replication (3).

The starting material for amplification can be a small amount of bacterial cells containing a plasmid, an isolated plasmid, intact M13 phage, or any circular DNA sample. Bacterial colonies can be picked from agar plates and added directly to the TempliPhi reaction. Alternatively, microliter quantities of a saturated bacterial culture or a glycerol stock can serve as starting material. Depending on the source of starting material, amplification is completed in 4–18 hours at 30°C with no need for thermal cycling. The product of the TempliPhi reaction is high molecular weight, double-stranded concatemers of the circular template. Note that when starting with M13 clones, the TempliPhi product is double-stranded DNA and can be sequenced with forward and reverse primers. DNA amplified by the TempliPhi method can be used directly in cycle sequencing reactions without any purification.

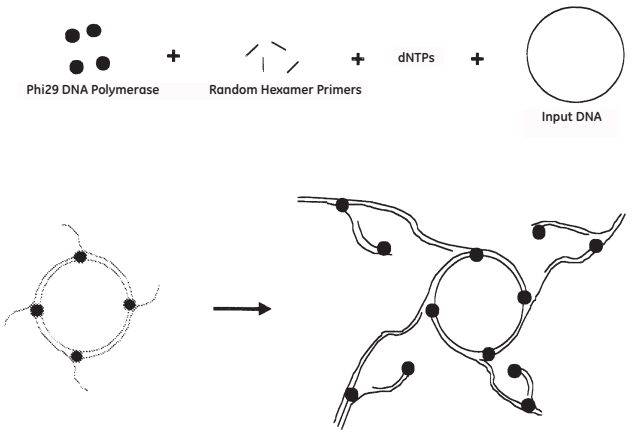

Fig 1. Schematic of the TempliPhi process. Random hexamer primers anneal to the circular template DNA at multiple sites. ø29 DNA polymerase extends each of these primers. When the DNA polymerase reaches a downstream extended primer, strand displacement synthesis occurs. The displaced strand is rendered single-stranded and available to be primed by more hexamer primer. The process continues, resulting in exponential, isothermal amplification

7. Protocol

7.1. Overview

The kit consists of three components–sample buffer, reaction buffer, and enzyme mix. Sample buffer contains random hexamers that prime DNA synthesis nonspecifically and is used to resuspend bacterial cells from a colony or cell culture or any other input DNA. If purified DNA is the starting material, a small volume of this DNA is added to sample buffer. The reaction buffer contains salts and deoxynucleotides, and is adjusted to a pH that supports DNA synthesis. The enzyme mix contains ø29 DNA polymerase and random hexamers in 50% glycerol.

Briefly, a maximum of 1 µl of the template to be amplified is added to 5 µl of sample buffer. The sample is heated to 95°C for three minutes to gently lyse the bacteria and release the plasmid DNA. The sample is cooled and combined with 5 µl of reaction buffer and 0.2 µl of enzyme mix and incubated at 30°C for 4–18 hours. At the end of this incubation, the DNA polymerase is inactivated by heating at 65°C for 10 minutes. The tube should now contain approximately 1–1.5 µg of amplified DNA that can be used directly in a DNA sequencing reaction.

When selecting amplification products for sequencing experiments, please note the following. In the absence of input DNA, there will most likely be an amplification product that will not function as a template in a sequencing reaction. This issue is discussed in detail in the troubleshooting section under the heading, “No sequencing result.”

7.2. TempliPhi DNA amplification: plasmids and M13

The steps outlined below describe a general protocol for amplifying sequencing templates. This protocol is a starting point for optimizing the reaction in your laboratory. Reaction parameters that can be manipulated for a particular type of template and laboratory workflow are discussed, as are the limitations of the method. The TempliPhi method can efficiently amplify small amounts of plasmid DNA, but is very sensitive to inhibitors. The most effective troubleshooting step is to use less input sample by reducing input volume or diluting the sample.

Thaw TempliPhi kit components (sample buffer [red cap], reaction buffer [blue cap]) on ice or at room temperature and place on ice once thawed.

1. Transfer 5 µl aliquots of sample buffer to appropriate reaction tubes or a microwell plate.
2. Transfer samples to the dispensed sample buffer. Depending on the source of the starting material, follow the recommendations below.

For liquid bacterial culture, transfer between 0.2–0.5 µl of saturated overnight culture directly to the dispensed sample buffer. Transfer only a small amount of the culture. Please refer to the note regarding liquid culture below.

For bacterial colonies, transfer a small portion of a colony directly to the dispensed sample buffer. A gentle touch of a straight needle on the colony surface provides sufficient starting material. Avoid transferring agar from the plate or excess cell material into the TempliPhi reaction. Transfer only a small portion of a colony. Please refer to the note regarding colonies and plaques on page 12.

For M13 phage in liquid culture, transfer 0.2–0.5 µl of phage supernatant directly into the TempliPhi sample buffer.

For M13 from a plaque, transfer a minute portion of the plaque using a straight needle to the dispensed sample buffer. A gentle touch of the plaque with a straight needle provides sufficient starting material. Transferring the entire plaque directly to the sample buffer might inhibit amplification reaction kinetics due to carry-over of excess cellular debris and/or soft agar.

For bacterial glycerol stocks, first dilute 1 µl of the glycerol stock into 50 µl of TE or water. Transfer 0.2–0.5 µl of the diluted glycerol stock to the dispensed TempliPhi sample buffer.

For purified plasmid or M13 DNA, transfer 1 pg–10 ng of DNA (volume < 0.5 µl) into the dispensed sample buffer.

Notes for liquid cultures and glycerol stocks: The TempliPhi method is very sensitive to inhibitors–it is crucial to add the smallest possible volume of culture or supernatant to the TempliPhi reaction. Components of some culture media can inhibit the TempliPhi reaction. Amplification is faster and more reproducible when template DNA is prepared from cultures that are not grown in extremely rich media; LB media supplemented with the appropriate antibiotic is recommended. If the source culture was grown to high cell densities in a rich medium, dilute the culture 10- to 100-fold in water or TE and transfer 1 µl of this diluted material. Dilution ensures complete amplification in the shortest time.

Notes for colonies or plaques: Attempt to transfer 1/10 to 1/100 ofthe colony (approximately 10^2–10^4 cells). It is crucial not to transfer too much material to the TempliPhi reaction. Use of more than 10^5 cells will decrease the TempliPhi reaction rate and produce incomplete reactions after reasonable incubation times. Alternately, transfer cell material to a tube containing 50 µl of TE or water, vortex, and transfer 0.2–0.5 µl of the suspension to the dispensed TempliPhi sample buffer. This dilution step ensures complete amplification in the shortest time.

3. Denature the sample. After the sample is added to sample buffer, seal the reaction tubes with an appropriate lid. Heat at 95°C for 3 minutes, and then cool to room temperature or 4°C. The rate of cooling is not critical.

Note: This step lyses bacterial cells or phage particles sufficiently to release the circular template into the liquid. Avoid heating at higher temperatures or for longer times, which can release bacterial chromosomal DNA that competes with the desired template during amplification.

4. Prepare TempliPhi premix. In a separate tube, combine 5 µl of reaction buffer and 0.2 µl enzyme mix for each TempliPhi reaction. It is convenient to make a master mix sufficient for there quired number of TempliPhi reactions just prior to use. Once made, the master mix must be used the same day and not stored for future use.

This premix contains all components necessary to generate non-specific amplification product, and must be kept on ice until ready for use.

5. Transfer 5 µl of the TempliPhi premix to the cooled, denatured sample prepared in step 3.

6. Incubate at 30°C for 4–18 hours.

Note: If minimal amounts of inhibitory material were transferred along with the template, the reaction will typically be complete after a 4 hour incubation and produce 1.25–1.75 µg of DNA. If amplification is incomplete after 4 hours (< 1.25 µg of DNA produced) or if the yield is not consistent from sample to sample, there are two solutions.

- Increase the incubation period from 4 hours to overnight (typically 18 hours). This allows sufficient time for the inhibited reaction to consume all the deoxynucleotides and produce the maximum amount of DNA.

- Transfer a smaller amount of starting material (and thus inhibitory components) into the amplification reaction.

7. Heat-inactivate the enzyme by incubating at 65°C for 10 minutes. Cool to 4°C.

Note: Inactivating the ø29 DNA polymerase activity prevents potential interference with subsequent cycle sequencing reactions. Inactivating the proofreading exonuclease activity prevents degradation of template DNA during storage. The heat inactivation step can be eliminated if the amplified DNA sample will not be stored.

8. Perform cycle sequencing. An aliquot of the amplified DNA can be added directly into the cycle sequencing reaction without further purification.

Option A: Transfer 1–2 µl (150–500 ng) of amplified product to a 20 µl cycle sequencing reaction.

Option B: Because the amplified product can be viscous, a dilution step is recommended prior to transfer. Dilute the amplified product with 40 µl of water or TE, and mix well by pipetting up and down three times. After mixing, use 2–10 µl of diluted sample per 20 µl sequencing reaction.

Notes for cycle sequencing: This TempliPhi protocol typically generates 1.25–1.75 µg of DNA in a volume of 10 µl. The buffer system employed in the TempliPhi product is compatible with DYEnamic™ ET Dye Terminator Sequencing Kits and other sequencing methods. Unused amplified template can be stored at -20°C for at least one month with no loss of performance in cycle sequencing. TempliPhi-amplified DNA does not alter or adversely affect most popular post-sequencing reaction cleanup methods. Researchers may need to optimize the cleanup and sequencing reactions if they deviate from recommended sequencing protocols.

7.3. Control DNA amplification

The control DNA consists of 50 µl of pUC19 (2 ng/µl).

1. Transfer 0.5 µl (1 ng) of control DNA to a reaction tube containing 5 µl of sample buffer. Perform protocol steps 3 through 7. The control reaction should generate > 0.5 µg of product within 4 hours.

7.4. TempliPhi DNA amplification: single- or low-copy number BACs and fosmids

Bacterial artificial chromosomes (BACs) are cloning vectors based on the F factor plasmid of *Escherichia coli*. They can maintain cloned DNA fragments up to 300 kb. Sequencing BAC inserts, and in particular, the ends of BAC inserts, represents a key step in large-scale chromosomal sequencing projects.

The preparation and sequencing of BAC DNA can be challenging; clones constructed from cosmid and fosmid vectors have similar difficulties. Success rates depend quite heavily on the purity and quantity of DNA in the reactions. Much effort has been devoted to develop reliable methods to isolate and purify BAC recombinants.

Unlike high-copy number plasmids and M13, amplification of BAC or fosmid constructs directly from colonies and cultures is relatively inefficient because the cell lysis sufficient to release the BAC also releases chromosomal DNA. Additionally, BAC and fosmid-based constructs are only present as a single copy in the host cell. Below, we describe a modified TempliPhi protocol to prepare large circular constructs for sequencing. The method is efficient, streamlined, and generates DNA that matches the sequencing success rates of the best available BAC DNA preparation methods.

The amplification reaction requires a minimum of 10 ng of purified BAC DNA. Partially purified BAC DNA is readily obtained from an overnight 1.5 ml culture, followed by a standard mini-alkaline lysis

protocol (below) or similar procedures commonly applied. Two additional methods for liberating sufficient BAC DNA from single colonies are provided in Protocol 4.

Mini-alkaline lysis method for BAC purification from a 1.5 ml culture

1. Transfer 1 ml of an overnight BAC culture to a 1.5 ml microcentrifuge tube. Centrifuge for 1 minute at maximum speed in a microcentrifuge to pellet the bacterial cells. Discard the supernatant.
2. Resuspend the bacterial pellet in 200 µl of RNase A-TEG at room temperature. Vortex to mix well.

Note: Addition of RNase A to TEG buffer is essential to degrade bacterial RNA.

3. Add 200 µl of freshly prepared lysis solution. Mix gently by inversion (4–5 times) and incubate at room temperature for no more than 5 minutes. Do not vortex.
4. Add 200 µl of ice cold 3 M KOAc. Invert and mix 4–5 times. Place on ice for 10 minutes.
5. Centrifuge at maximum speed for 10 minutes to clear the lysate. Discard the pellet. If necessary, perform an additional centrifugation step to clarify the supernatant. Transfer the supernatant to a fresh tube.
6. Add 1 ml of 95% ethanol, and invert to mix. Centrifuge the tube in a 4°C microcentrifuge for 10 minutes. Remove the supernatant.
7. Wash the DNA pellet with 1 ml of 70% ethanol. Centrifuge at 15 000 x *g* (or maximum speed) for 5 minutes in a microcentrifuge. Discard the supernatant.
8. Air-dry the pellet at room temperature for 5–10 minutes. Gently dissolve the pellet in 50 µl of TE. Incubate the solution for at least 10 minutes at room temperature and mix the contents by gently

tapping the tube with a finger. Quantify the DNA concentrationby UV absorption at 260 nm and prepare a working stock of 10 ng/µl for use in TempliPhi reactions.

TempliPhi amplification protocol for BAC and other large constructs

The partially purified BAC DNA generated by the mini alkaline lysis method is ready for addition to TempliPhi sample buffer and amplification according to the procedure below. However, the procedure toamplify BAC templates differs significantly from protocols to amplify plasmid DNA or M13:

- To generate larger amounts of DNA, each reaction uses ten-fold greater quantities of TempliPhi reagents.
- An 18 hour incubation (instead of 4 hours) is required to achieve complete amplification.
- Amplified BAC DNA must be precipitated by the addition of ethanol or isopropanol and dissolved in water or TE prior to cycle sequencing. This essential step prevents inhibition of the sequencing reaction by TempliPhi components present when large amounts of DNA (2–5 µg) are required in the sequencing reactions.

The final yield of amplified DNA from this modified protocol is typically 10–15 µg per reaction tube.

Note: Scaled-down versions of this reaction are hypothetically feasible but must be optmized by the researcher.

9. Transfer 1 µl (10 ng/µl) of partially purified BAC DNA from step 8, page 16 to a tube containing 50 µl of sample buffer.

10. Heat-denature the sample at 95°C for 3 minutes, and then incubate at 4°C until ready to use.

The heating and cooling steps promote the association of random hexamers (from the sample buffer) to the denatured BAC DNA, thus forming replication forks.

11. Prepare the TempliPhi premix. For each BAC amplification, combine 50 µl of reaction buffer with 2 µl of enzyme mix in a tube set on ice. The TempliPhi premix should be kept on ice until ready for use.

It is convenient to prepare a master mix by combining sufficient reagents for the required number of amplification reactions. Any unused premix must be discarded, not stored for reuse.

12. Transfer 50 µl of prepared TempliPhi premix to the denatured sample from step 10, page 17.

13. Incubate the reaction tubes at 30°C for 18 hours.

Note: A minimum incubation period of 18 hours is essential to ensure complete DNA amplification for BAC and other large constructs.

14. Incubate the tubes at 65°C for 10 minutes to inactivate the ø29 DNA polymerase. Store the reaction tubes at 4°C or -20°C until ready for use.

15. Precipitate the BAC amplified products

Note: 10–15 µg of amplified DNA is typically produced by TempliPhi amplification using Protocol 3. It is essential to precipitate amplified BAC DNA to ensure the removal of salt, excess dNTPs, and random hexamers present in this scaled-up TempliPhi reaction. Precipitation also concentrates the amplified products, a critical step when sequencing large constructs, where sequencing reactions require greater quantities (2–5 µg) of input DNA.

15.1. Add a volume of isopropanol equal to the completed TempliPhi reactions (1:1 ratio).

15.2. Invert several times. The DNA pellet should be very clearly visible.

15.3. Pellet the DNA by centrifugation in a microcentrifuge at 15 000 x *g* for 5–10 minutes.

15.4. Carefully remove the supernatant. Wash the pellet with 500 µl of 70% ethanol. Vortex thoroughly.

15.5. Centrifuge at 15 000 x *g* for 2 minutes. Remove as much supernatant as possible.

15.6. Air-dry the pellet for 5–10 minutes. Do not overdry, or the pellet will be difficult to resuspend. Check pellets frequently to avoid overdrying.

15.7. Resuspend the pellet in TE in 1/4 of the original amplification reaction volume (e.g. if your reaction volume was 100 µl, resuspend precipitated DNA in 25 µl).

16. Utilize 2–5 µg of the amplified material in a sequencing reaction based on protocols specific for BAC and other large constructs. Guidelines for sequencing BAC and other large constructs are provided in a separate application note; please contact your GE Healthcare representative for more information.

Note: Sequencing BAC and other large constructs is not fool proof. Most researchers report success rates of 30–40%, even when ultra-purified BAC DNA is utilized. If your sequencing results are unsatisfactory, consider the following modifications to the cycle sequencing reaction:

- Increase the quantity of template in the sequencing reaction (typically 1–5 µg)
- Increase the quantity of sequencing primer
- Double the volume of the sequencing reaction to 40 µl, utilizing 16 µl of sequencing reagent premix
- Increase the number of amplification cycles
- Increase the duration of the polymerase extension step, and perform a rigorous post-sequencing terminator removal step.

7.5. Amplification of BAC DNA directly from an individual colony

Option A: Heat-lysis method

Amplification directly from colonies for BACs and other large constructs is inefficient for various reasons stated above. However, the protocol shown below has been tested with varying success rates. The user should optimize a small sample set before commencing large-scale studies.

1.(a) Pick an entire single colony of BAC-transformed cells and place into a reaction tube containing 20 µl of deionized water. Vortex to resuspend the cells.

Note: The whole colony must be picked in order to liberate sufficient starting material.

2.(a) Heat at 95°C for 3 minutes.

3.(a) Freeze the cell suspension at -20°C or colder for 2–3 hours.

4.(a) Centrifuge the frozen cell suspension at 4°C in a microcentrifuge at 15 000 x *g* for 15 minutes.

Note: This step preferentially precipitates any released chromosomal DNA while retaining BAC DNA in solution. This step also pellets cell debris.

5.(a) Transfer the BAC-containing supernatant to a new tube and vacuum-dry the entire sample, taking care not to overdry.

Note: Use the entire dried sample in subsequent TempliPhi amplification reactions.

6.(a) Add 50 µl of sample buffer to the dried DNA and proceed to Protocol 3, step 10, page 17.

Option B: Mini-alkaline lysis method

As with the heat-lysis method, the entire amount of BAC DNA recovered from a single colony must be utilized in a TempliPhi amplification reaction. Reagents supplied by commercial vendors

such as Qiagenare appropriate for this mini-alkaline lysis method. Alternatively, researchers may prepare their own reagents.

1.(b) Pick an entire BAC-containing colony and place into a tube containing 50 µl of RNase-TEG. Resuspend cells by mixing thoroughly. Vortex if necessary.

2.(b) Add 50 µl of lysis solution. Mix gently by inverting the tubes 5–6 times.

Note: Do not vortex. This step ensures the release of cellular contents. Leave at room temperature for 5 minutes. Cell lysis timeis critical—do not exceed 5 minutes.

3.(b) Add 50 µl of ice-cold 3 M KOAc and mix gently by inverting the tubes 5–6 times (do not vortex). Incubate on ice for 10 minutes to precipitate chrosomosomal DNA and cellular debris.

4.(b) In a microfuge, centrifuge at maximum speed for 30 minutes. Transfer the supernatant (containing the BAC DNA) to a clean tube, and discard the pellet.

5.(b) Centrifuge the supernatant a second time to remove residual particulates. Transfer the cleared supernatant to a new tube.

6.(b) Add 0.6 volumes (90 µl) of room temperature isopropanol to the cleared supernatant and mix by gentle inversion. Do not vortex.

7.(b) Recover the BAC-containing DNA pellet by centrifugation at 15 000 x g for 10 minutes. Centrifugation can be performed at 4°C or room temperature.

8.(b) Wash the pellet with 200 µl of 70% ethanol by centrifugation for 15 minutes at 4°C. Discard the supernatant and air-dry the pellet.

9.(b) Dissolve the DNA in 50 µl of TempliPhi sample buffer. Use this entire volume in an amplification reaction.

10.(b) Proceed to Protocol 3, step 10, page 17.

8. Troubleshooting

Problem	Possible causes/solutions
1. No amplification It is unusual to achieve no amplification. Confirm the success of the amplification reaction by analyzing a small amount of the sample (1 µl) on a low percentage (0.6%) agarose gel.	**1.** *Too much culture or starting material was added to the amplification reaction:* The best troubleshooting advice is to use less input tem-plate material—the TempliPhi method requires small amounts of input material. Components of some culture media can inhibit the TempliPhi reaction. Depending on the host cell and the type of culture medium, bacterial-spent media can also inhibit the TempliPhi reaction. Ideally, the volume of culture or supernatant added to the TempliPhi reaction should be < 1 µl to minimize carry-over of potential inhibitors. If you have difficulty transferring small volumes, dilute a portion of the liquid culture in TE buffer or water and add up to 1 µl of the diluted material. Avoid adding more than 1 µl of media. An excellent optimization step is the use of a dilution series of starting material. Add 1 µl of two-fold serially diluted material and monitor amplification by agarose gel electrophoresis at the most convenient incubation time for your workflow. Perform the control reaction.

Problem	Possible causes/solutions
	2. *Inefficient denaturation*: Although full lysis of the cells in Step 3. Page 13, is not necessary, denaturation must be sufficient to allow release of plasmid as well as primer annealing. Denature at 95°C for only 3 minutes. Longer denaturing times are not recommended because the template might become nicked, decreasing the efficiency of rolling circle amplification.
	3. *Enzyme inactivated:* It is critical to store properly the ø29 DNA polymerase. The ideal storage temperature is -70°C. If the components will be consumed within 2 months, -20°C storage may be used. A frost-free freezer must not be used. Perform the control reaction to confirm performance of the premix.
	4. *Insufficient DNA:* Research at GE Healthcare demonstrates robust amplification with as little as 1 picogram of plasmid DNA as the starting material. Perform a broad titration of your starting material to determine the range that produces optimal amplification.
	5. *DNA not circular:* The kinetics of the TempliPhi reaction strongly favor circular templates. Templates that have been digested with restriction enzymes or that are severely nicked will be poor substrates for TempliPhi. Start with circular templates.

Problem	Possible causes/solutions
2. No sequencing result When selecting amplification products for sequencing experiments, please note the following: • In the absence of input DNA, there *will most likely be an amplification product* that will not function as a sequencing template. • Like any other highly sensitive, exponential amplification strategy, minute quantities of contaminant DNA can serve as an efficient amplification substrate, but will not perform in the sequencing reaction.	**1.** *No amplification:* Confirm that a product has been generated by analyzing a small sample (1 µl) on a low percentage (0.6%) agarose gel. If no product is observed after staining with a double-stranded DNA-sensitive dye such as ethidium bromide, refer to the troubleshooting discussion above, "Problem: No amplification." **2.** *Nonspecific amplification, no input DNA:* The product of the TempliPhi reaction is double-stranded. It can be digested with a restriction enzyme appropriate to your sample to yield a characteristic banding pattern on an agarose gel. If you do not observe the expected pattern, nonspecific amplification caused by insufficient amounts of template DNA or insufficient denaturation could be the problem. Titrate your sample to determine the quantity that produces optimal amplification of your template. Confirm that denaturation occurred at 95°C for 3 minutes. **3.** *Nonspecific amplification, contaminating input DNA:* The TempliPhi reaction is very sensitive; minute amounts of any input circular DNA will be efficiently amplified. It is therefore important to use clean laboratory materials.

Problem	Possible causes/solutions
	4. *Poor sequencing results:* A common explanation for a failed sequencing reaction is excessive volume reduction and sequencing premix dilution. TempliPhi-amplified products have been tested under a wide range of conditions and found to be compatible with GE Healthcare and other sequencing chemistries, without the need for post-amplification purification/cleanup. However, excessive dilution of the sequencing premix could unbalance the essential components in the sequencing reaction. Avoid dilution of the sequencing premix. Poor performance attributed to sequencing premix dilution will not be supported GE Healthcare.
	5. *BAC amplification product did not generate a satisfactory sequencing result:* Typical success rates are 30–40%, whether or not templates are produced by TempliPhi. Even this degree of success requires numerous precautions by the researcher. Sequencing success with BAC and other large constructs, even when using purified DNA prepared by conventional means, is affected by many factors. Construct size, quantities of sequencing primer and template, number of cycles performed, amount of premix and volume of the sequencing

Problem	Possible causes/solutions
	5. *Continued.* reaction are some of the factors that play a vital role. Additionally, the complexity of the region of the genome being sequenced can affect the results. Follow the guidelines provided in the GE Healthcare application notes for sequencing pure and TempliPhi-amplified BAC DNA. If an amplified BAC does not generate satisfactory sequencing data using the recommended protocol, it is probably due the inherent nature of the construct being examined. Such a construct is probably not amenable to sequencing even when the DNA is purified by conventional methods. Answers to other frequently asked questions and protocol updates can be obtained by visiting the GE Healthcare Home Page followed by searching using keyword “TempliPhi.”

9. References

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