applied biosystems
by life technologies

# Cells-to-CpG™ Bisulfite Conversion Kit (50)

## Protocol

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# Contents

# About This Guide

## Purpose

The *Cells-to-CpG™ Bisulfite Conversion Kit (50) Protocol* provides instructions and troubleshooting information for using the Cells-to-CpG™ Bisulfite Conversion Kit (50).

## Safety information

**Note:** For general safety information, see this section and Appendix D, "Safety" on page 39. When a hazard symbol and hazard type appear by an instrument hazard, see the "Safety" Appendix for the complete alert on the instrument.

### Safety alert words

Four safety alert words appear in Applied Biosystems user documentation at points in the document where you need to be aware of relevant hazards. Each alert word—**IMPORTANT, CAUTION, WARNING, DANGER**—implies a particular level of observation or action, as defined below:

**IMPORTANT!** – Indicates information that is necessary for proper instrument operation or accurate chemistry kit use.

**CAUTION!** – Indicates a potentially hazardous situation that, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

**WARNING!** – Indicates a potentially hazardous situation that, if not avoided, could result in death or serious injury.

**DANGER!** – Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury. This signal word is to be limited to the most extreme situations.

## SDSs

The Safety Data Sheets (SDSs) for any chemicals supplied by Applied Biosystems or Ambion are available to you free 24 hours a day. For instructions on obtaining SDSs, see "SDSs" on page 40.

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**IMPORTANT!** For the SDSs of chemicals not distributed by Applied Biosystems or Ambion contact the chemical manufacturer.

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# Cells-to-CpG™ Bisulfite Conversion Kit (50)

## Product information

### Purpose of the product

Use the Cells-to-CpG™ Bisulfite Conversion Kit (50) (PN 4445555) as the first step in your methylation study. This kit allows you to perform direct and fast conversion of unmethylated cytosines to uracil in purified genomic DNA, cultured cells, or blood, in a single-tube format.

You can use the converted DNA in your methylation study to:

- Perform high-resolution melting (HRM) analysis of the bisulfite-converted samples and converted methylation standards to determine the percentage of methylated cytosines in the samples.
- Perform cycle sequencing analysis of converted and untreated samples to determine which cytosines are methylated.

### Kit contents and storage

Sufficient materials are supplied in the Cells-to-CpG™ Bisulfite Conversion Kit (50) to perform bisulfite conversion of 50 samples. Store all kit components at room temperature.

| Component | Amount |
|---|---|
| Binding Buffer | 1 × 50-rxn bottle |
| Binding Columns | 50 columns |
| Conversion Buffer | 2 × 1.5 mL |
| Conversion Reagent | 5 × 10-rxn tubes |
| Denaturation Reagent | 0.5 mL |
| Desulfonation Reagent | 1 × 50-rxn bottle |
| Elution Buffer | 2 × 1 mL |
| Elution Tubes | 50 tubes |
| Lysis Enhancer | 0.5 mL |
| Wash Buffer | 1 × 50-rxn bottle |

# Materials and equipment required but not included

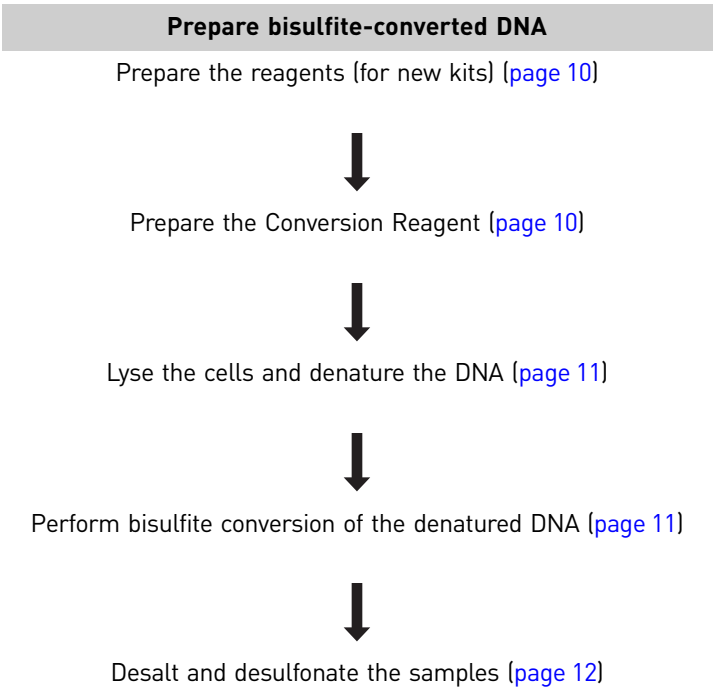
For optional materials and equipment and more ordering information, see Appendix A, "Ordering Information" on page 19.

For the SDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the SDS provided by the manufacturer, and observe all relevant precautions.

| Item | Source |
|---|---|
| Veriti® 96-Well Thermal Cycler | Applied Biosystems PN 4375786 |
| MicroAmp® Clear Adhesive Film, 100 films | Applied Biosystems PN 4306311 |
| MicroAmp® Optical Film Compression Pad, 5 each | Applied Biosystems PN 4312639 |
| Nuclease-free Water (not DEPC-treated) (1 × 500 mL) | Applied Biosystems PN AM9930 |
| Reaction plates compatible with your thermal cycler:<br>• MicroAmp® Fast 96-Well Reaction Plate, 0.1 mL, 10 plates<br>• MicroAmp® Optical 96-Well Reaction Plate, 10 plates | Applied Biosystems<br>• PN 4346907<br>• PN N8010560 |
| Reaction tubes or strips and caps compatible with your thermal cycler:<br>• MicroAmp® Reaction Tube with Cap, 0.2 mL, 1000 tubes<br>• MicroAmp® 8-Tube Strip, 0.2 mL, 125 strips<br>• MicroAmp® 8-Tube Strip, Assorted Colors, 0.2 mL, 120 strips | Applied Biosystems<br><br>• N8010540<br>• N8010580<br>• N8010838 |
| Centrifuge with plate adapters | Major laboratory suppliers (MLS) |
| Ethanol, molecular biology grade, ≥99.5% or 200 proof | MLS |
| Isopropyl alcohol, ACS reagent grade, ≥99.5% | MLS |
| Microcentrifuge | MLS |
| Microcentrifuge tubes | MLS |
| Pipette tips, nuclease-free | MLS |
| Pipettors, positive displacement or air-displacement | MLS |
| Vortexer | MLS |
| Water bath at 60 °C | MLS |

# Worfklow

This protocol provides instructions for preparing converted DNA using the Cells-to-CpG™ Bisulfite Conversion Kit (50).



# Prepare bisulfite-converted DNA

## Acceptable sample types and recommended input amounts

| Input type | Optimal input amount | Minimum input amount | Maximum input amount |
|---|---|---|---|
| Purified gDNA | 100 ng to 1 µg | 50 pg | 5 µg |
| Cultured cells | 5000 cells to $10^5$ cells | 10 cells | $10^5$ cells |
| Blood | 2.5 µL | 1 µL | 5 µL |

**Note:** Use the Unconverted Human Male gDNA (Control Sample 1) from the Cells-to-CpG™ Bisulfite Conversion and Quantitation Control Kit as an external sample control for bisulfite conversion. For instructions, see page 26.

## Prepare the reagents (for new kits)

Before you use a new kit, prepare the Binding Buffer, Wash Buffer, and Desulfonation Reagent.

1. Add 14 mL isopropyl alcohol (≥99.5%) to the bottle of Binding Buffer, then swirl the bottle to mix.
2. Add 44 mL ethanol (≥99.5% or 200 proof) to each bottle of Wash Buffer, then swirl the bottles to mix.
3. Add 10 mL ethanol (≥99.5% or 200 proof) to each bottle of Desulfonation Reagent, then swirl the bottles to mix.

After you prepare the reagents, you will have sufficient reagents for 50 reactions.

## Prepare the Conversion Reagent

For optimal results, prepare the Conversion Reagent immediately before performing the bisulfite conversion. Each tube of prepared Conversion Reagent contains sufficient reagent for 10 bisulfite conversion reactions.

1. Add Denaturation Reagent and water to the powder in one Conversion Reagent tube, then mix well:

| Component | Volume |
|---|---|
| Denaturation Reagent | 26 µL |
| Water | 0.8 mL |

2. Add 50 µL Conversion Buffer to the Conversion Reagent tube, then mix again.
3. To improve solubility of the powder, place the Conversion Reagent tube in a 60 °C water bath for 10 minutes.

4. Mix by vortexing 2 or 3 times during the 10-minute incubation.

**Note:** It is normal to see trace amounts of undissolved powder in the Conversion Reagent.

After you prepare the bisulfite conversion reactions, you can store any remaining Conversion Reagent at –20 °C for up to 1 month. Before use, thaw the Conversion Reagent at 50 °C for 10 minutes, then vortex.

## Lyse the cells and denature the DNA

If you are starting with purified gDNA, skip the cell lysis step.

1. Set up the PCR tubes or reaction plate. For each sample:
   - **Purified gDNA**: Pipet 45 µL into a PCR tube or reaction plate, then skip cell lysis and proceed with denaturing the DNA.
   - **Cultured cells, blood, tissue, and FFPE samples**: Pipet 40 µL into a PCR tube or reaction plate.

2. (Cultured cells, blood, tissue, and FFPE samples only) To lyse the cells in the PCR tube or reaction plate, add 5 µL Lysis Enhancer to 40 µL of sample, then swirl the tube or pipet up and down to mix.

3. Denature the DNA in the PCR tube or reaction plate:
   a. Add 5 µL Denaturation Reagent to 45 µL lysed cells or purified gDNA, then swirl the tube or pipet up and down to mix.
   b. Incubate at 50 °C for 10 minutes.

## Perform bisulfite conversion of the denatured DNA

Convert unmethylated cytosines to uracil in the denatured DNA samples.

1. Add 100 µL prepared Conversion Reagent to each denatured sample for a total reaction volume of 150 µL, then mix the reaction.

   **Note:** The reaction should not contain any undissolved Conversion Reagent powder after mixing.

2. Incubate the sample in a thermal cycler, selecting the appropriate thermal cycling conditions for your samples:
   - For gDNA input between 100 ng and 2 µg, use general thermal cycling conditions with acceptable recovery and conversion:

| Temp | Time |
|---|---|
| 65 °C | 30 minutes |
| 95 °C | 1.5 minutes |
| 65 °C | 30 minutes |
| 95 °C | 1.5 minutes |

| Temp | Time |
|---|---|
| 65 °C | 30 minutes |
| 4 °C | Up to 4 hours |

- For crude samples and gDNA input ≥2 µg, use thermal cycling conditions optimal for conversion but with slightly decreased recovery for long amplicons:

| Temp | Time |
|---|---|
| 95 °C | 3 minutes |
| 65 °C | 60 minutes |
| 95 °C | 3 minutes |
| 65 °C | 30 minutes |
| 4 °C | Up to 4 hours |

- For gDNA input between 50 pg and 100 ng, use thermal cycling conditions optimal for recovery but with decreased conversion rates for high sample input:

| Temp | Time |
|---|---|
| 65 °C | 30 minutes |
| 95 °C | 0.5 minutes |
| 65 °C | 30 minutes |
| 95 °C | 0.5 minutes |
| 65 °C | 30 minutes |
| 4 °C | Up to 4 hours |

STOPPING POINT If you cannot proceed with desalting and desulfonation immediately, you can store the converted sample at -20 °C for up to 2 days. Thaw the samples at room temperature, then proceed with desalting and desulfonation.

## Desalt and desulfonate the samples

Remove salts from the DNA samples, then desulfonate the DNA to remove the sulfonic groups.

1. Spin the Binding Column with a microcentrifuge tube at 10,000 rpm for 1 minute.
2. Load the Binding Column:
   a. Add 600 µL of Binding Buffer to the column.
   b. Add the converted sample (150 µL) to the column containing the Binding Buffer.

c. Close the cap, then invert the column several times to mix.

d. Spin the column at 10,000 rpm for 1 minute.

e. Discard the flowthrough.

3. Wash the sample to remove salts:

a. Return the column to the microcentrifuge tube.

b. Add 600 µL of Wash Buffer to the column.

c. Spin the column at 10,000 rpm for 1 minute, or until all the Wash Buffer passes through the column.

d. Discard the flowthrough.

4. Desulfonate the DNA:

a. Return the column to the microcentrifuge tube.

b. Add 200 µL of Desulfonation Reagent to the column.

c. Close the cap, then let the column stand at room temperature (20–30 °C) for 15 minutes.

**IMPORTANT!** It is important that you close the cap before the 15-minute incubation.

d. Spin the column at 10,000 rpm for 1 minute.

e. Discard the flowthrough.

5. Wash the DNA:

a. Return the column to the microcentrifuge tube.

b. Add 400 µL of Wash Buffer to the column.

c. Spin the column at 10,000 rpm for 2 minutes, or until all the Wash Buffer passes through the column.

d. Discard the flowthrough.

6. Remove any residual Wash Buffer:

a. Return the column to the microcentrifuge tube.

b. Spin the column at 10,000 rpm for 1 minute.

**IMPORTANT!** Trace amounts of Wash Buffer could inhibit downstream reactions.

c. Discard the flowthrough and the microcentrifuge tube.

7. Elute the DNA:

a. Place the column onto a new Elution Tube.

b. Add 40 µL Elution Buffer directly to the center of the column.

**Note:** If you started with less than 10 ng gDNA, you can reduce the elution volume to 10 µL.

c. Let the column stand at room temperature (20–30 °C) for 2 minutes.

d. Spin the column at 10,000 rpm for 1 minute.

STOPPING POINT Store the converted DNA at 4 °C for up to 6 months. For long-term storage, store at –20 °C or –70 °C. Store aliquots to avoid multiple freeze-thaw cycles.

## Assess the yield and quality of the bisulfite-converted DNA

Use the Cells-to-CpG™ Bisulfite Conversion and Quantitation Control Kit to assess the quality of the converted DNA. For instructions on how to use the control kit, see Appendix B on page 25.

1. Determine the efficiency of bisulfite conversion (page 27).
2. Quantify the amount of bisulfite-converted DNA (page 32).
3. Determine the integrity of the bisulfite-converted DNA (page 35).

# After bisulfite conversion

After you prepare the converted DNA, you can proceed with:

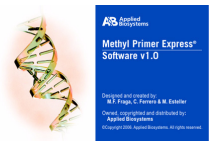
- High resolution melting (HRM) analysis and sequencing analysis (page 15)
  or
- Sequencing analysis (page 16)

## HRM analysis and sequencing by capillary electrophoresis

For instructions, see the *High Resolution Melting Getting Started Guide* (PN 4393102).

**Perform high resolution melting analysis**

Design the primers using Methyl Primer Express® Software v1.0

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Optimize the HRM reactions


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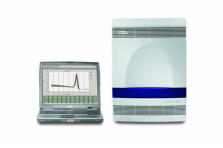
Prepare the HRM reactions using MeltDoctor™ HRM Master Mix or the MeltDoctor™ HRM Reagent Kit



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Amplify and melt the DNA using the 7500 Fast Real-Time PCR System or the 7900HT Fast Real-Time PCR System

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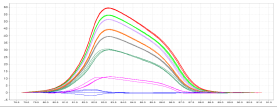
Review the high-resolution melting data using High Resolution Melting Software v2.0



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**Sequence the variants (optional)**

Purify the PCR product using ExoSAP-IT® and an Applied Biosystems thermal cycler

↓

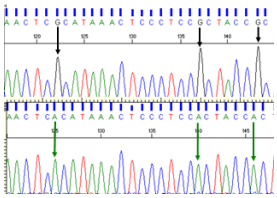
Perform cycle sequencing reactions using the BigDye® Terminator v1.1 Cycle Sequencing Kit and an Applied Biosystems thermal cycler

↓

Clean up the sequencing reaction using the BigDye XTerminator® Purification Kit

↓

Perform capillary electrophoresis using an Applied Biosystems DNA Sequencer



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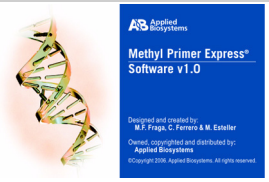
Review the sequencing data using SeqScape® Software or Variant Reporter® Software

## Sequencing by capillary electrophoresis

**Sequence the samples**

Design the primers using Methyl Primer Express® Software v1.0




↓

Amplify the DNA using AmpliTaq Gold® DNA Polymerase and an Applied Biosystems thermal cycler

↓

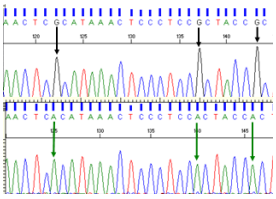
Perform cycle sequencing reactions using the BigDye® Terminator v1.1 Cycle Sequencing Kit or BigDye® Terminator v3.1 Cycle Sequencing Kit and an Applied Biosystems thermal cycler

↓

Clean up the sequencing reaction using the BigDye XTerminator® Purification Kit

↓

Perform capillary electrophoresis using an Applied Biosystems DNA Sequencer



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Review the sequencing data using SeqScape® Software or Variant Reporter® Software

# Troubleshooting

| Observation | Possible cause | Recommended action |
|---|---|---|
| Incomplete conversion:<br>• Conversion rate is <99% using the Unconverted Control Sample 1 provided with the kit<br>or<br>• DNA sequence after bisulfite conversion shows Cs instead of Ts at unmethylated Cs | Too much DNA template added to the bisulfite conversion reactions | Repeat the reactions using the recommended amounts of DNA template (page 10). |
| | Temperature variability during the bisulfite conversion reactions | Verify the performance of your thermal cycler. |
| | Insufficient amounts of denaturing or conversion reagents added to the samples | Repeat the denaturation or the bisulfite conversion using the harsher conversion conditions. |

| Observation | Possible cause | Recommended action |
|---|---|---|
| $C_T$ values much higher than expected when quantifying the amount of converted DNA using the control kit (page 33) | Low rate of bisulfite conversion | Determine the efficiency of bisulfite conversion (page 27). |
| | Recovery rate is low | Check the OD 260 reading of the converted DNA. |
| | PCR reactions contain carryover contaminants such as isopropyl alcohol or ethanol that inhibit the PCR and shift the Tm in melt curves | Use less converted DNA sample in the PCR reactions or use a vacuum centrifuge to evaporate the isopropyl alcohol and ethanol. |
| All PCR reactions using the conversion and quantitation kit failed | PCR master mix contains uracil-N-glycosylase (UNG) | Use PCR master mix with no UNG. |
| | PCR reactions do not contain a dye for real-time PCR detection | • Run the PCR products on an agarose gel to check whether the samples amplified.<br>• Repeat the PCR reactions, making sure that you add a dye for real-time PCR detection. |
| Recovery rate is <50% | Poor quality of the starting gDNA template | Check the quality of the starting gDNA template. |
| | Insufficient isopropyl alcohol in the Binding Buffer or insufficient ethanol in the Wash Buffer | Verify that isopropyl alcohol was added to the Binding Buffer and that ethanol was added to the Wash Buffer before desalting and desulfonation. |
| | Dirty NanoDrop spectrophotometer | Check the OD reading at 260 nm. If the OD 260 is ≥0.1, clean the NanoDrop spectrophotometer and repeat the OD reading. |
| Unsuccessful amplification of long amplicons | Degraded converted DNA degraded | Repeat the bisulfite conversion using milder conversion conditions. |
| | PCR conditions not optimized for longer amplicons | Perform PCR optimization (extension time, number of cycles, $MgCl_2$ concentrations, and so on). |
| | Insufficient amounts of converted DNA in the PCR reactions | Add ≥10 ng of converted DNA to the PCR reactions. |
| | PCR reactions do not contain sufficient primer concentrations | Add up to 600 µM of each primer to the PCR reactions. |
| Amplification observed with negative controls | Template contamination in reagents, in the laboratory, or on equipment | Clean up the lab and use new reagents for PCR. Make sure you follow best practices in PCR to avoid contamination. |
| | Primer-dimer formation | Check the primer design and if needed, design new primers. |
| | | Reduce the primer concentrations in the PCR reactions. |
| | | Reduce the number of cycles in the amplification run. |

| Observation | Possible cause | Recommended action |
|---|---|---|
| Failed PCR amplifications for direct-converted DNA from FFPE samples | DNA degraded in long-term archived FFPE samples | • Check the quality of the converted DNA and quantify the amount of bisulfite-converted DNA (page 32).<br>• Combine converted DNA from different slides.<br>• Perform preamplification of the targets of interest. |
| | Insufficient converted DNA in the PCR reactions | |
| Long amplicons cannot be sequenced successfully using capillary electrophoresis | Long stretches of Ts after conversion causes slippage of the DNA polymerase during single strand extension | • Sequence the opposite strand using the reverse primer.<br>or<br>• Redesign the sequencing primers to avoid long Ts. |

# Ordering Information

For the SDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the SDS provided by the manufacturer, and observe all relevant precautions.

## How to order Cells-to-CpG™ kits

For information on the Cells-to-CpG™ Bisulfite Conversion Kits, go to the Applied Biosystems website at **www.appliedbiosystems.com** and select:

**Products ▸ Gene Expression ▸ Controls, Reagents, & Kits ▸ DNA Methylation Analysis Kits (DNA Sequencing Based) ▸ Cells-to-CpG™ Bisulfite Conversion Kit**

| Item | Source |
|---|---|
| Cells-to-CpG™ Bisulfite Conversion Kit (2x96) | Applied Biosystems PN 4445554 |
| Cells-to-CpG™ Bisulfite Conversion Kit (50) | Applied Biosystems PN 4445555 |
| Cells-to-CpG™ Bisulfite Conversion and Quantitation Control Kit | Applied Biosystems PN 4445553 |
| Cells-to-CpG™ Methylated and Unmethylated gDNA Control Kit | Applied Biosystems PN 4445552 |

## Materials and equipment for using the control kits

| Item | Source |
|---|---|
| E-Gel® 4% High-Resolution Agarose 18-Pak | Invitrogen PN G501804 |
| MeltDoctor™ HRM Master Mix, 5 mL bottle | 4415440 |
| MeltDoctor™ HRM Master Mix, 5×5 mL bottle | 4415452 |
| MeltDoctor™ HRM Master Mix, 10×5 mL bottle | 4415450 |
| MeltDoctor™ HRM Master Mix, 50 mL bottle | 4409535 |
| Real-time PCR instrument (96-well system):<br>• 7500 Fast Real-Time PCR System<br>• 7900HT Fast Real-Time PCR System<br>• StepOnePlus™ Real-Time PCR System | Applied Biosystems |

| Item | Source |
|---|---|
| TrackIt™ 50 bp DNA Ladder | Invitrogen PN 10488-043 |
| UltraPure™ DNase/RNase-Free Distilled Water, 500 mL | Invitrogen PN 10977-015 |
| M13 forward and reverse sequencing primers:<br>• M13 Forward (–20), 2 µg<br>• M13 Reverse, 2 µg<br>**Note:** Use only if the HRM PCR product contains M13 sequences. | Invitrogen<br>• PN N520-02<br>• PN N530-02 |
| Deionized Water | MLS |

# Materials and equipment for high resolution melting analysis

## MeltDoctor™ HRM reagents

| Item | Applied Biosystems part number |
|---|---|
| MeltDoctor™ HRM Master Mix, 5 mL bottle | 4415440 |
| MeltDoctor™ HRM Master Mix, 5×5 mL bottle | 4415452 |
| MeltDoctor™ HRM Master Mix, 10×5 mL bottle | 4415450 |
| MeltDoctor™ HRM Master Mix, 50 mL bottle | 4409535 |
| MeltDoctor™ HRM Positive Control Kit:<br>• MeltDoctor™ HRM Allele A DNA (20X), 150 µL<br>• MeltDoctor™ HRM Allele G DNA (20X), 150 µL<br>• MeltDoctor™ HRM Allele A/G DNA (20X), 150 µL<br>• MeltDoctor™ HRM Primer Mix (20X), 500 µL | 4410126 |
| MeltDoctor™ HRM Reagent Kit:<br>• AmpliTaq Gold® 360 DNA Polymerase<br>• AmpliTaq Gold® 360 Buffer<br>• 360 GC Enhancer<br>• GeneAmp® dNTP Blend<br>• MeltDoctor™ HRM Dye (20X) | 4425557 |

## Equipment and software

| Item | Source |
|---|---|
| 7500 Fast Real-Time PCR System with Notebook Computer | Applied Biosystems PN 4351106 |
| 7500 Fast Real-Time PCR System with Tower Computer | Applied Biosystems PN 4351107 |

| Item | Source |
|---|---|
| 7900HT Fast Real-Time PCR System with 384-Well Block Module | Applied Biosystems PN 4329001 |
| 7900HT Fast Real-Time PCR System with Fast 96-Well Block Module | Applied Biosystems PN 4351405 |
| High Resolution Melting (HRM) Software v2.0 | Applied Biosystems PN 4397808 |
| Methyl Primer Express® Software v1.0 | Applied Biosystems PN 4376041 |
| Centrifuge with plate adapters | Major laboratory suppliers (MLS) |
| Lab equipment | MLS |
| Microcentrifuge | MLS |
| Microcentrifuge tubes | MLS |
| Pipettors and pipette tips | MLS |
| Vortexer | MLS |

## Supplies

| Item | Source |
|---|---|
| Appropriate reaction plate for your instrument:<br>• MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL<br>• MicroAmp® Fast Optical 48-Well Reaction Plate | Applied Biosystems<br>• PN 4346906 and 4366932<br>• PN 4375816 |
| MicroAmp® 96- & 384-Well Optical Adhesive Film:<br>• 25 films<br>• 100 films | Applied Biosystems<br>• PN 4360954<br>• PN 4311971 |
| MicroAmp® 48-Well Optical Adhesive Film:<br>• 25 films<br>• 100 films | Applied Biosystems<br>• PN 4375928<br>• PN 4375323 |
| Microcentrifuge tubes | MLS |
| Pipettors and pipette tips | MLS |

# Materials and equipment for sequencing

## Equipment

| Item | Source |
|---|---|
| Applied Biosystems DNA sequencer:<br>• Applied Biosystems 3500/3500*xl* DNA Analyzer<br>• Applied Biosystems 3730/3730*xl* DNA Analyzer<br>• Applied Biosystems 3130/3130*xl* DNA Analyzer<br>• Applied Biosystems 3100/3100-*Avant* DNA Analyzer | Applied Biosystems |
| Veriti® 96-Well Fast Thermal Cycler | Applied Biosystems |
| Centrifuge with plate adapters | MLS |
| Microcentrifuge | MLS |
| Vortexer | MLS |

## Supplies

| Item | Source |
|---|---|
| MicroAmp® Clear Adhesive Film, 100 films | Applied Biosystems<br>PN 4306311 |
| MicroAmp® Optical Film Compression Pad, 5 each | Applied Biosystems<br>PN 4312639 |
| Wide-bore (>1 mm) pipette tips:<br>• Wide-Orifice Tips<br>• Clear Wide Bore Tips | <br>• Rainin Instrument LLC<br>• Axygen Scientific Inc. |
| Microcentrifuge tubes | MLS |
| Pipettors and pipette tips | MLS |

## Reagents

| Item | Source |
|---|---|
| AmpliTaq Gold® 360 Master Mix, 1 mL | Applied Biosystems<br>PN 4398876 |
| AmpliTaq Gold® 360 Master Mix, 5 mL | Applied Biosystems<br>PN 4398881 |
| AmpliTaq Gold® 360 Master Mix, 10 × 5 mL | Applied Biosystems<br>PN 4398901 |
| AmpliTaq Gold® 360 Master Mix, 50 mL | Applied Biosystems<br>PN 4398886 |

| Item | Source |
|---|---|
| AmpliTaq Gold® 360 Master Mix Protocol | Applied Biosystems<br>PN 4398944 |
| AmpliTaq Gold® 360 Master Mix Quick Reference Card | Applied Biosystems<br>PN 4398954 |
| BigDye® Terminator v1.1 Cycle Sequencing Kit, 100 reactions | Applied Biosystems<br>PN 4337450 |
| BigDye XTerminator® Purification Kit, 2 mL (~100 20-µL reactions) | Applied Biosystems<br>PN 4376486 |
| M13 forward and reverse sequencing primers:<br>• M13 Forward (–20), 2 µg<br>• M13 Reverse, 2 µg<br>**Note:** Use only if the HRM PCR product contains the M13 sequences. | Invitrogen<br>• PN N520-02<br>• PN N530-02 |
| UltraPure™ DNase/RNase-Free Distilled Water, 500 mL | Invitrogen PN 10977-015 |
| ExoSAP-IT®, 100 reactions | USB Corporation<br>PN 78200 |
| Deionized Water | MLS |

# Use Controls for Bisulfite Conversion

## Product information

### Purpose of the control kit

When you perform methylation studies, you can use the components of the Cells-to-CpG™ Bisulfite Conversion and Quantitation Control Kit (PN 4445553) to serve as controls during the bisulfite conversion and amplification of the bisulfite-converted DNA:



### Kit components and storage

Store kit components at –20 °C for long-term storage or at 4 °C for short-term storage.

| Component | Amount |
|---|---|
| Unconverted Human Male gDNA (Control Sample 1) (100 ng/µL) | 50 µL |
| Converted Human Male gDNA (Control Sample 2) (10 ng/µL) | 20 µL |
| 20× Non-Conversion Control Primer Mix | 20 µL |
| 20× Conversion Control Primer Mix | 20 µL |
| 20× Quantitation Control Primer Mix | 20 µL |
| 20× Long Amplicon Control Primer Mix | 20 µL |

# Use an external sample control for bisulfite conversion

Use the Unconverted Human Male gDNA (Control Sample 1) (100 ng/µL) as an external sample control during the bisulfite conversion.

## Perform bisulfite conversion of Control Sample 1

1. Prepare 45 µL sample:
   - 1 µL Unconverted Human Male gDNA (Control Sample 1) (100 ng/µL)
   - 44 µL deionized water
2. Prepare the bisulfite-converted DNA:
   a. Prepare the Conversion Reagent (page 10)
   b. Lyse the cells and denature the DNA (page 11) (skip the cell lysis step)
   c. Perform bisulfite conversion of the denatured DNA (page 11)
   d. Desalt and desulfonate the samples (page 12)
   e. Assess the yield and quality of the bisulfite-converted DNA (page 14)

## How to use the bisulfite-converted Control Sample 1

After you complete bisulfite conversion of the Unconverted Human Male gDNA and your DNA samples, you can use the Unconverted Human Male gDNA as an external sample control when you:

- Determine the efficiency of bisulfite conversion (page 29)
- Quantify the amount of bisulfite-converted human genomic DNA (page 32)
- Determine the integrity of bisulfite-converted human genomic DNA (page 35)

# Determine the efficiency of bisulfite conversion

After you perform the bisulfite conversion procedures of Control Sample 1 and of your human genomic DNA samples, perform real-time PCR of the samples and controls to determine:

- The amount of unconverted human genomic DNA using 20× Non-Conversion Control Primer Mix.
- The amount of converted human genomic DNA using 20× Conversion Control Primer Mix.

Based on the difference in the $C_T$ values, you can determine the conversion efficiency.

## Required materials

- Unconverted Human Male gDNA (Control Sample 1) (100 ng/µL)
- Bisulfite-converted Control Sample 1
- Converted Human Male gDNA (Control Sample 2) (10 ng/µL)
- Your bisulfite-converted human genomic DNA samples
- 20× Non-Conversion Control Primer Mix
- 20× Conversion Control Primer Mix
- MeltDoctor™ HRM Master Mix
- Deionized water
- Real-Time PCR System
- E-Gel® 4% High-Resolution Agarose Gel
- TrackIt™ 50 bp DNA Ladder

## Amplify the unconverted and converted human genomic DNA

**Note:** For detailed instructions on how to set up and run your real-time PCR instrument, refer to the appropriate guide for your instrument.

1. Amplify the samples and controls in two sets of reactions: one set of reactions with 20× Non-Conversion Control Primer Mix and the other set of reactions with 20× Conversion Control Primer Mix:

| Component | Volume per reaction |
|---|---|
| DNA sample or control | 1.0 µL |
| One Control Primer Mix:<br>• 20× Non-Conversion Control Primer Mix<br>or<br>• 20× Conversion Control Primer Mix | 0.5 µL |
| MeltDoctor™ HRM Master Mix | 5.0 µL |
| Deionized water | 3.5 µL |
| **Total Volume** | **10 µL** |

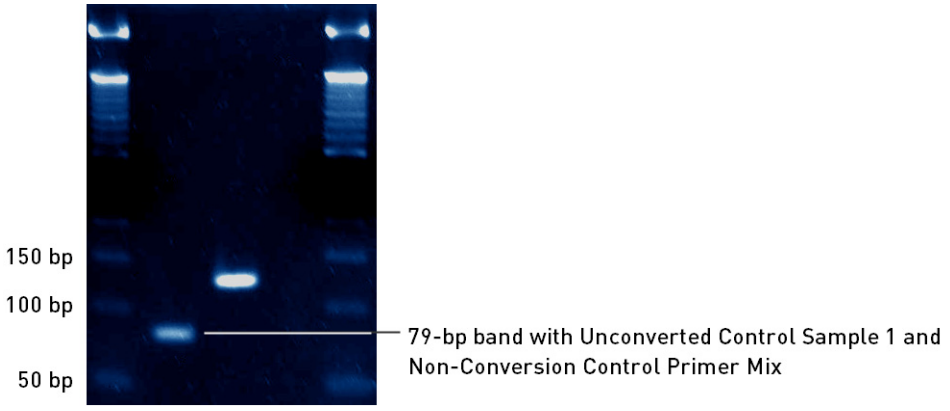
2. Run the reactions in a Real-Time PCR System:

| Stage | Step | Temp | Time |
|---|---|---|---|
| Holding | Enzyme activation | 95 °C | 10 min |
| Cycling (40 or 45 cycles) | Denature | 95 °C | 15 sec |
| | Anneal | 60 °C | 1 min |
| Melt curve/dissociation | Denature | 95 °C | 15 sec |
| | Anneal | 60 °C | 15 sec |
| | Melt | 95 °C | 15 sec |

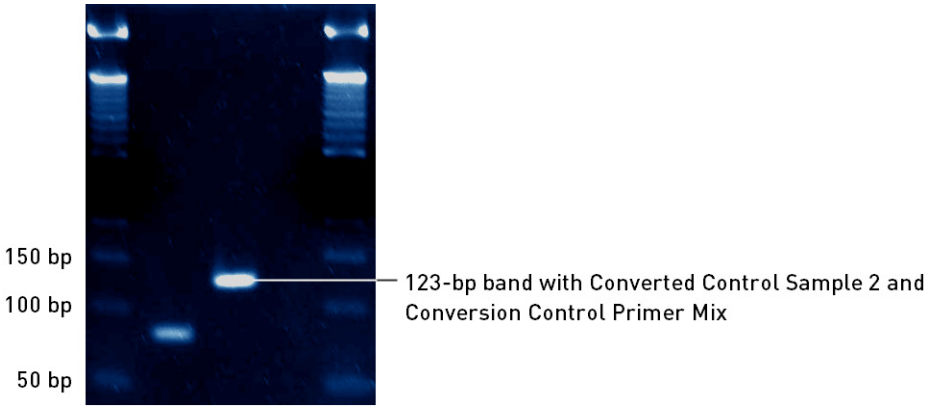
## Review the size of the PCR products

Run a 4%E-Gel® agarose gel to determine whether the PCR products you obtained are the expected size and to compare the amounts of converted and unconverted DNA.

1. Review the lanes with samples and controls amplified using the Non-Conversion Control Primer Mix:
   - In the lanes with the bisulfite-converted DNA samples, do you see no detectable bands? If you see a 79-bp band, the bisulfite conversion may be incomplete. If you see the 79-bp band in the lane with Converted Control Sample 2, the reactions may be contaminated.
   - In the lane with Unconverted Control Sample 1, do you see a 79-bp band? If you do not see the 79-bp band, the amplification reactions were not successful.

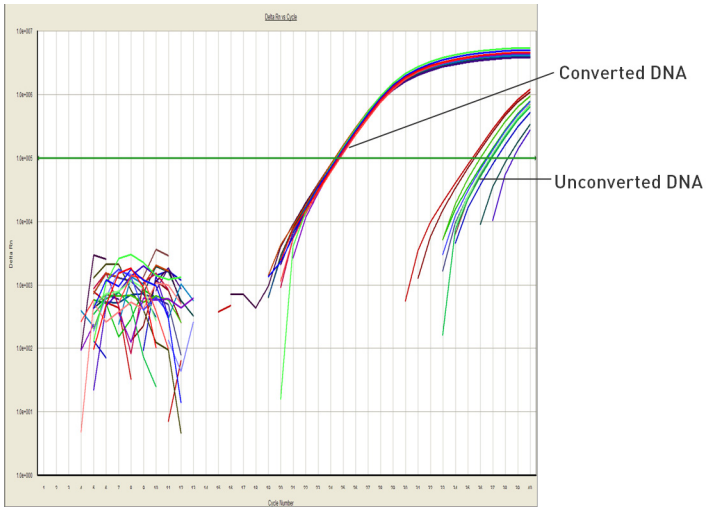


2. Review the lanes with samples and controls amplified using the Conversion Control Primer Mix:
   - In the lane with Unconverted Control Sample 1, do you see no detectable bands? If you see the 123-bp band, the reactions may be contaminated.
   - In the lanes with the bisulfite-converted DNA samples, do you see a distinct, strong 123-bp band in each lane? If the band is not clear or is not strong, the bisulfite conversion may be incomplete.
   - In the laneswith Converted Control Sample 2, do you see a distinct, strong 123-bp band? If you do not see the 123-bp band, the amplification reactions were not successful.

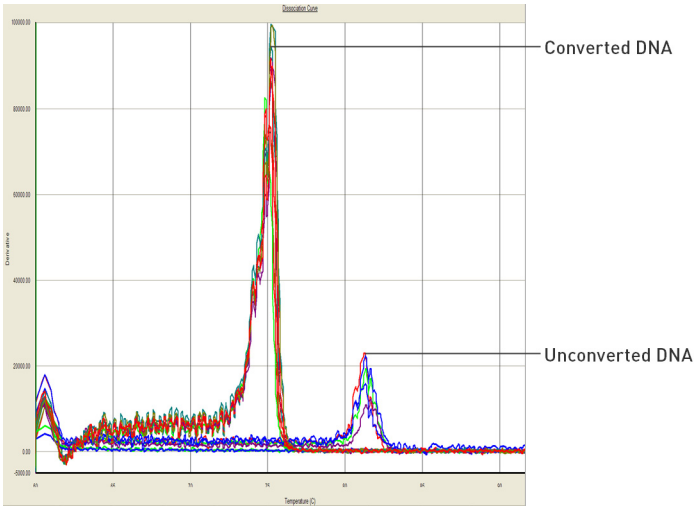



## Determine the efficiency of bisulfite conversion

1. Review the amplification plots and $C_T$ values:
   - For the bisulfite-converted DNA samples, are the $C_T$ values for the amplification reactions using the 20× Conversion Control Primer Mix low? If you see high $C_T$ values, then the bisulfite conversion may not been successful.
   - For the bisulfite-converted DNA samples, are the $C_T$ values for the amplification reactions using the 20× Non-Conversion Control Primer Mix high? If you see low $C_T$ values, then the bisulfite conversion may not been successful.



2. Review the Tm peaks in the Melt Curve:
   - Do you see the Tm peak with your samples that were amplified using the 20× Conversion Control Primer Mix?
   - Do the control samples show the expected Tm peaks?




3. Compare the fluorescence generated in the Control Primer Mix 1 reactions to the fluorescence generated in the Control Primer Mix 2 reactions:
   - For the Control Primer Mix 2 reactions, do the fluorescence levels exceed the threshold between cycles 8 and 35?
   - For the Control Primer Mix 2 reactions, is there an exponential increase in fluorescence?

4. Calculate the efficiency of bisulfite conversion for each sample:

$$\text{Conversion efficiency} = \left( \frac{2^{(-\Delta C_T + 1)}}{2^{(-\Delta C_T + 1)} + 1} \right) \times 100$$

**Note:** $\Delta C_T = C_{T\,(\text{converted})} - C_{T\,(\text{unconverted})}$

**Note:** To use this formula to calculate the conversion efficiency, you need to use at least 10 ng of converted DNA sample in the PCR amplification reactions. If you are performing bisulfite conversion with small samples such as FFPE samples, we recommend that you perform bisulfite sequencing to calculate the conversion efficiency.

## Expected results

Bisulfite conversion is successful if $\Delta C_T = C_{T\ (converted)} - C_{T\ (unconverted)}$ value is less than –7 and the percentage of bisulfite conversion is ≥ 99.5%.

| Sample | Amount of unconverted DNA: Non-Conversion primer mix target (79 bp) | Amount of bisulfite-converted DNA: Conversion primer mix target (123 bp) | Expected % of bisulfite conversion |
|---|---|---|---|
| Unconverted Control Sample 1 | Amplification | No amplification | 0% |
| Bisulfite-converted Control Sample 1 | Little or no amplification | Amplification | ≥ 99.5% |
| Converted Control Sample 2 | No amplification | Amplification | 100% |
| Unknown sample | Little or no amplification | Amplification | ≥ 99.5% |

# Quantify the amount of bisulfite-converted DNA

Perform a standard curve experiment on a Real-Time PCR System using the Converted Human Male gDNA (Control Sample 2) (10 ng/µL) and 20× Quantitation Control Primer Mix to quantify the amount of bisulfite-converted human genomic DNA in your samples.

## Required materials

- Converted Human Male gDNA (Control Sample 2) (10 ng/µL)
- Bisulfite-converted Control Sample 1
- Your bisulfite-converted human genomic DNA samples
- 20× Quantitation Control Primer Mix
- MeltDoctor™ HRM Master Mix
- Deionized water
- Real-Time PCR System

## Guidelines for preparing the standard dilution series

Prepare a standard dilution series using the Converted Human Male gDNA (Control Sample 2) (10 ng/µL) to generate the standard curve. Follow these Applied Biosystems recommendations:

- Perform 1:2 serial dilutions, and prepare at least 5 standards.
- Select the standards so that the range of standard concentrations is within the expected range of converted DNA concentrations. The expected range is 0.5–10 ng/µL.
- Prepare sufficient standard so that you can prepare 3 replicate reactions for each dilution point

## Example of standard dilution series

In the example below, the range of DNA concentrations is 0.3125 ng/µL–10 ng/µL.

| Standard | Source | Source volume | Volume of deionized water | Standard concentration (ng/µL) |
|---|---|---|---|---|
| Standard 1 (undiluted) | Control Sample 2 (10 ng/µL) | 10 µL | 0 µL | 10 ng/µL |
| Standard 2 (1:2) | Standard 1 | 5 µL | 5 µL | 5 ng/µL |
| Standard 3 (1:4) | Standard 2 | 5 µL | 5 µL | 2.5 ng/µL |
| Standard 4 (1:8) | Standard 3 | 5 µL | 5 µL | 1.25 ng/µL |
| Standard 5 (1:16) | Standard 4 | 5 µL | 5 µL | 0.625 ng/µL |
| Standard 6 (1:32) | Standard 5 | 5 µL | 5 µL | 0.3125 ng/µL |

## Amplify and melt the bisulfite-converted human genomic DNA

For detailed instructions on how to set up and run your Real-Time PCR Instrument, refer to the appropriate guide for your instrument.

1. Prepare a dilution series of the Converted Human Male gDNA (Control Sample 2) (10 ng/µL) to generate the standard curve.

2. Prepare the reactions:

| Component | Volume per reaction |
|---|---|
| Converted DNA sample, standard, or control | 1.0 µL |
| 20× Quantitation Control Primer Mix | 0.5 µL |
| MeltDoctor™ HRM Master Mix | 5.0 µL |
| Deionized water | 3.5 µL |
| **Total Volume** | **10 µL** |

3. Run the reactions in a Real-Time PCR System:

| Stage | Step | Temp | Time |
|---|---|---|---|
| Holding | Enzyme activation | 95 °C | 10 min |
| Cycling (40 or 45 cycles) | Denature | 95 °C | 15 sec |
| | Anneal | 60 °C | 1 min |
| Melt curve/dissociation | Denature | 95 °C | 15 sec |
| | Anneal | 60 °C | 15 sec |
| | Melt | 95 °C | 15 sec |

## Review the amplification results

For more information about reviewing and publishing the results for a standard curve experiment, refer to the appropriate guide or the Help for your instrument.

1. Verify that the samples amplified:
   - Are the $C_T$ values between 20 and 35?
   - Is there an exponential increase in fluorescence?

2. Look for outliers with $C_T$ values that differ from replicates by more than 2.
   If there are any outliers, omit them from the analysis, then reanalyze.

3. Look for one sharp Tm peak in the Melt Curve.

4. Review the amplification efficiency and the correlation coefficient in the Standard Curve:
   - Is the slope close to -3.3, indicating optimal, 100% PCR amplification efficiency?
   - Is the $R^2$ value >0.99, indicating that the $C_T$ data points of the standard reactions correlate well with the regression line in the standard curve?

5. Review the results table for quantities and flags:
   a. Group the results by replicates, and review whether the quantities are similar within each replicate group.
   b. If available, group the results according to flags, and review the flags that were applied.
   c. If available, group the results according to the $C_T$ value, and review which reactions produced $C_T$ values outside the expected range.
   d. If the data are acceptable, review the quantities for your samples.

# Determine the integrity of the bisulfite-converted DNA

After you perform bisulfite conversion of Control Sample 1 and your samples, perform PCR amplification using the 20× Long Amplicon Control Primer Mix to determine the integrity of the bisulfite-converted DNA.

## Required materials

- Converted Human Male gDNA (Control Sample 2) (10 ng/µL)
- Bisulfite-converted Control Sample 1
- Your bisulfite-converted human genomic DNA samples
- 20× Long Amplicon Control Primer Mix
- Use a PCR Master Mix that does not contain a fluorescent dye
- Deionized water
- Thermal cycler
- E-Gel® 4% High-Resolution Agarose Gel
- TrackIt™ 50 bp DNA Ladder

## Amplify the unconverted and converted human genomic DNA

**Note:** For detailed instructions on how to set up and run your thermal cycler, refer to the appropriate guide for your instrument.

1. Prepare the reactions:

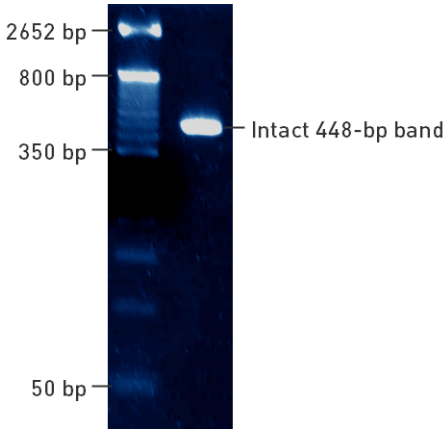
| Component | Volume per reaction |
|---|---|
| DNA sample or control | 1.0 µL |
| 20× Long Amplicon Control Primer Mix | 0.5 µL |
| PCR master mix (2×) | 5.0 µL |
| Deionized water | 3.5 µL |
| **Total Volume** | **10 µL** |

2. Run the reactions in a thermal cycler:

| Stage | Step | Temp | Time |
|---|---|---|---|
| Holding | Enzyme activation | 95 °C | 10 min |
| Cycling (40 or 45 cycles) | Denature | 95 °C | 15 sec |
| | Anneal | 60 °C | 1 min |

## Review the size of the PCR products

Run a 4% E-Gel® agarose gel to determine whether the PCR products you obtained are 448 bp and that the DNA is not degraded.



# Prepare Methylated DNA Standards for HRM Analysis

## Product information

### Purpose of the kit

Use the Cells-to-CpG™ Methylated and Unmethylated gDNA Control Kit (PN 4445552) to prepare methylated DNA standards with known levels of methylation:

- The Methylated Human Male Genomic DNA is 100% methylated
- The Unmethylated Human Male Genomic DNA is 0% methylated

Perform bisulfite conversion of the methylated DNA standards, then use the converted methylated DNA standards when you perform high resolution melting (HRM) analysis to determine the percentage of methylated DNA in your samples.

### Kit components and storage

Store kit components at –20 °C for long-term storage or at 4 °C for short-term storage.

| Component | Amount |
|---|---|
| Methylated Human Male gDNA (100 ng/µL) | 50 µL |
| Unmethylated Human Male gDNA (100 ng/µL) | 50 µL |

# Prepare methylated DNA standards

You need to convert a sufficient amount of methylated DNA standards to perform HRM analysis for each HRM assay.

1. Using the Methylated Human Male gDNA and the Unmethylated Human Male gDNA controls, prepare methylated DNA standards that contain the same amount of DNA but different ratios of 100% methylated and 0% methylated DNA, for example: 0%, 1%, 2%, 5%, 10%, 15%, 20%, 25%, 50%, and 100%.

   **Note:** To detect low levels of methylation, add more standards between 0% and 2% methylation, for example: 0.0%, 0.1%, 0.5%, 1%, 2%, 5%, 10%, and 100%.

2. For each ratio, prepare 45 µL sample:
   - 100 ng to 1 µg of 100% methylated DNA and 0% methylated DNA mixed accordingly
   - Sufficient deionized water for a total sample volume of 45 µL

   **Note:** If you are performing multiple HRM assays, consider increasing the amount of mixed DNA for each ratio. Make sure that each sample contains the same amount of DNA.

3. Proceed with denaturing the DNA (page 11), then complete the bisulfite conversion procedures:
   a. Prepare the Conversion Reagent (page 10)
   b. Perform bisulfite conversion of the denatured DNA (page 11)
   c. Desalt and desulfonate the samples (page 12)
   d. Assess the yield and quality of the bisulfite-converted DNA (page 14)

**Note:** For information on how to perform HRM analysis using the bisulfite-converted methylated DNA standards, refer to the *Applied Biosystems High Resolution Melting Getting Started Guide* (PN 4393102).

# Safety

This appendix covers:



## Chemical safety

### General chemical safety

Chemical hazard warning

**WARNING! CHEMICAL HAZARD.** Before handling any chemicals, refer to the Safety Data Sheet (SDS) provided by the manufacturer, and observe all relevant precautions.

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See "About SDSs" on page 40.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the SDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the SDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

# SDSs

## About SDSs

Chemical manufacturers supply current Safety Data Sheets (SDSs) with shipments of hazardous chemicals to new customers. They also provide SDSs with the first shipment of a hazardous chemical to a customer after an SDS has been updated. SDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new SDS packaged with a hazardous chemical, be sure to replace the appropriate SDS in your files.

## Obtaining SDSs

The SDS for any chemical supplied by Applied Biosystems is available to you free 24 hours a day. To obtain SDSs:

1. Go to **www.appliedbiosystems.com**, click **Support**, then select **SDS**.
2. In the Keyword Search field, enter the chemical name, product name, SDS part number, or other information that appears in the SDS of interest. Select the language of your choice, then click **Search**.
3. Find the document of interest, right-click the document title, then select any of the following:
   - **Open** – To view the document
   - **Print Target** – To print the document
   - **Save Target As** – To download a PDF version of the document to a destination that you choose

**Note:** For the SDSs of chemicals not distributed by Applied Biosystems, contact the chemical manufacturer.

## Biological hazard safety

### General biohazard

**WARNING! BIOHAZARD.** Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (stock no. 017-040-00547-4; **www.cdc.gov/biosafety/publications/index.htm**)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030; **www.access.gpo.gov/ nara/cfr/waisidx_01/ 29cfr1910a_01.html**).
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.

Additional information about biohazard guidelines is available at:

**www.cdc.gov**

# Documentation and Support

## Related documentation

The following related documents are available:

| Document | Part number |
|---|---|
| *Cells-to-CpG™ Bisulfite Conversion Kit (2x96) Protocol* | 4449006 |
| *Cells-to-CpG™ Bisulfite Conversion Kit (2x96) Quick Reference Card* | 4448996 |
| *Cells-to-CpG™ Bisulfite Conversion Kit (50) Protocol* | 4448998 |
| *Cells-to-CpG™ Bisulfite Conversion Kit (50) Quick Reference Card* | 4449007 |

Portable document format (PDF) versions of these documents are available on the Applied Biosystems web site at **www.appliedbiosystems.com**.

## Obtaining support

For the latest services and support information for all locations, go to:

**www.appliedbiosystems.com**

At the Applied Biosystems web site, you can:

- Access worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities.
- Search through frequently asked questions (FAQs).
- Submit a question directly to Technical Support.
- Order Applied Biosystems user documents, SDSs, certificates of analysis, and other related documents.
- Download PDF documents.
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- Download software updates and patches.

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