epi BiSKit™

A complete kit for the preparation of purified, bisulfite-converted DNA

Instructions for Use

Epi BiSKit (M7-01-001)

For *in vitro* diagnostic use only.

Read and follow these Instructions for Use prior to using this product. The current revision of this document can be found at epigenomics.com.

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REF M7-01-001

IVD CE

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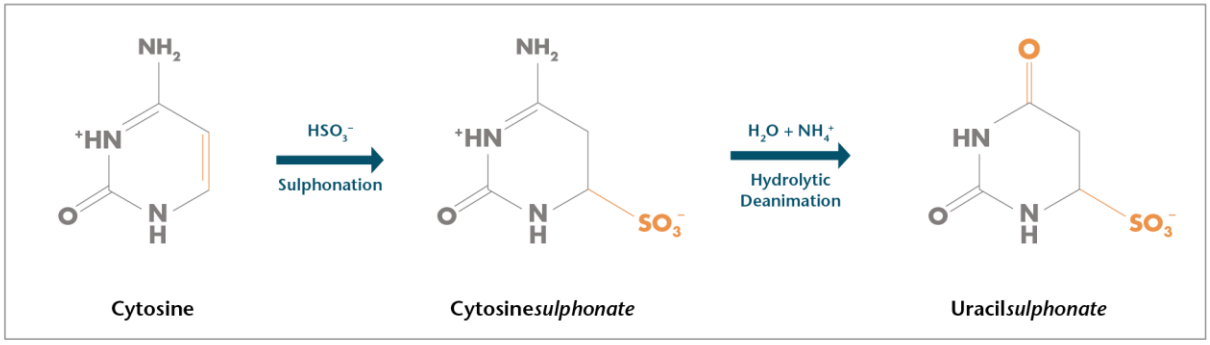
1. Intended Use

The Epi BiSKit provides a complete set of reagents for the preparation of bisulfite-converted DNA from plasma derived from human whole blood samples. Using the reagents provided, each Epi BiSKit is intended to process 32 preparations, providing purified, sulfonated bisulfite-converted DNA for use in diverse, downstream molecular applications.

2. Summary and Principles

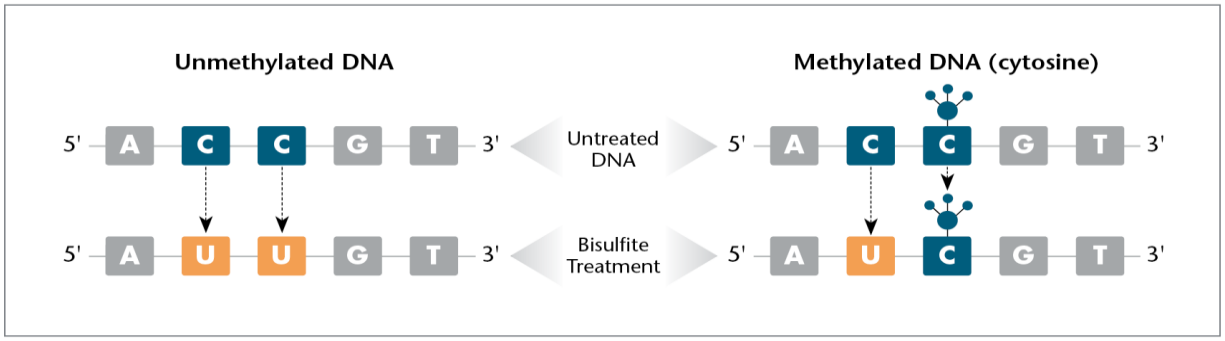
DNA, extracted from cell-free plasma and treated with bisulfite using the Epi BiSKit (M7-01-001), is extracted from plasma by binding to magnetic beads. Impurities are removed from the magnetic beads by a wash step. The purified DNA is then released from the beads by suspension in an elution buffer, and treated with bisulfite reagents to produce sulfonated bisulfite converted DNA (bisDNA). The bisDNA is then re-purified using magnetic beads and cleansing steps to produce the finished product of pure, sulfonated bisulfite-converted DNA.

Figure 1: Biochemical process to yield sulfonated bisulfite-converted DNA



Bisulfite conversion is the method of choice for analyzing DNA methylation. It is based on the nucleophilic addition of a bisulfite ion to a cytosine nucleotide and a subsequent deamination reaction to yield uracil sulfonate; 5-methylcytosine (methylated cytosine) is protected from deamination by the methyl group. Thus, the sequence of bisDNA differs from the input DNA by the substitution of uracil nucleotides for unmethylated cytosines, while retaining methyl cytosine bases.

Figure 2: Bisulfite conversion



3. Kit Contents for Thirty Two Plasma Preparations

Reagent	Reference Number	Containers	Volume	Storage Temperature
Lysis Binding Buffer	H003710	1 bottle	125.0 mL	15°C to 30°C
Wash A Concentrate	H003720	1 bottle	60.0 mL	15°C to 30°C
Wash B Concentrate	H003730	1 bottle	7.0 mL	15°C to 30°C
Magnetic Beads	H003740	1 bottle	4.0mL	15°C to 30°C
Bisulfite Solution	H003750	4 tubes	1.9 mL each	15°C to 30°C
Elution Buffer	H003760	1 tube	6.0 mL	15°C to 30°C
Protection Buffer	H003770	1 tube	1.0 mL	15°C to 30°C

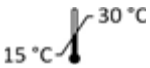
Note: Protection Buffer color can vary from clear to a brownish color and grow more intense over time; color does not affect kit performance.

4. Storage and Stability

Reagents provided with the Epi BiSKit (M7-01-001) are stable until the expiration date when stored and handled as directed. Do not use material past expiration date. Do not mix components from different kit lots.

- Store all Epi BiSKit reagents at 15°C to 30°C, prior to use.
- Bisulfite Solution is sensitive to oxygen exposure.

  Use only unopened tubes of Bisulfite Solution. Discard previously opened and used tubes and contents.
- Store reconstituted Wash A and/or Wash B buffers at 15°C to 30°C for up to six weeks.
- After first use of remaining reagents, store at 15°C to 30°C for up to six weeks.



5. Precautions and Safety

Laboratory Precautions

- This procedure is for professional laboratory use only and assumes familiarity with DNA extraction methods. Good technique is essential and failure to follow instructions provided may produce inaccurate results.
- Compliance with good laboratory practices is essential to minimize the risk of cross-contamination between samples during and after the DNA extraction, bisulfite conversion, and purification procedure.
- Use only single-use pipettes and filter tips to prevent cross-contamination of the sample.
- Use of reference pipettes for pipetting extracted and bisulfite treated DNA is strongly recommended.
- **Do not freeze extracted DNA prior to bisulfite conversion.**
- Bisulfite Solution is sensitive to oxygen contact; use only unopened tubes of Bisulfite Solution; do not store or re-use but discard any left-over solution.
- When removing liquid from microtubes in multiple steps in the procedure, take care not to remove magnetic beads.
- In step 8.13, the drying step is very important. If the remaining droplets are not removed from the beads before drying and elution of the DNA, the remaining Wash B Buffer can be the cause of invalid results occurring in downstream applications.
- For molecular downstream applications such as PCR, strict separation of pre-PCR activities (e.g., plasma DNA extraction and purification, PCR setup) and post-PCR activities (e.g., Real-Time PCR) is highly recommended to prevent contamination by amplicons generated from previous PCR testing. To prevent the release of any PCR product, used PCR plates should never be opened. Place the PCR plate in a re-sealable plastic bag immediately after removal from the PCR instrument, close and dispose the bag in a dedicated PCR waste container.
- Use of external quality controls that monitor quality and quantity of DNA generated using this DNA preparation procedure are recommended for all downstream applications.

Additional Precautions and Considerations

- A minimum of 3.5 mL of plasma, post-centrifugation, is required to yield optimal DNA using this extraction protocol. Multiple blood collection tubes might be required to supply 3.5 mL of plasma depending on the fill volume of the tubes selected.
- All blood collection tubes for generating cell-free plasma should be validated for compatibility with your specific molecular methods and requirements.
- Never mix kit components between kit lots or use the kits or kit components beyond their stated expiration date.
- Epi BiSKit generates sulfonated bisulfite-converted DNA (Figure 1). For molecular applications that require a desulfonated bisulfite converted DNA template, an additional desulfonation procedure will be needed.
- Use of Epi BiSKit for DNA extraction should be evaluated for compatibility with any intended downstream application.
- The Epi BiSKit components do not contain infectious substances or agents that may cause disease in humans or animals.

Safety Information

When working with chemicals, always wear a laboratory coat and disposable gloves. Clean contaminated surfaces with water. For more information, please refer to the respective Safety Data Sheets (SDS) available for the product at epigenomics.com.

Lysis Binding Buffer and Wash A Concentrate: Contain TRITON X-100 and guanidinium thiocyanate.

Hazard statements: Harmful if swallowed. Harmful in contact with skin. Harmful if inhaled. Causes skin irritation. Causes serious eye damage.

Precautionary statements:

Prevention: Avoid breathing mist/vapors/spray. Wash thoroughly after handling. Do not eat, drink or smoke when using this product. Use only outdoors or in a well-ventilated area. Wear protective gloves/protective clothing/eye protection/face protection.

DANGER

Response: If swallowed: Call a poison center/doctor if you feel unwell. Rinse mouth. If on skin: Wash with plenty of water. If skin irritation occurs: Get medical advice/attention. Take off contaminated clothing and wash it before reuse. If in eyes: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Immediately call a poison center/doctor. If inhaled: Remove person to fresh air and keep comfortable for breathing. Call a poison center/doctor if you feel unwell.

Storage: Store away from incompatible materials.

Disposal: Dispose of contents/container in accordance with local/ regional/ national/ international regulations.

Bisulfite Solution: Contains aqueous solution of ammonium bisulfite (Ammonium hydrogen sulfite).

Hazard statements: Causes serious eye irritation.

Precautionary statements:

WARNING

Prevention: Wash thoroughly after handling. Wear eye protection/face protection.

Response: If in eyes: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. If eye irritation persists: Get medical advice/attention.

Storage: None.

Disposal: None.

Protection Buffer: Contains 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, tetrahydrofurfurylalcohol.

Hazard statements: Combustible liquid. Causes skin irritation. Causes serious eye irritation. May damage fertility or the unborn child.

Precautionary statements:

Prevention: Obtain special instructions before use. Do not handle until all safety precautions have been read and understood. Keep away from heat/sparks/open flames/hot surfaces. - No smoking. Wash thoroughly after handling. Wear protective gloves/protective clothing/eye protection/face protection.

DANGER

Response In case of fire: Use foam, carbon dioxide, dry powder or water fog for extinction. If on skin: Wash with plenty of water. If skin irritation occurs: Get medical advice/attention. Take off contaminated clothing and wash it before reuse. If in eyes: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. If eye irritation persists: Get medical advice/attention.

Storage Store in a well-ventilated place. Keep cool. Store locked up.

Disposal Dispose of contents/container in accordance with local/ regional/ national/ international regulations.

Wash B Concentrate, Elution Buffer, Magnetic Beads, are not harmful.

6. Materials Required But Not Provided

The following general laboratory equipment is required to perform the Epi BiSKit. All laboratory equipment should be installed, calibrated, operated, and maintained according to the manufacturer's recommendations. The following tables display required and special equipment along with the list of consumables and recommended suppliers.

General Laboratory Equipment

Required Equipment	Suggested Supplier
Tube racks for 15.0 mL tubes and 2.0 mL tubes	VWR or Fisher Scientific, or equivalent
Rotator with adjustable angle	VWR International, or equivalent
Vortex Mixer	Thermo Scientific MaxiMix II vortex mixer, or equivalent
Thermoshaker for 2.0 mL tubes	Thermomixer® C with dry heating block 2.0 mL Eppendorf, or equivalent; *Equipment accuracy for shaking at 1000 rpm ± 100 rpm; temperature accuracy of 23°C ± 2° and 80°C ± 2°*
Magnetic Separator	DynaMag™-15 magnet, Thermo Fisher Scientific
Magnetic Separator	DynaMag™-2 magnet, Thermo Fisher Scientific
Reference Pipettes with adjustable volumes of the following ranges 10-100 µL, 100-1000 µL	Eppendorf Research® Plus, adjustable-volume pipette, Eppendorf, or equivalent
Repeat Pipettor capable of repetitively dispensing volumes in an adjustable range	Eppendorf Multipette® M4, Eppendorf, or HandyStep electronic, Brandtech®, or equivalent
Bench Top Centrifuge with rotor for 1.5/2.0 mL tubes	Centrifuge 5418 or 5430 with Rotor FA-45-30-11, Eppendorf, or equivalent; *accuracy for 1000 rcf ± 150 rcf*
100.0 mL graduated cylinder (PP)	Brandtech®, Carl Roth, Thermo Fisher, or equivalent

General Laboratory Consumables and Reagents

Consumables and Reagents	Suggested Consumables and Reagents
Ethanol absolute, 200 proof, for molecular biology, ≥99.5 %	Merck KGaA or Sigma-Aldrich Co., or equivalent
15.0 mL polypropylene (PP) centrifuge tubes with conical bottom, sterile	Sarstedt, or equivalent
2.0 mL microtubes with round bottom and with attached PP cap with lid seal mechanism	Sarstedt SafeSeal of Eppendorf Safe-Lock™ or equivalent
Pipette tips with aerosol barrier	Eppendorf ep Dualfilter T.I.P.S®: • 2-100 µL, or equivalent • 50-1000 µL, or equivalent
Repeat Pipettor Tips for volumes of 0.5 mL, 1.0 mL, 10.0 mL, 2.05 mL	Eppendorf Combitips Advanced®, or equivalent For 0.5 mL, 1.0 mL , 10.0 mL, 25.0 mL
Disposable Transfer Pipettes with reference lines, non-sterile bulk packaged, length about 15.0 cm,stem diameter 5.0 mm, capacity about 5.0 mL	VWR or Thermo Scientific Samco™ brand, or equivalent
Disposable Transfer Pipettes, non-sterile bulk packaged, length about 23 cm, stem diameter 5.0 mm, capacity about 5.0 mL	Carl Roth graduated or Samco ™ extra-long transfer pipettes, Thermo Scientific, or equivalent

7. Specimen Collection and Handling

Blood Collection, Storage and Handling

This product is optimized for use with a plasma sample volume of 3.5 mL. The following recommendations and instructions apply to use of 3.5 mL of plasma for generation of purified, sulfonated bisulfite-converted cell-free DNA (cfDNA) for downstream applications.

Blood should be collected according to standard procedures for venipuncture.

Note: Do not freeze whole blood samples.

8. DNA Bisulfite Conversion Procedure

A repeat pipettor is recommended for repetitive dispensing of the following reagents:

- Lysis Binding Buffer, step 8.3
- Ethanol in the DNA binding, step 8.4

Reference pipettes are recommended for the following:

- Magnetic Bead suspension
- Wash A Buffer
- Wash B Buffer
- Elution Buffer
- Bisulfite Solution
- Protection Buffer

Use of reference pipettes for pipetting extracted and bisulfite treated DNA is strongly recommended.

8.1. Preparation of Working Solutions

Preparation of Wash A Buffer

- Add 60.0 mL of Absolute Ethanol (for molecular biology, ≥99.5 %) to the Wash A Concentrate using a sterile graduated cylinder or a serological pipette
- Close lid, mix thoroughly by inverting the bottle five times, avoiding formation of foam; label the bottle with date of dilution
- Store reconstituted Wash A Buffer at 15°C to 30°C for up to six weeks

Preparation of Wash B Buffer

- Add 40.0 mL of Absolute Ethanol (for molecular biology, ≥99.5 %) to the Wash B Concentrate using a sterile graduated cylinder or a serological pipette
- Close lid, mix thoroughly by inverting the bottle five times; label the bottle with date of dilution
- Store reconstituted Wash B Buffer at 15°C to 30°C for up to six weeks

8.2. DNA Extraction and Bisulfite Conversion in Plasma

The Epi BiSKit contains sufficient reagents to process up to 32 preparations including quality controls. It is recommended that positive and negative controls suitable to the application be included in each independent test run. Epi BiSKit includes four single-use tubes of Bisulfite Solution that will accommodate a maximum of four independent test runs (e.g., four runs of eight samples each).

Note: Brief centrifugation of microtubes (stated as 'Briefly spin down the tubes') is required in several steps of this instruction to remove drops from the lid and/or to collect remaining liquid. It is recommended to centrifuge for 10 to 20 sec at 1,000 ± 150 rcf using a Bench-Top centrifuge. Avoid stronger centrifugation to prevent the compacting of magnetic bead pellets in these steps. For conversion of RPM (revolutions per minute) to rcf (relative centrifugal force), refer to the manufacturer's centrifuge user manual.

Note: Vortexing of tubes and vials is required in several steps of this instruction to ensure homogeneous mixing of liquid. It is recommended to vortex samples for 5 to 10 sec at medium speed.

Note: If frozen plasma samples are used, thaw samples for about 30 min at 15°C to 30°C. Begin the lysis step within 60 min of thawing plasma samples.

8.3. Lysis

Prior to use, briefly shake the Lysis Binding Buffer and visually check for precipitates. If precipitates are present, heat the Lysis Binding Buffer in a water bath at 37°C for 60 min and shake gently until the precipitate is completely dissolved. Equilibrate Lysis Binding Buffer to room temperature before use.

- Add the following to a labelled 15.0 mL conical centrifuge tube:
    - 3.5 mL plasma sample, Positive Control, or Negative Control
- 3.5 mL Lysis Binding Buffer to each 15.0 mL centrifuge tube
- Cap the tube and vortex for 5 to 10 sec
- Incubate tubes on the bench top at 15°C to 30°C for 10 ± 1 min

8.4. DNA Binding

Note: A homogeneous suspension of beads in the Magnetic Beads is essential for proper performance. Deviations from the specified amount of beads may lead to false results. To ensure correct magnetic bead concentration, the bottle should be mixed thoroughly just before pipetting. There should be no visible sediment at the bottom of the bottle. **Mix to ensure a homogeneous suspension between the pipetting steps.**

- Add to the 15.0 mL centrifuge tube in the following order:
    - 90 µL Magnetic Beads (freshly suspended)
    - 2.5 mL of Absolute Ethanol (for molecular biology, ≥99.5 %)
- Cap the tube and mix by inverting the tube five to six times
- Place 15.0 mL tubes into a rotator and rotate at room temperature for 45 ± 5 min at medium speed (approximately 10 to 20 rpm); adjust rotator angle to 35 to 45 degrees

8.5. DNA Washing

Before starting the wash procedure set the thermoshaker to 80 ± 2°C for later use in the elution and bisulfite conversion steps.

- Place the 15.0 mL tubes into the DynaMag™-15 magnetic racks for 5 to 10 min
- Pour off the supernatant carefully, taking care not to remove magnetic beads and add 1.5 mL Wash A Buffer
- Resuspend magnetic beads completely by vortexing for 5 to 10 sec
- Using an extra-long (22.5 cm) disposable transfer pipette, transfer magnetic beads suspension into a labelled 2.0 mL microtube
- Place disposable transfer pipette back into 15 mL tube to collect remaining magnetic beads and transfer them into the 2.0 mL microtube
- Place the microtubes into the DynaMag-2 magnetic racks for 2 to 6 min
- Using disposable transfer pipette (15 cm), remove as much buffer as possible while microtubes are still in the DynaMag-2 magnetic rack, taking care not to remove magnetic beads
- Briefly spin down the microtubes and place the 2.0 mL microtubes into the DynaMag-2 magnetic racks for 2 to 6 min
- Using a 10-100 µL reference pipette, remove as much residual buffer as possible while microtubes are still in magnetic rack

8.6. Elution

- Transfer the microtubes into a non-magnetic rack
- Vortex Elution Buffer for 5 to 10 sec
- Add 100 µL Elution Buffer to each microtube and close the microtubes and resuspend the magnetic beads by vortexing for 5 to 10 sec
- Place microtubes into a thermoshaker set to 1,000 ± 100 rpm and incubate at 80 ± 2°C for 10 ± 1 min
- Briefly spin down the microtubes and place microtubes into the DynaMag-2 magnetic racks for 2 to 6 min
- Transfer the complete eluate, while microtubes are still in the magnetic rack, (~100 µL DNA solution) into fresh 2.0 mL microtubes
- Discard the 2.0 mL microtubes containing the magnetic beads

8.7. Storage of Extracted DNA

Note: If extracted DNA is not used immediately, store material at 2°C to 8°C for up to 24 hours.
Do not freeze the extracted DNA.

8.8. Bisulfite Conversion

Note: Bisulfite Solution is sensitive to oxygen contact. Use only unopened tubes of Bisulfite Solution; do not store and discard any left-over solution.

Note: The color of the Protection Buffer can vary from a clear to a brownish color.

- Add the following reagents to the 2.0 mL microtubes containing the eluate (~100 µL DNA solution):
  - 25 µL Protection Buffer
  - 150 µL Bisulfite Solution
- Cap the microtubes and mix the bisulfite reaction by vortexing for 5 to 10 sec
- Briefly spin down the microtubes and place microtubes into thermoshaker and incubate for 45 ± 5 min at 80 ± 2°C without shaking
- Remove microtubes from the thermoshaker immediately after 45 ± 5 min
- Reset thermoshaker temperature to 23 ± 2°C, or set up a second thermoshaker to 23 ± 2°C for later use

8.9. Binding Step

Note: A homogeneous suspension of beads in the Magnetic Beads is essential for proper performance. Deviations from the specified amount of beads may lead to false results. To ensure correct magnetic bead concentration, the bottle should be mixed thoroughly just before pipetting. There should be no visible sediment at the bottom of the bottle. **Mix to ensure a homogeneous suspension between the pipetting steps.**

- Briefly spin down the 2.0 mL microtubes containing the bisulfite reaction
- Add the following components to the microtube:
  - 1000 µL Wash A Buffer
  - 20 µL Magnetic Beads (freshly suspended)
- Mix by vortexing for 5 to 10 sec
- Wait until thermoshaker reaches 23 ± 2°C and place the microtubes in the thermoshaker at 1000 ± 100 rpm and incubate for 45 ± 5 min at 23 ± 2°C
- Briefly spin down the microtubes and place microtubes on the DynaMag™-2 magnetic racks for 2 to 6 min
- Using a fresh 6 inch disposable transfer pipette, remove as much liquid as possible while tubes are still in the magnetic rack, taking care not to remove magnetic beads

8.10. First Wash

- Remove the sample rack from the magnet for washing and vortexing and add 800 µL Wash A Buffer
- Resuspend by vortexing for 5 to 10 sec
- Briefly spin down the microtubes and place microtubes on the DynaMag™-2 magnetic racks for 2 to 6 min
- Using a fresh 6 inch disposable transfer pipette, remove as much liquid as possible while tubes are still in the magnetic rack, taking care not to remove magnetic beads

8.11. Second Wash

- Remove the sample rack from the magnet for washing and vortexing and add 800 µl Wash B Buffer
- Resuspend by vortexing for 5 to 10 sec
- Briefly spin down the microtubes
- Place microtubes on the DynaMag™-2 magnetic racks for 2 to 6 min
- Using a fresh 15 cm disposable transfer pipette, remove as much liquid as possible while tubes are still in the magnetic rack, taking care not to remove magnetic beads

8.12. Third Wash

- Remove the sample rack from the DynaMag™-2 magnet for washing and vortexing and add 400 µl Wash B Buffer
- Resuspend by vortexing for 5 to 10 sec

  Briefly spin down the microtubes and place microtubes on the DynaMag™-2 magnetic racks for 2 to 6 min
- Using a fresh 15cm disposable transfer pipette, remove as much liquid as possible while tubes are still in the magnetic rack, taking care not to remove magnetic beads
- Briefly spin down the microtubes and place microtubes on the DynaMag™-2 magnetic racks for 2 to 6 min
- Using a 10-100 µL reference pipette, remove as much remaining liquid as possible while tubes are still in the magnetic rack, taking care not to remove magnetic beads

Note: This drying step is very important. If the remaining droplets are not removed from the beads, remaining Wash B Buffer can be the cause of an invalid result in downstream applications.

8.13. Drying

Note: Do not increase drying time or temperature as over-drying might reduce bisDNA recovery

- Open microtube lid
- Place open microtubes into thermoshaker
- Allow the pellet to dry for 10 ± 1 min at 23 ± 2°C without shaking

8.14. Elution

- Transfer microtubes into a non-magnetic rack and add 60 µL Elution Buffer
- Close the microtubes and resuspend the magnetic beads by vortexing for 5 to 10 sec
- Incubate for 10 ± 1 min at 23 ± 2°C in a thermoshaker at 1000 ± 100 rpm
- Briefly spin down the microtubes and place microtubes on the DynaMag™-2 magnetic racks for 2 to 6 min
- Using a 10-100 µL reference pipette, transfer the complete eluate (~ 60 µL DNA solution) into 2.0 mL microtubes

8.15. Final Product: bisDNA

The Epi BiSKit generates sulfonated bisulfite DNA (bisDNA) for downstream applications. For molecular applications that require a desulfonated bisulfite DNA template, an additional desulfonation procedure will be needed.

If purified bisDNA is not used immediately, store material at 2°C to 8°C for up to 24 hours or frozen at -25°C to -15°C for up to three days.

9. Quality Control

Quality Control procedures are designed to monitor the integrity of reagent performance and ensure the accuracy (validity) of reported test results. As with any control product or feature, results should not be evaluated if control results fail to yield the expected results. Each laboratory should refer to the guidelines and procedures established by their laboratory, appropriate regulatory agencies, or accrediting organizations in accordance to the requirements of each laboratory's Quality Assurance program and accrediting organization.

It is recommended for an external positive and negative control to be included in each independent run for the chosen downstream application.

10. Limitations of the Procedure

- The Epi BiSKit generates sulfonated bisulfite DNA. For molecular applications that require a desulfonated bisulfite DNA template, an additional desulfonation procedure will be needed.
- The Epi BiSKit has been validated for use with 3.5 mL of plasma derived from whole blood samples.
- The use of blood collection tubes for generating cell-free plasma should be validated for compatibility for your specific molecular methods and requirements.
- Other sample types such as serum, urine or other body fluids have not be validated for use with this product.
- The Epi BiSKit components are not interchangeable or replaceable with other manufacturer's products.
- Use of this product is limited to personnel experienced and trained in molecular diagnostic methods

11. Meaning of Symbols

	Consult instructions for use
	Reference number
	*In Vitro* Diagnostic Medical Device
	Lot number
	Use-by date
	Manufacturer
	Temperature limits
	Contains sufficient for <n> tests
	Do not re-use
	CE mark (made in compliance with 98/79/EC Directive on IVDs)
	Data Matrix

12. Contact Information

For questions, information or Customer Support, please contact Epigenomics in one of the following ways:

Email	Support@products.epigenomics.com
On-Line	Epigenomics.com
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