

Supporting Data

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Product name: MCRCA DNA Amplification Kit

Cat #: PPK-100, PPK-200

Description:

MCRCA DNA amplification kits are novel products developed specifically to prepare templates for DNA sequencing. As illustrated in Figure, the MCRCA method utilizes bacteriophage Phi29 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 Phi29 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 MCRCA 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 MCRCA reaction is high molecular weight, double-stranded concatemers of the circular template.

Note that when starting with M13 clones, the MCRCA product is double-stranded DNA and can be sequenced with forward and reverse primers. DNA amplified by the MCRCA method can be used directly in cycle sequencing reactions without any purification.

Figure:

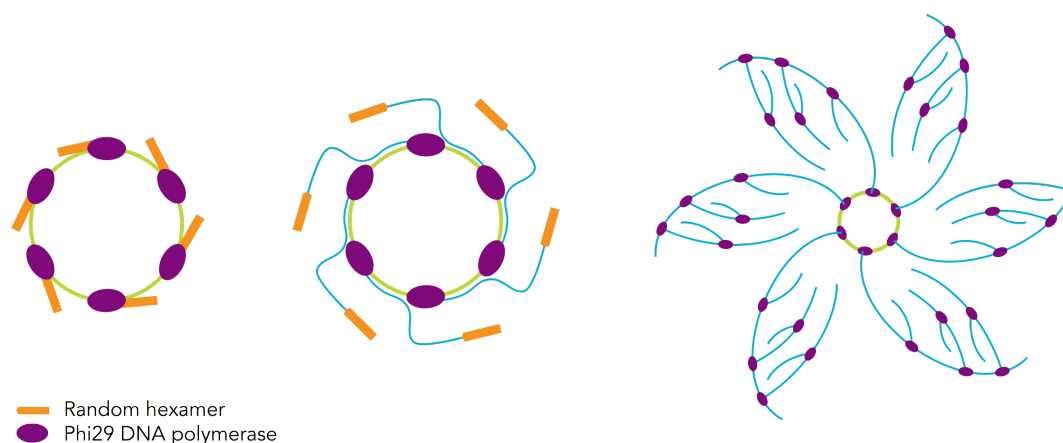


Figure 1, Schematic of the MCRCA process. Random hexamer primers anneal to the circular template DNA at

multiple sites. Phi29 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.

Protocols:

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 Phi29 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 min. 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.”

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 MCRCA 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 MCRCA kit components on ice or at room temperature and place on ice once thawed, transfer 5 µl aliquots of sample buffer to appropriate reaction tubes or a microwell plate.

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 MCRCA reaction. Transfer only a small

portion of a colony.

For M13 phage in liquid culture, transfer 0.2–0.5 µl of phage supernatant directly into the MCRCA 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 MCRCA 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 MCRCA method is very sensitive to inhibitors—it is crucial to add the smallest possible volume of culture or supernatant to the MCRCA reaction. Components of some culture media can inhibit the MCRCA 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 of the colony (approximately 102–104 cells). It is crucial not to transfer too much material to the MCRCA reaction. Use of more than 105 cells will decrease the MCRCA 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 MCRCA sample buffer. This dilution step ensures complete amplification in the shortest time.

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 min, 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.

Prepare MCRCA premix. In a separate tube, combine 5 µl of reaction buffer and 0.2 µl enzyme mix for each MCRCA reaction.

It is convenient to make a master mix sufficient for the required number of MCRCA 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 nonspecific amplification product, and must be

kept on ice until ready for use.

Transfer 5 µl of the MCRCA premix to the cooled, denatured sample prepared in previous step.

Incubate at 30 °C for 4–18 h.

Note: If minimal amounts of inhibitory material were transferred along with the template, the reaction will typically be complete after 4 h incubation and produce 1.25–1.75 µg of DNA.

If amplification is incomplete after 4 h (< 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 h to overnight (typically 18 h). 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.

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

Note: Inactivating the Phi29 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.

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

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

Option 2: 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 MCRCA protocol typically generates 1.25–1.75 µg of DNA in a volume of 10 µl. Unused amplified template can be stored at -20 °C for at least one month with no loss of performance in cycle sequencing. MCRCA-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.

Control DNA amplification

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

Transfer 0.5 µl (1 ng) of control DNA to a reaction tube containing 5 µl of sample buffer. Perform amplification protocol steps.

The control reaction should generate > 0.5 µg of product within 4 h.

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 MCRCA 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 Amplification of BAC DNA directly from an individual colony

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

Transfer 1 ml of an overnight BAC culture to a 1.5 ml microcentrifuge tube. Centrifuge for 1 min at maximum speed in a microcentrifuge to pellet the bacterial cells. Discard the supernatant.

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.

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 min. Do not vortex.

Add 200 µl of ice cold 3 M KOAc. Invert and mix 4–5 times. Place on ice for 10 min.

Centrifuge at maximum speed for 10 min 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.

Add 1 ml of 95% ethanol, and invert to mix. Centrifuge the tube in a 4 °C microcentrifuge for 10 min. Remove the supernatant.

Wash the DNA pellet with 1 ml of 70% ethanol. Centrifuge at 15 000.g (or maximum speed) for 5 min in a microcentrifuge. Discard the supernatant.

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 min at room temperature and mix the contents by gently tapping the tube with a finger.

Quantify the DNA concentration by UV absorption at 260 nm and prepare a working stock of 10 ng/μl for use in MCRCA reactions.

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 MCRCA sample buffer and amplification according to the procedure below. However, the procedure to amplify 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 MCRCA reagents.
- An 18 h incubation (instead of 4 h) 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 MCRCA 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 optimized by the researcher.

Transfer 1 μl (10 ng/μl) of partially purified BAC DNA from previous step to a tube containing 50 μl of sample buffer.

Heat-denature the sample at 95 °C for 3 min, 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.

Prepare the MCRCA premix. For each BAC amplification, combine 50 μl of reaction buffer with 2 μl of enzyme mix in a tube set on ice. The MCRCA 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.

Transfer 50 μl of prepared MCRCA premix to the denatured sample from Heat-denature step.

Incubate the reaction tubes at 30 °C for 18 h.

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

Incubate the tubes at 65 °C for 10 min to inactivate the Phi29 DNA polymerase. Store the reaction tubes at 4 °C or -20 °C until ready for use.

Precipitate the BAC amplified products

Note: 10–15 μg of amplified DNA is typically produced by MCRCA 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 MCRCA 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.

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

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

Pellet the DNA by centrifugation in a microcentrifuge at 15 000•g for 5–10 min.

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

Centrifuge at 15 000 •g for 2 min. Remove as much supernatant as possible.

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

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).

Utilize 2–5 µg of the amplified material in a sequencing reaction based on protocols specific for BAC and other large constructs.

Amplification of BAC DNA directly from an individual colony

Option 1: 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.

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.

Heat at 95 °C for 3 min.

Freeze the cell suspension at -20 °C or colder for 2–3 h.

Centrifuge the frozen cell suspension at 4 °C in a microcentrifuge at 15 000 •g for 15 min.

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

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 MCRCA amplification reactions.

Add 50 µl of sample buffer to the dried DNA.

Option 2: 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 MCRCA amplification reaction. Reagents supplied by commercial vendors are appropriate for this mini-alkaline lysis method. Alternatively, researchers may prepare their own reagents.

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.

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 min. Cell lysis time is critical—do not exceed 5 min.

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 min to precipitate chromosomal DNA and cellular debris.

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

Centrifuge the supernatant a second time to remove residual particulates. Transfer the cleared supernatant to a new tube.

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

Recover the BAC-containing DNA pellet by centrifugation at 15 000 g for 10 min. Centrifugation can be performed at 4 °C or room temperature.

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

Dissolve the DNA in 50 µl of MCRCA sample buffer. Use this entire volume in an amplification reaction.

Troubleshooting:

Problem: 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.

Possible causes/solutions

1. Too much culture or starting material was added to the amplification reaction: The best troubleshooting advice is to use less input template material—the MCRCA method requires small amounts of input material.

Components of some culture media can inhibit the MCRCA reaction. Depending on the host cell and the type of culture medium, bacterial-spent media can also inhibit the MCRCA reaction. Ideally, the volume of culture or supernatant added to the MCRCA 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.

2. Inefficient denaturation: Although full lysis of the cells is not necessary, denaturation must be sufficient to allow release of plasmid as well as primer annealing. Denature at 95 °C for only 3 min. 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 Phi29 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 MCLAB demonstrates robust amplification with as little as 1 picogram of plas-

mid 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 MCRCA reaction strongly favors circular templates. Templates that have been digested with restriction enzymes or that are severely nicked will be poor substrates for MCRCA. Start with circular templates.

Problem: 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.

Possible causes/solutions

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 MCRCA 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 min.

3. Nonspecific amplification, contaminating input DNA: The MCRCA reaction is very sensitive; minute amounts of any input circular DNA will be efficiently amplified. It is therefore important to use clean laboratory materials.

4. Poor sequencing results: A common explanation for a failed sequencing reaction is excessive volume reduction and sequencing premix dilution. MCRCA-amplified products have been tested under a wide range of conditions and found to be compatible with MCLAB 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 MCLAB.

5. BAC amplification product did not generate a satisfactory sequencing result: Typical success rates are 30–40%, whether or not templates are produced by MCRCA. 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 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 MCLAB application notes for sequencing pure and MCRCA-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.

References:

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2. Lizardi, P. et al., *Nat. Genet.* 19, 225–232 (1998).
3. Estaban, J. A. et al., *J. Biol. Chem.* 268, 2719–2726 (1993).