AGENCOURT® COSMCPREP®

HIGH AND LOW COPY PLASMID PURIFICATION

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Agencourt CosMCPrep: Table of Contents

Introduction

The Agencourt CosMCPrep 96-well plasmid purification system utilizes Agencourt's patented SPRI® paramagnetic bead technology for high-throughput preparation of high-copy or low-copy plasmid DNA from *E.coli* cells. Agencourt CosMCPrep can also be used with fosmid and BAC vector-based constructs ranging from 5kb to 150kb. The system uses alkaline lysis followed by a SPRI purification to differentially bind plasmid DNA to paramagnetic beads. While the DNA is bound to the beads, contaminants can be rinsed away using a simple washing procedure. Because Agencourt CosMCPrep utilizes magnetic separation technology, the protocol does not require vacuum filtration. This makes Agencourt CosMCPrep extremely amenable to automation. Plasmid DNA purified with this system can be used in a variety of molecular biology applications, such as:

- Fluorescent DNA sequencing (including capillary electrophoresis)
- PCR[1] amplification
- Transformation
- Restriction enzyme digestion

[1] *The PCR process is covered by patents owned by Roche Molecular Systems, Inc., and F. Hoffman-La Roche, Ltd.*

- In Vitro transposon insertions

When used with high-copy plasmids, Agencourt CosMCPrep yields approximately 500 ng – 2 µg of DNA per purification. For low-copy plasmids and larger constructs, an average yield of approximately 300 ng - 1 µg is typical. These yields vary slightly depending on the cell line, vector type, and size of the construct.

Agencourt CosMCPrep has an additional recovery benefit when working with high molecular weight (HMW) DNA. Since SPRI utilizes a negatively charged bead, HMW DNA can be effectively and rapidly eluted in aqueous solutions. Traditional methods for isolation utilize solid-phase supports that generally hinder elution of DNA from the support. In fact, most anion-exchange systems use positively charged supports which require large amounts of salt to facilitate the release of HMW DNA. Elution in aqueous solutions makes SPRI purified DNA a good choice for enzymatic reactions.

Automation:

The Agencourt CosMCPrep plasmid purification system was designed for use with automated liquid handling robotics. The purification utilizes magnetic separation in place of traditional centrifugation or vacuum filtration steps. For this reason, the protocol can be run on various robotic platforms without human intervention. Automation is also strongly recommended due to the numerous pipetting/liquid handling steps required for sample processing.
For basic Agencourt CosMCPrep automation guidelines please visit:
http://www.agencourt.com/technical/reagent_information/cosmcprep/.

Agencourt recommends the following robot platforms for optimal automation of Agencourt CosMCPrep: Beckman Coulter Biomek® FX, Zymark Sciclone™, or PerkinElmer DNATrak™. As a minimum requirement the automation platform needs to be a 96-tip system compatible with standard 200 µL – 250 µL pipette tips (wide bore tips can also be used for the Agencourt CosMCPrep protocol, as they may make the lysate transfer step easier to perform). An orbital shaker is also strongly recommended for some of the mixing steps. To completely eliminate human intervention steps, a plate gripper/mover is required. Automated plate wash systems are not compatible with Agencourt CosMCPrep, as the narrow aspiration-tips can be easily clogged with flocculent from the alkaline lysis during the ethanol wash cycles.

Additional consideration should be given to the layout of the deck. The Agencourt CosMCPrep process uses the four Agencourt CosMCPrep kit reagents plus isopropanol and ethanol. A total of six reagents may need to be on the deck at the same time. However, the protocol can be split up into separate alkaline lysis and plasmid purification sections to conserve deck space.

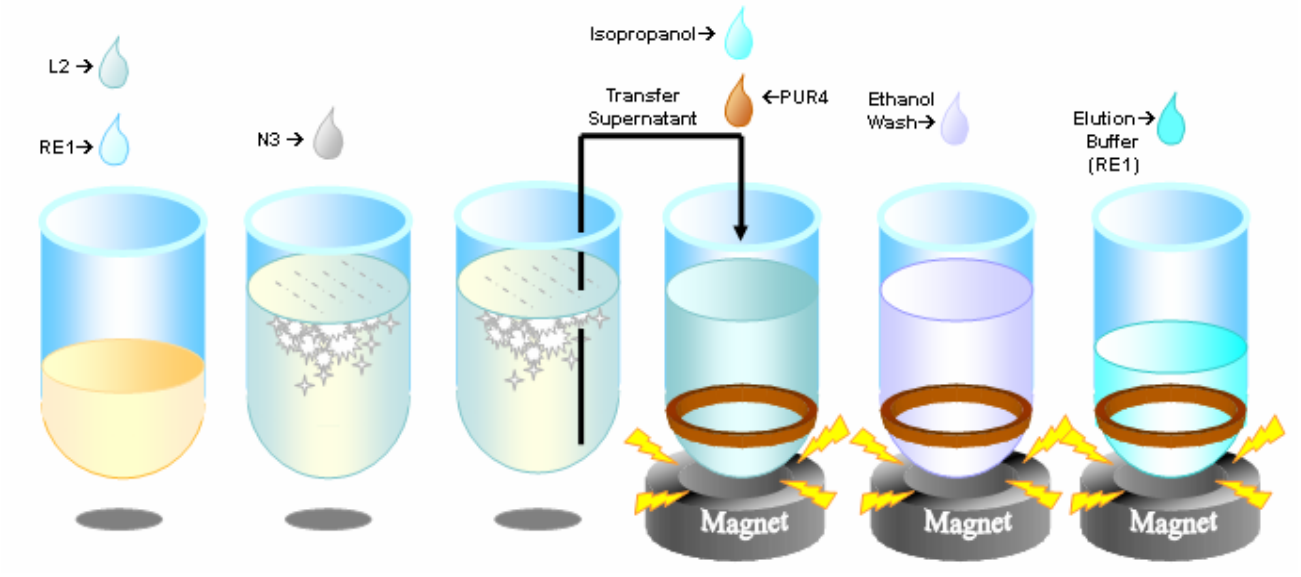
Also, to make efficient use of processing time it is necessary to have more than one magnetic Agencourt SPRIPlate® on the deck. A set of four Agencourt SPRIPlates is a typical setup, but a very fast robot may further increase throughput by using up to eight magnet plates.

Ideally deck layout and scripting should be optimized to process a set of samples within a two hour time frame. To find out the optimal number of magnet plates, determine the cycle time of the process for your robot (the time that elapses between two subsequent plates as they go through the same point in the process). It is important to determine the cycle time when the process is in a steady state (all magnets are in use), as opposed to the ramp-up or ramp-down part of the sequence. The number of plates that can then incubate simultaneously is the incubation time divided by the cycle time.

Process Overview:

The Agencourt CosMCPrep procedure is performed in the following stages:

1. **Pelleted *E.coli* cells are resuspended in RE1 solution, L2 solution is added to lyse the bacterial cultures.**
2. **Addition of N3 solution neutralizes the high pH and maintains DNA integrity. During neutralization *E.coli* chromosomal DNA and cellular contaminants coagulate and form a flocculent in the lysate.**
3. **Clear plasmid-enriched lysate is transferred out from under the flocculent and moved into a fresh 96-well round bottom microtiter plate.**
4. **Addition of isopropanol and PUR4 solution binds the DNA of interest to the magnetic beads. A magnet plate is used to separate the beads from solution.**
5. **Beads with captured plasmid are washed with ethanol to remove salts and other contaminants.**
6. **Purified plasmid is eluted from the magnetic beads using RE1 solution.**



Materials Supplied in the Agencourt CosMCPrep Kit:

Agencourt CosMCPrep reagents have a shelf life of 6 months if stored as directed. As part good laboratory practice, gloves should be worn when using the Agencourt CosMCPrep kit.

Kit Components:

Agencourt CosMCPrep RE1 Solution - Resuspension and elution solution

- Store at 4°C. RE1 solution has RNAse activity.

Agencourt CosMCPrep L2 Solution - Lysis solution

- Store at room temperature. (Or at 37°C to prevent precipitation in colder labs.)
- If white precipitate forms due to cold lab conditions, warm solution, shake to redissolve precipitate.

Agencourt CosMCPrep N3 Solution - Neutralization solution

- Store at room temperature.

Agencourt CosMCPrep PUR4 Solution – Plasmid bind solution

- Store at 4°C. DO NOT FREEZE.
- Shake well to resuspend magnetic particles before using. The reagent should be homogenous prior to use.

Materials Supplied by the User:

Consumables and Hardware:

- Agencourt SPRIPlate® 96R ring magnetic plate [APN 000219]
- Source Plate:
  - 96-well 2.2 mL deepwell culture block (ABGene/Marsh # DW9622 http://www.abgene.com/) for BACs **OR**
  - 96-well 1.1 mL culture block (ABGene/Marsh # DW9611 http://www.abgene.com/) for plasmids/fosmids (see individual protocols for details)
- Destination Plate:
  - 300 µL round bottom microtiter plate (96-well (300 µL well capacity) round bottom plate [Costar # 07-200-105; www.fishersci.com]
- Gas permeable seals (ABGene/Marsh # TR-25B http://www.abgene.com/) to cover plates during shaking
- Liquid handling robotics

Reagents:

- 2xYT bacterial growth media containing the appropriate antibiotic (American Bioanalytical # AB15063-01000 http://www.americanbio.com/)
- LB with 10% Glycerol growth media containing the appropriate antibiotic (American Bioanalytical # CU08048-01000 http://www.americanbio.com/) (optional)
- 100% Isopropanol.
- 70% Ethanol (EtOH)

Note: 70% EtOH is hygroscopic. Fresh 70% EtOH should be prepared regularly for optimal results.

2xYT (1 Liter):	LB (1 Liter):
16 g Tryptone	10 g Tryptone
10 g Yeast Extract	5 g Yeast Extract
5 g NaCl	10 g NaCl
pH to 7.0	pH to 7.0

Agencourt CosMCPrep for Plasmids, Fosmids & Cosmids:

(Alternative protocol for BACs begins on page 13)

1. **Pipette 800 µL 2xYT bacterial growth media containing the appropriate antibiotic (see recommendations below) into each well of a 1.1 mL deep well culture block.**

For extra cost savings, high copy plasmids may be grown using only 600 µL of 2xYT. Agencourt strongly recommends reduced antibiotic concentrations for overnight cultures as conventional concentrations can delay the propagation of cells. Final concentration in media or glycerol: Chloramphenicol 12.5 µg/mL, Ampicillin 50µg/mL, Kanamycin 35 µg/mL, Carbenicillin 50 µg/mL, Zeocin 25 µg/mL, Tetracycline 2.5 µg/mL.

If using different media, antibiotic and/or growth parameters than recommended in this protocol, increase or decrease your growth times to meet the following final cell density averages in the overnight culture:

Plasmids in 800 µL: 1.32×10^9 cells total

Cosmids or Fosmids in 800 µL: 1.52×10^9 cells total

See '**Calculation of Cell Density**' for quantitation methods.

2. **Innoculate each well with a single plasmid containing *E. coli* bacterial colony.**

   Overnight cultures can be inoculated directly from colony-containing agar lawns or from glycerol stock plates. At Agencourt, we use 3.5 µL of the glycerol pre-culture. (For recommendations on growth of glycerol stocks, see protocol section 'Growth of Glycerol Preculture Plates'.)

3. **Cover the plate with a gas permeable seal and shake at 300 rpm at 37°C. Grow plasmids for 17. 5 hours and fosmids/cosmids for 19 hours.**

   Do not allow culture blocks to grow longer than recommended. If the plates grow too long, the cells may begin to die and the Agencourt CosMCPrep purification process will not yield optimum results.

4. **Pellet bacterial cultures by centrifuging culture plates at 2500 x g for 10 minutes.**

   The following are settings for two common centrifuges and rotors:

Beckman GH 3.8 rotor with MicroPlus carrier:	2500 x g = 3300 rpm
Jouan P60 rotor with microplate carrier:	2500 x g = 2900 rpm

5. **After centrifugation, remove the seal and invert the block to decant the media away from the cell pellets. Blot the inverted block on a paper towel to remove excess media.**

   Blot gently to avoid dislodging the cell pellet. If you need to stop the process at this point, pelleted blocks can be sealed and frozen at -20°C or -80°C.

6. **Add 100 µL of RE1 solution and thoroughly resuspend cell pellets by vortexing, shaking or pipette mixing.**

Pipette mixing and shaking are most often used for automated processes. Pipette mix 20 times (if possible, mix 5 times at 4 different locations in the well) for an even resuspension. Aspirate and dispense 80 µL each time at a mixing speed of 150 µL/sec. Alternatively, shake 4 minutes at 600-1200 RPM on a deck mounted shaker. Shaking speeds will vary depending on the orbit of the shaker. Vortexing will take 2-3 minutes on a high setting. The cell pellet should be completely resuspended so that the mixture appears homogenous and has no cell clumps.

7. Add 100 µL of L2 solution, gently mix, and allow the samples to lyse for 5 minutes.

Shake 5 minutes at 300-600 RPM. Alternatively, gently pipette mix 2 times then allow the samples to sit for 5 minutes for complete lysis. Vigorous pipette mixing is not recommended, as large plasmids can be easily sheared. Do not allow samples to lyse for longer than 10 minutes.

If you notice a white precipitate in the L2 solution prior to addition, warm the bottle in a 37°C water bath or under hot running water, shaking periodically, until the precipitate dissolves. It is recommended that you wear gloves when handling L2, as it is a basic solution.

8. Add 100 µL of N3 solution, gently mix, and allow the samples to neutralize for 10 minutes.

Shake the samples for 10 minutes at 300-600 RPM to complete the neutralization. Alternatively, pipette mix very gently near the bottom of the plate, avoiding the flocculent at the top of the well. It is recommended that you wear gloves when handling N3. Addition of N3 neutralizes the solution and precipitates proteins and cellular debris, creating a white flocculent.

9. Transfer 110 µL of the clear lysate to a 300 µL round bottom microtiter plate. This transfer is the most critical step of the process.

The supernatant should be free of flocculent for optimal results. The method detailed below describes the steps involved in a manual transfer.

Manual Transfer:

For ease of manual transfer, Agencourt recommends centrifugation of the lysate to compact the flocculent prior to transfer.

- Following neutralization, centrifuge samples at 4700 x g for 20 minutes to pellet the flocculent.
- Slowly aspirate 110 µL of the clear lysate from the top of the well to avoid touching the pelleted flocculent.
- Dispense the clear lysate into a clean round bottom plate.
- After centrifugation the samples should be transferred within a few minutes, as the pellet will dislodge over time.

For questions regarding this protocol, call Technical Support at Agencourt 1-800-773-9186

10. Add 10 µL of Agencourt CosMCPrep PUR4 solution and 80 µL of 100% isopropanol. Pipette mix 10 times.

PUR4 contains magnetic particles in a plasmid binding buffer. As soon as both the magnetic particle solution and isopropanol are added, the beads may begin to fall out of solution if the sample is not pipette mixed immediately. The liquid should appear homogeneous after mixing. Shaking samples, instead of Pipette mixing, may result in reduced yield.

The final concentration of isopropanol in the well should be 40% for optimal results. If your initial transfer volume is higher or lower than specified due to robotic optimizations or limitations, the volume of isopropanol to be added can be determined by using the following equation:

$$Vol_{Iso} = 0.66 * (Vol_S + Vol_{PUR4})$$

Where:

Vol_{Iso} = volume of isopropanol to be added per well

Vol_S = volume of cleared lysate transferred

Vol_{PUR4} = volume of PUR4 solution (10 µL)

11. Place the round bottom microtiter plate on an Agencourt SPRIPlate 96R and allow the beads to separate for 15 minutes.

Once separation is complete, the supernatant solution should be clear and the beads should form a ring around the bottom of the plate. The supernatant may have a slight yellowish-brown tinge, but should otherwise be translucent. It should not be cloudy.

12. With the plate on the Agencourt SPRIPlate 96R, aspirate and discard the supernatant from the plate.

You should be able to move the tips to the bottom of the well in the center of the ring without disturbing the beads. If the magnetic particles are disturbed during aspiration, more separation time may be required before removing the supernatant. If your robotics system allows, it is best to aspirate the supernatant at a slow rate (less than 8 µL/sec with widebore tips, 5 µL/sec with narrow bore tips) just above the bottom of the plate. The discarded material should not contain magnetic beads.

13. With the plate on the Agencourt SPRIPlate 96R, dispense 200 µL of 70% ethanol into each well of the plate to wash the magnetic beads. Allow samples to incubate for 30 seconds, then remove and discard the ethanol wash solution. Repeat the wash twice, for a total of 3 washes.

Allow the wash solution to remain in the wells of the plate for at least 30 seconds. For best results make fresh 70% ethanol each day. If the concentration of the ethanol is less than

70%, some of the product may be washed away. Do not disturb the ring of beads during the wash step as beads have the target DNA bound to them.

14. Dry the plate at room temperature or 37°C until all ethanol has evaporated (~30 minutes).

Alternatively, a speedvac can be used to dry the plates using a no-heat setting. Once dry, the plates may be stored in a clean environment at room temperature for several days.

15. Add 40 µL of RE1 solution* to each well of the plate, incubate for 5 minutes at 37°C and shake to elute samples.

Vortex or shake the plate for 30 seconds after incubating the plates for 5 minutes at 37°C to fully elute the plasmid from the beads. For large templates, especially BACs, it is helpful to let the plates sit for 5-10 minutes after vortexing to allow the large templates extra time to dissociate from the beads. It is not necessary for the beads to go back into solution for complete elution to occur, however, it is extremely important for the elution buffer to completely cover the ring of beads for maximum recovery.

For long-term freezer storage it is helpful to transfer the eluant away from the beads. Simply place the round bottom microtiter plate back on the magnet for 1-3 minutes, and then transfer the samples to a new clean plate.

*RE1 solution is the optimal elution buffer for Agencourt CosMCPrep samples and is recommended for use in sequencing reactions. Other aqueous low-salt buffers may also be used for elution.

Quick Reference: Agencourt CosMCPrep for Plasmids, Fosmids & Cosmids

1. **Pipette 800 µL 2xYT bacterial growth media into each well of 1.1 mL deep well culture block.**
2. **Innoculate each well with a single plasmid containing *E. coli* bacterial colony.**
3. **Cover the plate with a gas permeable seal and shake at 300 rpm at 37°C. Grow plasmids for 17. 5 hours and fosmids/cosmids for 19 hours.**
4. **Pellet bacterial cultures by centrifuging culture plates at 2500 x g for 10 minutes.**
5. **After centrifugation, remove the seal and invert the block to decant the media away from the cell pellets. Blot the inverted block on a paper towel to remove excess media.**
6. **Add 100 µL of RE1 solution and thoroughly resuspend cell pellets by vortexing, shaking or pipette mixing.**
7. **Add 100 µL of L2 solution, gently mix, and allow the samples to lyse for 5 minutes.**
8. **Add 100 µL of N3 solution, gently mix, and allow the samples to neutralize for 10 minutes.**
9. **Transfer 110 µL of the clear lysate to a 300 µL round bottom microtiter plate. This transfer is the most critical step of the process. (For manual transfer, first centrifuge lysate for 20 minutes 4700 x g.)**
10. **Add 10 µL of Agencourt CosMCPrep PUR4 solution and 80 µL of 100% isopropanol. Pipette mix 10 times.**
11. **Place the round bottom microtiter plate on an Agencourt SPRIPlate 96R and allow beads to separate for 15 minutes.**
12. **With the plate situated on the Agencourt SPRIPlate 96R, aspirate and discard the supernatant from the plate.**
13. **With the plate situated on the Agencourt SPRIPlate 96R, dispense 200 µL of 70% ethanol into each well of the plate to wash the magnetic beads. Allow samples to incubate for 30 seconds, then remove and discard the ethanol wash solution. Repeat the wash twice, for a total of 3 washes.**

For questions regarding this protocol, call Technical Support at Agencourt 1-800-773-9186

14. Dry the plate at room temperature or 37°C until all ethanol has evaporated (~30 minutes).

15. Add 40 µL of RE1 solution to each well of the plate, incubate for 5 minutes at 37°C and shake to elute samples.

Pellet | RE1 & L2 | N3 | Pellet | Pur4 & Iso | Remove Supernatant | Wash 3x with Ethanol | Dry | Elute

Agencourt CosMCPrep for BACs:

(Alternative protocol for Plasmids, Cosmids and Fosmids begins on page 6)

1. **Pipette 1.7 mL 2xYT bacterial growth media containing the appropriate antibiotic (see recommendations below) into each well of a 2.2 mL deep well culture block.**

   Agencourt strongly recommends reduced antibiotic concentrations for overnight cultures as conventional concentrations can delay the propagation of cells. Final concentration in media or glycerol: Chloramphenicol 12.5 µg/mL, Ampicillin 50 µg/mL, Kanamycin 35 µg/mL, Carbenicillin 50 µg/mL, Zeocin 25 µg/mL, Tetracycline 2.5 µg/mL.

   If using different media, antibiotic and/or growth parameters than recommended in this protocol, increase or decrease your growth times to meet the following final cell density averages in the overnight culture:

   BACs (original media volume: 1700 µL): 3.21×10^9 cells total

   See '**Calculation of Cell Density**' for quantitation methods.

2. **Innoculate each well with a single plasmid containing *E. coli* bacterial colony.**

   Growth cultures can be inoculated directly from agar lawns or from glycerol stocks prepared according to the protocol above.

3. **Cover the plate with a gas permeable seal and shake at 300 rpm at 37°C for 19 hours.**

   Do not allow culture blocks to grow longer than 19 hours. If plates grow too long, the cells will begin to die and the Agencourt CosMCPrep purification process will not yield optimum results.

4. **Pellet bacterial cultures by centrifuging culture plates at 2500 x g for 10 minutes.**

   The following are settings for two common centrifuges and rotors:

Beckman GH 3.8 rotor with MicroPlus carrier:	2500 x g = 3300 rpm
Jouan P60 rotor with microplate carrier:	2500 x g = 2900 rpm

5. **After centrifugation, remove the seal and invert the block to decant the media away from the cell pellets. Blot the inverted block on a paper towel to remove excess media.**

   Blot gently to avoid dislodging the cell pellet. If you need to stop the process at this point, pelleted blocks can be sealed and frozen at -20°C or -80°C.

6. Add 100 µL of RE1 solution and thoroughly resuspend cell pellets by vortexing, shaking or pipette mixing.

Pipette mixing and shaking are most often used for automated processes. Pipette mix 20 times (if possible, pipette mix 5 times at 4 different locations in the well) for an even resuspension. Aspirate and dispense 80 µL each time at a mixing speed of 150 µL/sec. Alternatively, shake 4 minutes at 600-1200 RPM on a deck mounted shaker. Shaking speeds will vary depending on the orbit of the shaker. Vortexing will take 2-3

minutes on a high setting. The cell pellet should be completely resuspended so that the mixture appears homogenous and has no cell clumps.

7. Add 100 µL of L2 solution, gently mix, and allow the samples to lyse for 5 minutes.

Shake 5 minutes at 300-600 RPM. Alternatively, gently pipette mix 2 times then allow the samples to sit for 5 minutes for complete lysis. Vigorous pipette mixing is not recommended, as BACs can be easily sheared. Do not allow samples to lyse for longer than 10 minutes.

If you notice a white precipitate in the L2 solution prior to addition, warm the bottle in a 37°C water bath or under hot running water, shaking periodically, until the precipitate dissolves. It is recommended that you wear gloves when handling L2, as it is a basic solution.

8. Add 100µl of N3 solution and shake 10 minutes on an orbital or linear shaker to neutralize samples.

Addition of N3 neutralizes the solution and precipitates proteins and cellular debris, creating a white flocculent. BACs must be shaken to help release the large template from the flocculent. Shake on the same orbital shaker used for overnight culture (room temperature or 37°C are both fine), or on a deck mounted shaker for automated systems. It is recommended that you wear gloves when handling N3.

9. Following neutralization, centrifuge samples at 4700 x g for 20 minutes to pellet the flocculent.

Pelleting of the flocculent allows for transfer of a greater percentage of the lysate versus the Agencourt CosMCPrep method for plasmids, fosmids and cosmids, leading to higher yields. A 20 minute spin is usually sufficient to pellet the flocculent, but if you notice that the pellet is not tightly bound to the bottom of the well, increase the centrifugation time.

10. Transfer 175 µL of the clear lysate to a 300 µL round bottom microtiter plate. This transfer is the most critical step of the process.

The supernatant should be free of cellular contaminants for optimal results. For best results, use slow aspiration speeds (5 µL/sec) and remove the clear lysate from the top of the well. The aspirate height used for this transfer step will most likely need to be optimized to avoid disturbing the pellet. For some robotics, the use of wide bore pipette tips may help to prevent aspiration of flocculent.

11. Add 10 µL of Agencourt CosMCPrep PUR4 solution and 122 µL of 200-proof isopropanol. Pipette mix 10 times.

PUR4 contains magnetic particles in a plasmid binding buffer. As soon as both the magnetic particles and isopropanol are added, the beads may begin to fall out of solution if the sample is not pipette mixed immediately. The liquid should appear homogeneous after mixing. Shaking samples, instead of pipette mixing, may result in reduced yield.

The final concentration of isopropanol in the well should be 40% for optimal results. If your initial transfer volume is higher or lower than specified due to robotic optimizations or limitations, the volume of isopropanol to add to each reaction can be determined by the following equation:

$$Vol_{Iso} = 0.66 * (Vol_S + Vol_{PUR4})$$

Where:

Vol_{Iso} = volume of isopropanol to be added per well

Vol_S = volume of cleared lysate transferred

Vol_{PUR4} = volume of PUR4 solution (10 µL)

12. Place the round bottom microtiter plate on an Agencourt SPRIPlate 96R and allow beads to separate for 15 minutes.

Once separation is complete, the supernatant should be clear and the beads should form a ring around the bottom of the plate. The supernatant may have a slight yellowish-brown tinge, but should otherwise be translucent. It should not be cloudy.

13. With the plate on the Agencourt SPRIPlate 96R, aspirate and discard the supernatant from the plate.

You should be able to move the tips to the bottom of the well in the center of the ring without disturbing the beads. If the magnetic particles are disturbed during aspiration, more separation time may be required before removing the supernatant. If your robotics system allows, it is best to aspirate the supernatant at a slow rate (less than 8 µL/sec with widebore tips, 5 µL/sec with narrow bore tips) just above the bottom of the plate. The discarded material should not contain magnetic beads.

14. With the plate situated on the Agencourt SPRIPlate 96R, dispense 200 µL of 70% ethanol into each well of the plate to wash the magnetic beads. Allow samples to incubate for 30 seconds, then remove and discard the ethanol wash solution. Repeat the wash twice, for a total of 3 washes.

Allow the wash solution to remain in the wells of the plate for at least 30 seconds. For best results make fresh 70% ethanol each day. If the concentration of the ethanol is less than 70%, some of the product may be washed away. Do not disturb the ring of beads during the wash step as beads have the target DNA bound to them.

15. Dry the BAC samples at 37°C for 5 minutes.

Plates can also be dried at room temperature for 5-10 minutes. For best results, do not overdry BACs.

16. Add 40 µL of RE1 solution* to each well of the plate, incubate for 5 minutes at 37°C and shake to elute samples.

Vortex or shake the plate for 30 seconds after incubating for 5 minutes at 37°C to fully elute the plasmid from the beads. For large templates, especially BACs, it is helpful to let the plates sit for 5-10 minutes after vortexing to allow the large templates extra time to dissociate from the beads. It is not necessary for the beads to go back into solution for complete elution to occur, however, it is extremely important for the elution buffer to completely cover the ring of beads for maximum recovery.

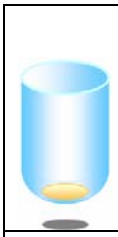
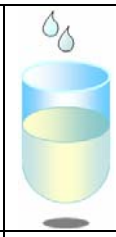
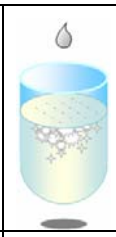
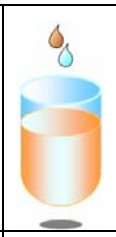
For long-term freezer storage it is helpful to transfer the eluant away from the beads. Simply place the round bottom microtiter plate back on the magnet for 1-3 minutes, and then transfer the samples to a new clean plate.

*RE1 solution is the optimal elution buffer for Agencourt CosMCPrep samples and is recommended for use in sequencing reactions. Other aqueous low-salt buffers may also be used for elution.

Quick Reference: Agencourt CosMCPrep for BACs

1. **Pipette 1.7 mL 2xYT bacterial growth media into each well of a 2.2 mL deep well culture block.**
2. **Innoculate each well with a single plasmid containing *E. coli* bacterial colony.**
3. **Cover the plate with a gas permeable seal and shake at 300 rpm at 37°C for 19 hours.**
4. **Pellet bacterial cultures by centrifuging culture plates at 2500 x g for 10 minutes.**
5. **After centrifugation, remove the seal and invert the block to decant the media away from the cell pellets. Blot the inverted block on a paper towel to remove excess media.**
6. **Add 100 µL of RE1 solution and thoroughly resuspend cell pellets by vortexing, shaking or pipette mixing.**
7. **Add 100 µL of L2 solution, gently mix, and allow the samples to lyse for 5 minutes.**
8. **Add 100 µL of N3 solution and shake 10 minutes to neutralize samples.**
9. **Following neutralization, centrifuge samples at 4700 x g for 20 minutes to pellet the flocculent.**
10. **Transfer 175 µL of the clear lysate to a 300 µL round bottom microtiter plate. This transfer is the most critical step of the process.**
11. **Add 10 µL of Agencourt CosMCPrep PUR4 solution and 122 µL of 200-proof isopropanol. Pipette mix 10 times.**
12. **Place the round bottom microtiter plate on an Agencourt SPRIPlate 96R and allow the beads to separate for 15 minutes.**
13. **With the plate situated on the Agencourt SPRIPlate 96R, aspirate and discard the supernatant from the plate.**
14. **With the plate situated on the Agencourt SPRIPlate 96R, dispense 200 µL of 70% ethanol into each well of the plate to wash the magnetic beads. Allow samples to incubate for 30 seconds, then remove and discard the ethanol wash solution. Repeat the wash twice, for a total of 3 washes.**
15. **Dry the BAC samples at 37°C for 5 minutes.**
16. **Add 40 µL of RE1 solution to each well of the plate, incubate for 5 minutes at 37°C and shake to elute samples.**

For questions regarding this protocol, call Technical Support at Agencourt 1-800-773-9186

								
Pellet	RE1 & L2	N3	Pellet	Pur4 & Iso	Remove Supernatant	Wash 3x with Ethanol	Dry	Elute

Growth of Glycerol Preculture Plates (optional):

Agencourt recommends picking colonies into glycerol stock plates, so that clones can be retrieved for further analysis after DNA purification. If you do not want to produce glycerol stock plates, pick your bacterial cultures directly into deepwell growth blocks (see "*Growth of Bacterial Cultures*"). Below are instructions for making 96 and 384-well glycerol stocks.

1. **A. 96-Well Glycerols:**

   Fill 96-well round bottom microtiter plates (Costar # 3795) with 220 µL of LB / 10% Glycerol / appropriate antibiotic.

   B. 384-Well Glycerols:

   Fill 384-well flat bottom microtiter plates (Marsh # N5-8113-WI) with 90 µL of LB / 10% Glycerol / appropriate antibiotic.

Note: **For low-copy plasmids, a much lower antibiotic concentration is required for healthy growth. Antibiotic concentrations for low-copy plasmids may be 2-5 fold less than for high-copy plasmids. Unusually high antibiotic concentrations may significantly hinder amplification during growth. (Sambrook and Russell, Molecular Cloning: A Laboratory Manual, 3rd Ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 2001. Vol. 3: A2.6.)**

2. **Inoculate each well with a single plasmid containing *E. coli* bacterial colony.**

   Use an automated picking machine to pick single colonies into each well of the plate. Automated picking ensures accuracy and consistency; however, sterile toothpicks may also be used to manually pick colonies from an agar lawn.

3. **Cover plates with lids, wrap plates in plastic film and grow statically (no shaking) in a 37°C warm room for 12 hours.**

If you do not have lids for the plates, use a blank plate in between the glycerol plates. Wrap each stack with Saran Wrap or plastic film to avoid evaporation. Do not allow glycerol plates to grow longer than 12 hours, as the cells will begin to die and will not yield healthy growth cultures. Overgrown glycerol precultures often have visible cell mass clustered at the bottom of the affected wells. A thick milky consistency or web-like veils of cells can sometimes coat the sides of wells that have been overgrown. The quality of the pre-culture is very important for single- or low-copy purification success and any one of the aforementioned symptoms may inhibit optimum downstream results. If you notice that a culture grows poorly (uneven pellet sizes; large, loose pellets; wells with no growth), you may want to examine the preculture.

Calculation of Cell Density:

Agencourt recommends quantitation of cell number via optical density at 600nm on a spectrophotometer. Perform the reading using a 1:20 dilution of the overnight culture, as the concentration of cells in non-diluted culture is typically too high to obtain an accurate OD 600nm reading. Clear media should be used to blank the system.

The following protocol uses a 96 well half area (150 μΛ) flat bottom plate (Costar #3695, item #29444320 from http://www.vwrsp.com/).

1. **Measure the total volume left in the well after overnight growth. This should be slightly less than the starting volume due to evaporation overnight. You need to know this final volume to determine the total number of cells in the well.**
2. **Fill the wells a half area flat bottom plate with 142.5 μL DiH_2O.**
3. **Add 7.5 μL of 2xYT (or sterile growth media used for the overnight culture) to each well and pipette to mix.**
4. **Take an OD 600 reading of the plate. These readings are the blank/background.**
5. **Fill the wells of a second half area flat bottom plate with 142.5 μL DiH_2O.**
6. **Add 7.5 μL of fresh overnight culture to each well and pipette to mix. Pipette mix the overnight culture before you take your sample and make sure no cells are clustered at the bottom of the well. Do not let the diluted sample plate sit too long after they are prepared, as the cells will begin to settle and the reading will be inaccurate.**
7. **Take an OD 600 reading of the plate. These readings are the raw ODs.**

Use these values to calculate the total number of cells in the overnight culture. When evaluating 96 samples it is helpful to program an Excel spreadsheet to automatically calculate these values, given the Blank and Raw OD values:

Subtract the blank: $OD_{Raw} - OD_{Blank} = OD_{Adjusted}$

Multiply by path length (1.1 for Costar half area plate with 150 µL total volume) **and dilution factor** (20 for 1:20 dilution): $OD_{Adjusted}$ x Path Length x Dilution Factor = OD of culture in half area plate

Multiply by 1 OD (5.0×10^8 cells/mL): OD x (5.0×10^8) cells/mL = Number of cells in 1 mL of overnight culture

Multiply by the volume (in milliliters) of the overnight culture: No. Cells in 1 mL x Total Overnight Culture Volume (mL) = Total Number Cells in the Overnight Culture

Simplified:

$(OD_{Raw} - OD_{Blank})$ x Path Length x Dilution Factor x (5.0×10^8) x Final Overnight Culture Volume (mL)

$(OD_{Raw} - OD_{Blank})$ x 1.1 x 20 x (5.0×10^8) x Final Overnight Culture Volume (mL)

$(OD_{Raw} - OD_{Blank})$ x (1.1×10^{10}) x Total Overnight Culture Volume (mL) = Total Number Cells in Overnight Culture