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ZYMO RESEARCH

THE
Epigenetics
COMPANY™

EZ-96 DNA Methylation-Direct™ Kit (Shallow-Well Format)

High throughput bisulfite conversion of DNA directly from the source

Highlights

- High throughput (96-well), complete *bisulfite conversion* of DNA directly* from blood, tissue, or cells.
- Compatible with small sample inputs – as few as 10 cells or 50 pg DNA.
- Well-suited for FFPE and LCM-derived samples.

Catalog Numbers:
D5022



Scan with your smart-phone camera to view the online protocol/video.

*Patent pending.



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Product Contents

| EZ-96 DNA Methylation-Direct™ Kit | D5022 2 x 96 Rxns.) | Storage Temperature |
|---|------------------------|------------------------|
| Proteinase K and Storage Buffer* | 20 mg Set | -20°C (after mixing) |
| M-Digestion Buffer (2X) | 15 ml | Room Temp. |
| CT Conversion Reagent** | 2 Bottles | Room Temp. |
| M-Dilution Buffer | 7 ml | Room Temp. |
| M-Solubilization Buffer | 18 ml | Room Temp. |
| M-Reaction Buffer | 4 ml | Room Temp. |
| M-Binding Buffer | 125 ml | Room Temp. |
| M-Wash Buffer*** | 2 x 36 ml | Room Temp. |
| M-Desulphonation Buffer | 40 ml | Room Temp. |
| M-Elution Buffer | 8 ml | Room Temp. |
| Silicon-A™ Binding Plates | 2 Plates | Room Temp. |
| Conversion Plates w/Pierceable Cover Film | 4 Plates/Films | Room Temp. |
| Collection Plates | 2 Plates | Room Temp. |
| Elution Plates | 2 Plates | Room Temp. |
| Instruction Manual | 1 | - |

* Add 1040 µl **Proteinase K Storage Buffer** to the tube of **Proteinase K** prior to use. The final concentration of **Proteinase K** after the addition of **Proteinase K Storage Buffer** is 20 mg/ml.

** 7.9 ml **M-Solubilization Buffer** and 3 ml **M-Dilution Buffer** are added per bottle of **CT Conversion Reagent**, mixed, and then 1.6 ml **M-Reaction Buffer** is added prior to use.

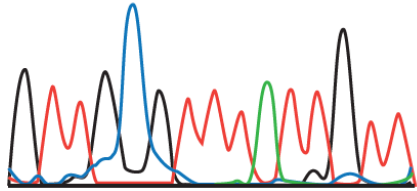
*** Add 144 ml of 100% ethanol to the 36 ml **M-Wash Buffer** concentrate before use.

Introduction to DNA Methylation

DNA methylation is a naturally occurring event in both prokaryotic and eukaryotic organisms. In prokaryotes DNA methylation provides a way to protect host DNA from digestion by restriction endonucleases that are designed to eliminate foreign DNA, and in higher eukaryotes DNA methylation functions in the regulation/control of gene expression (1). It has been demonstrated that aberrant DNA methylation is a widespread phenomenon in cancer and may be among the earliest changes to occur during oncogenesis (2). DNA methylation has also been shown to play a central role in gene imprinting, embryonic development, X-chromosome gene silencing, and cell cycle regulation. In many plants and animals, DNA methylation consists of the addition of a methyl group to the fifth carbon position of the cytosine pyrimidine ring via a methyltransferase enzyme (3). The majority of DNA methylation in mammals occurs in 5'-CpG-3' dinucleotides, but other methylation patterns do exist. In fact, about 80 percent of all 5'-CpG-3' dinucleotides in mammalian genomes are found to be methylated, whereas the majority of the twenty percent that remain unmethylated are within promoters or in the first exons of genes.

The ability to detect and quantify DNA methylation efficiently and accurately has become essential for the study of cancer, gene expression, genetic diseases, as well as many other important aspects of biology. To date, a number of methods have been developed to detect/quantify DNA methylation including: high-performance capillary electrophoresis (4) and methylation-sensitive arbitrarily primed PCR (5). However, the most common technique used today remains the bisulfite conversion method (6). This technique involves treating methylated DNA with bisulfite, which converts unmethylated cytosines into uracil. Methylated cytosines remain unchanged during the treatment. Once converted, the methylation profile of the DNA can be determined by PCR amplification followed by DNA sequencing (see next page).

| | | | | | | | | | | | | | | | | |
|--|---|---|---|---|---|------------------|---|---|---|----|----|----|----|----|----|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | |
| Original DNA with methylated C ^m pG | ▶ | G | T | T | G | C ^m G | C | T | C | A | C | T | G | C | C | |
| DNA Sequencing after CT conversion | ▶ | G | T | T | G | C | G | T | T | T | A | T | T | G | T | T |



DNA sequencing results following bisulfite treatment. DNA with methylated C^mpG at nucleotide position #5 was processed using the **EZ DNA Methylation™ Kit**. The recovered DNA was amplified by PCR and then sequenced directly. The methylated cytosine at position #5 remained intact while the unmethylated cytosines at positions #7, 9, 11, 14 and 15 were completely converted into uracil following bisulfite treatment and detected as thymine following PCR.

References:

1. Costello JF, Plass CJ. *Med. Genet.* 2001; 38(5): 285-303.
2. Stirzaker C. *Cancer Res.* 1997; 57(11): 2229-2237.
3. Adams RL. *Bioessays.* 1995; 17(2): 139-145.
4. Fraga MF, *et al.* *Electrophoresis.* 2000; 21(14): 2990-2994.
5. Gonzalgo ML. *Cancer Res.* 1997; 57(4): 594-599.
6. Frommer M. *Proc. Natl. Acad. Sci. USA.* 1992; 89(5): 1827-1831.

Specifications

- **Starting Materials:**

Cells: Compatible with cells from solid tissue, tissue culture, whole blood, buffy coat, biopsies, LCM (Laser-Capture Micro-Dissection) and FFPE samples, etc. The number of cells per standard treatment can range from 10^3 - 10^5 cells. For optimal results, the cell number should be from 1×10^3 - 8×10^4 cells.

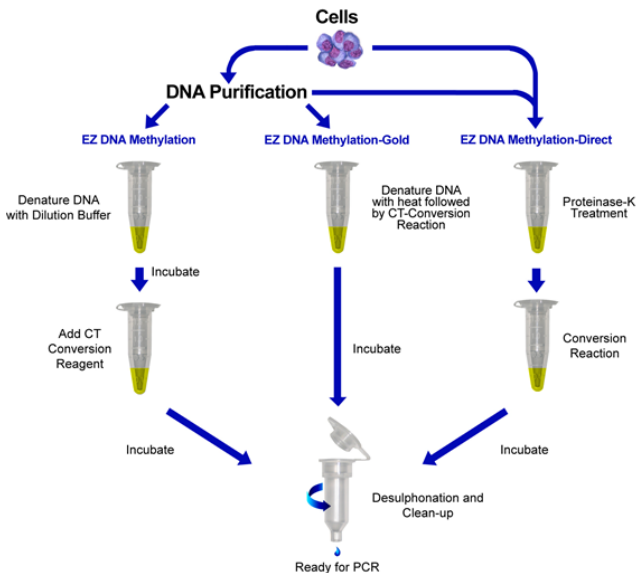
Purified DNA: Samples containing 50 pg - 2 µg of DNA. For optimal results, the amount of input DNA should be from 200 to 500 ng.

- **Conversion Efficiency** – > 99.5% of non-methylated C residues are converted to U; > 99.5% protection of methylated cytosines.
- **DNA Recovery** – > 80%
- **Sensitivity of Detection (Lower Limit)** – 10 cells for successful PCR amplification.

Product Description

The **EZ DNA Methylation-Direct™ Kit** is a further refinement of our popular **EZ DNA Methylation™** and **EZ DNA Methylation-Gold™ Kits**. The **EZ DNA Methylation-Direct™ Kit** features simple and reliable DNA bisulfite conversion directly from blood, tissue, and cells without the prerequisite for DNA purification. The increased sensitivity of this kit makes it possible to amplify bisulfite converted DNA from as few as 10 cells or 50 pg DNA. Like the **EZ DNA Methylation-Gold™ Kit**, DNA denaturation and bisulfite conversion processes are combined into a single step (see below). All kits streamline the three step process of bisulfite conversion of nonmethylated cytosine in DNA into uracil. In addition the methylation kits share innovative in-column desulphonation technology that eliminates otherwise cumbersome DNA precipitation steps while ensuring researchers consistent results every time. The kits have been designed to minimize template degradation, loss of DNA during treatment and clean-up, and to provide complete conversion of unmethylated cytosines. Recovered DNA is ideal for PCR amplification for downstream analyses including restriction endonuclease digestion, sequencing, microarrays, etc.

An outline comparing the **EZ DNA Methylation-Direct™ Kit** procedure to Zymo Research's other methylation kits is shown below.



Outline of the **EZ DNA Methylation™**, **EZ DNA Methylation-Gold™** and **EZ DNA Methylation-Direct™ Kit** procedures.

Note: 96-Well spin-plate formats are available for processing larger numbers of samples. Also, MagPrep kits are available (p. 16) for adaptation to liquid handling robots (e.g., Tecan – Freedom EVO®) and automated sample prep.

Selected EZ DNA Methylation™ Kit Citations:

1. Ehrich M, *et al.* Nuc. Acids Res. 2007; 35 (5): e29
2. Kaneda M, *et al.* Nature. 2004; 429: 900-903
3. Zhang F, *et al.* Proc. Natl. Acad. Sci. USA. 2007; 104 (11): 4395-4400.
4. Oda M, *et al.* Genes & Dev. 2006; 20: 3382-3394.
5. England RPM, *et al.* Nature Meth. 2005; 2: 1-2.

Protocol

Reagent Preparation

Preparation of Proteinase K

- ✓ Add 1040 µl of **Proteinase K Storage Buffer** to the tube containing **Proteinase K**. Dissolve completely then store at -20°C.

Preparation of CT Conversion Reagent

- ✓ The **CT Conversion Reagent** supplied within this kit is a solid mixture and must be prepared prior to first use. Prepare as follows:
 1. Add 7.9 ml of **M-Solubilization Buffer** and 3 ml of **M-Dilution Buffer** to a bottle of **CT Conversion Reagent**.
 2. Mix at room temperature with frequent vortexing or shaking for 15 minutes.
 3. Add 1.6 ml of **M-Reaction Buffer** and mix an additional 4 minutes.

Note: It is normal to see trace amounts of undissolved reagent in the **CT Conversion Reagent**. Each bottle of **CT Conversion Reagent** is designed for 96 separate DNA treatments.

Storage: The **CT Conversion Reagent** is light sensitive, so minimize its exposure to light. For best results, the **CT Conversion Reagent** should be used immediately following preparation. If not used immediately, the **CT Conversion Reagent** solution can be stored overnight at room temperature, one week at 4°C, or up to one month at -20°C. Stored **CT Conversion Reagent** solution must be warmed to 37°C, then vortexed prior to use.

Preparation of M-Wash Buffer

- ✓ Add 144 ml of 100% ethanol to the 36 ml **M-Wash Buffer** concentrate before use.

Sample Processing

Either blood, tissue, cells, or purified DNA can be used as the starting material for the **EZ DNA Methylation-Direct™ Kit**. If purified DNA is used, then proceed directly to **Section II** (page 9).

If blood, tissue, or cells are used, see **Appendix I** (page 11) for sample-specific recommendations (e.g., FFPE and LCM samples). For optimal results, the cell number should be between 1×10^3 - 8×10^4 per treatment, although the cell number can range from 10 - 10^5 cells. Using more cells than the recommended limit may result in incomplete bisulfite conversion of the DNA.

Section I: Sample Digestion with Proteinase K

Digestions should be performed in a **Conversion Plate** (provided) using procedure **A** or **B** (below) based on the number of cells and/or tissue type. Digestions are scalable to facilitate multiple samples or to increase the ease of manipulation. Sufficient volumes of reagents are included with this kit to increase the overall **Proteinase K** digestion volume 5-fold.

- A.** Setup the following digestion for samples containing up to 2×10^3 cells.

| | |
|----------------------------|--------------------------------------|
| 10 μ l | M-Digestion Buffer (2X) |
| Up to 9 μ l | Sample ($\leq 2 \times 10^3$ cells) |
| 1 μ l | Proteinase K |
| <u>X μl</u> | <u>H₂O</u> |
| 20 μ l | Total Volume |

Important! “Difficult to digest” samples result in the formation of visible debris following digestion. These should be processed according to procedure **B**.

- B.** Setup the following digestion for samples containing up to 1×10^5 cells. This should also include all “difficult to digest” samples that form debris or precipitate following **Proteinase K** digestion— see **Appendix I**.

| | |
|----------------------------|-----------------------------|
| 13 μ l | M-Digestion Buffer (2X) |
| Up to 12 μ l | Sample ($\leq 10^5$ cells) |
| 1 μ l | Proteinase K |
| <u>X μl</u> | <u>H₂O</u> |
| 26 μ l | Total Volume |

Section I: Sample Digestion with Proteinase K (continued)

2. Seal the **Conversion Plate** with **Cover Film** and incubate the samples for 20 minutes at 50°C.

Note: For FFPE, LCM, and other “fixed” tissue samples, adjust the incubation time to 4 hours (see **Appendix I**).

3. If following procedure **A**, proceed directly to **Section II**.

If following procedure **B**, mix the contents the **Conversion Plate** thoroughly, then mount on a **Collection Plate** and centrifuge for 10 minutes at 1,000 x *g*. Pierce or remove film, then transfer 20 µl of the supernatant to a new **Conversion Plate** and proceed to **Section II**.

Note: Proteinase K digested material can be stored for several months at -20°C.

Section II: Bisulfite Conversion of DNA

1. Add 130 µl of **CT Conversion Reagent** solution to each 20 µl sample in the **Conversion Plate** from **Section I**. Mix the samples by pipetting up and down.

Note: If procedure A is used (p. 8), the **CT Conversion Reagent** can be added directly to the samples in the **Conversion Plate** by either piercing the Cover Film or carefully removing it. Use a new Cover Film to re-seal the plate.

Note: If purified DNA is used, add up to 20 µl of DNA to 130 µl of **CT Conversion Reagent** solution. If the volume of DNA is less than 20 µl, compensate with water.

2. Seal the **Conversion Plate** with **Cover Film**. Place the plate in a thermal cycler and perform the following steps:

1. 98°C for 8 minutes
2. 64°C for 3.5 hours
3. 4°C storage for up to 20 hours

Note: The 4°C storage step is *optional*.

3. Add 400 µl of **M-Binding Buffer** to the wells of a **Silicon-A™ Binding Plate** on a provided **Collection Plate**.

Note: The capacity of each well of the collection plate is approximately 1 ml. Empty the collection plate whenever necessary to prevent contamination with the flow-through.

Section II: Bisulfite Conversion of DNA (continued)

4. Load the samples (from Step 2) into the wells of the **Silicon-A™ Binding Plate** containing the **M-Binding Buffer**. Mix by pipetting up and down.
5. Centrifuge at $\geq 3,000 \times g$ ($5,000 \times g$ max.) for 5 minutes. Discard the flow-through and reuse the **Collection Plate** for the following wash steps.
6. Add 400 μl of **M-Wash Buffer** to each well of the plate. Centrifuge at $\geq 3,000 \times g$ for 5 minutes.
7. Add 200 μl of **M-Desulphonation Buffer** to each well and let the plate stand at room temperature (20°C - 30°C) for 15-20 minutes, then centrifuge at $\geq 3,000 \times g$ for 5 minutes. Discard the flow-through.
8. Add 400 μl of **M-Wash Buffer** to each well of the plate. Centrifuge at $\geq 3,000 \times g$ for 5 minutes. Add another 400 μl of **M-Wash Buffer** and centrifuge for an additional 5 minutes.
9. Place the **Silicon-A™ Binding Plate** onto a provided **Elution Plate**. Add 30 μl of **M-Elution Buffer** directly to each well. Wait 5 minutes, then centrifuge for 3 minutes at $\geq 3,000 \times g$ to elute the DNA.

Note: Alternatively, water or TE (pH ≥ 6.0) can be used for elution if required for your experiments.

The DNA is ready for immediate analysis or can be stored at or below -20°C for later use. For long term storage, store at $\leq -70^{\circ}\text{C}$. We recommend using 1-4 μl of eluted DNA for each PCR, however, up to 30 μl can be used if necessary. The elution volume can be $> 30 \mu\text{l}$ depending on the requirements of your experiments, but small elution volumes will yield higher concentrations of DNA.

Appendices

Appendix I: Recommendations for Specific Cells and Tissues

The following guidelines are provided as recommendations when sampling specific cell and tissue sources. *Most importantly*, the optimal amount of DNA used for bisulfite treatment (**Section II**) should be from 1×10^3 - 8×10^4 cells, although DNA from as few as 10 to as many as 10^5 cells may be used. **Caution:** using more cells than the recommended maximum may result in incomplete bisulfite conversion of the DNA.

Important! “Difficult to digest” samples result in the formation of visible debris following digestion and should be processed according to digestion procedure **B** on page 8. This can occur with samples that are large or resistant to **Proteinase K** digestion, including: connective tissue (e.g., cartilage), adipose tissue, some fixed tissue, etc. If debris is not removed by centrifugation, it may interfere with the bisulfite conversion process resulting in incomplete conversion of the DNA.

Whole Blood:

Use up to 0.5 μ l whole blood per **Proteinase K** digestion (procedure **A** or **B**, page 8) However, the volume of the **Proteinase K** digestion can be adjusted when processing multiple samples or for convenient sample manipulation. For example, to increase the sample volume 5-fold for digestion procedure **A**: add 2.5 μ l of blood to 50 μ l **M-Digestion Buffer**, 42.5 μ l H_2O , and 5 μ l of **Proteinase K**.

Solid Tissue (Fresh or Frozen):

Use up to 0.1 mg or 0.1 μ l tissue per **Proteinase K** digestion (procedure **A** or **B**). However, the volume of the **Proteinase K** digestion can be adjusted when processing multiple samples or for convenient sample manipulation. For example, to increase the sample volume 5-fold for digestion procedure **B**: add 0.5 mg or 0.5 μ l of tissue to 65 μ l **M-Digestion Buffer**, 59.5 μ l H_2O , and 5 μ l of **Proteinase K**.

Cultured Cells and Other Cell-Containing Liquids:

Both monolayer and cells in suspension may be processed either directly from the culture container or after harvesting. Small amounts of culture medium do not adversely affect the procedure but should be kept to a minimum. Ideally, cells should be suspended in PBS or Tris-buffered solutions prior to **Proteinase K** digestion.

Other cell-containing liquids (e.g., those derived from FACS or buffy coat) may also be used directly as sample sources. If the composition of the liquid is not “defined”, then pellet the cells by centrifugation and remove the supernatant. Cells should be resuspended in PBS or Tris-buffered solutions. Generally, cells in body fluids can be used directly for **Proteinase K** digestion.

FFPE (Formalin-Fixed Paraffin-Embedded) and Other “Fixed” Tissues:

Paraffin-embedded tissues must be deparaffinized prior to use. This can be accomplished according to conventional xylene-ethanol protocols. The **Proteinase K** digestion must be extended from 20 minutes to 4 hours for FFPE and any other fixed tissue samples.

LCM (Laser Capture Micro-Dissection):

Tissue samples from LCM should be in PBS or Tris-buffered solutions. The **Proteinase K** digestion must be extended from 20 minutes to 4 hours for LCM and any other fixed tissue samples.

- 4. PCR Conditions.** Usually, 35 to 40 cycles are required for successful PCR amplification of bisulfite converted DNA. Optimal amplicon size should be between 150-300 bp; however larger amplicons (up to 1 kb) can be generated by optimizing the PCR conditions. Annealing temperatures between 55-60°C typically work well.

As most non-methylated cytosine residues are converted into uracil, the bisulfite-treated DNA usually is AT-rich and has low GC composition. Non-specific PCR amplification is relatively common with bisulfite treated DNA due to its AT-rich nature. PCR using “hot start” polymerases is strongly recommended for the amplification of bisulfite-treated DNA.

Note: **ZymoTaq™** is a “hot start” DNA polymerase specifically designed for the amplification of bisulfite treated DNA.

- 5. Quantifying Bisulfite Treated DNA.** Following bisulfite treatment of genomic DNA, the original base-pairing no longer exists since non-methylated cytosine residues are converted into uracil. Recovered DNA is typically A, U, and T-rich and is single stranded with limited non-specific base-pairing at room temperature. The absorption coefficient at 260 nm resembles that of RNA. Use a value of 40 µg/ml for $Ab_{260} = 1.0$ when determining the concentration of the recovered bisulfite-treated DNA.

Frequently Asked Questions

Q: Should the input DNA be dissolved in TE, water, or some other buffer prior to its conversion?

A: Water, TE or modified TE buffers can be used to dissolve the DNA and do not interfere with the conversion process.

Q: Which Taq polymerase(s) do you recommend for PCR amplification of converted DNA?

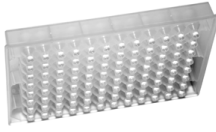
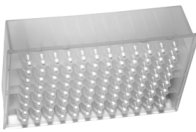
A: We recommend a “hot start” DNA polymerase (e.g., ZymoTaq™ DNA Polymerase).

Q: Why am I not getting “complete” conversion of the DNA?

A: 1) If sampling solid tissue, then it is most likely that too much sample was processed, resulting in incomplete DNA conversion. 2) If sampling FFPE tissue, then it is probable that the DNA was extensively damaged and/or cross-linked resulting in incomplete DNA conversion. 3) If debris is not removed by centrifugation from the Proteinase K digestion, it may interfere with the bisulfite conversion process resulting in incomplete conversion of the DNA.

Q: Why are there two different catalog numbers for the EZ-96 DNA Methylation-Direct™ Kit?

A: The two different catalog numbers are used to differentiate between the binding plates that are included in the kit. Deep and shallow-well binding plates are available to accommodate most rotors and microplate carriers. Below is a comparison of the two binding plates. See table on next page.

| | | |
|--|---|---|
| |  |  |
| Binding Plate | Silicon-A™ Plate | Zymo-Spin™ I-96 Plate |
| Style | Shallow-Well | Deep-Well |
| Height of Binding Plate | 19 mm (0.75 inches) | 35 mm (1.38 inches) |
| Binding Plate/Collection Plate Assembly | 43 mm (1.69 inches) | 60 mm (2.36 inches) |
| Binding Cap./Minimum Elution Volume | 5 µg/30 µl | 5 µg/15 µl |
| Catalog Numbers | D5022 | D5023 |

Ordering Information

| Product Description | Catalog No. | Size |
|---|----------------|------------------------------|
| EZ DNA Methylation-Direct™ Kit | D5020 D5021 | 50 Rxns. 200 Rxns. |
| EZ-96 DNA Methylation- Direct™ Kit (Shallow-Well) | D5022 | 2 x 96 Rxns. |
| EZ-96 DNA Methylation- Direct™ Kit (Deep-Well) | D5023 | 2 x 96 Rxns. |
| EZ-96 DNA Methylation- Direct™ MagPrep* | D5044 D5045 | 4 x 96 Rxns. 8 x 96 Rxns. |

* MagPrep kits are adaptable to liquid handling robots (e.g., Tecan – Freedom EVO®) making them ideal for automated sample prep.

| Individual Kit Components | Catalog No. | Amount |
|---------------------------|--|---------------------------------|
| CT Conversion Reagent | D5001-1 D5003-1 | 1 Tube 1 Bottle |
| M-Dilution Buffer | D5005-2 D5006-2 | 1.5 ml 7 ml |
| M-Binding Buffer | D5005-3 D5006-3 D5040-3 | 30 ml 125 ml 250 ml |
| M-Wash Buffer | D5001-4 D5002-4 D5007-4 D5040-4 | 6 ml 24 ml 36 ml 72 ml |
| M-Desulphonation Buffer | D5001-5 D5002-5 D5040-5 | 10 ml 40 ml 80 ml |
| M-Elution Buffer | D5001-6 D5002-6 D5007-6 D5041-6 | 1 ml 4 ml 8 ml 40 ml |
| M-Solubilization Buffer | D5020-7 D5021-7 | 4.5 ml 18 ml |
| M-Reaction Buffer | D5020-8 D5021-8 | 1 ml 4 ml |

| | | |
|---|--------------------------------------|-----------------------------------|
| M-Digestion Buffer | D5020-9 D5021-9 | 4 ml 15 ml |
| Proteinase K and Storage Buffer | D3001-2-5 D3001-2-20 | 5 mg Set 20 mg Set |
| Zymo-Spin™ IC Columns (capped) | C1004-50 C1004-250 | 50 Pack 250 Pack |
| Collection Tubes | C1001-50 C1001-500 C1001-1000 | 50 Pack 500 Pack 1,000 Pack |
| MagBinding Beads | D4100-5-3 D4100-5-8 D4100-5-16 | 6 ml 8 ml 16 ml |
| Zymo-Spin™ I-96 Binding Plates | C2004 | 2 Plates |
| Silicon-A™ Binding Plates | C2001 | 2 Plates |
| Conversion Plates w/ Pierceable Cover Film | C2005 | 2 Plates /Films |
| Collection Plates | C2002 | 2 Plates |
| Elution Plates | C2003 | 2 Plates |



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Integrity of kit components is guaranteed for up to one year from date of purchase. Reagents are routinely tested on a lot-to-lot basis to ensure they provide the highest performance and reliability.

This product is for research use only and should only be used by trained professionals. It is not for use in diagnostic procedures. Some reagents included with this kit are irritants. Wear protective gloves and eye protection. Follow the safety guidelines and rules enacted by your research institution or facility.

EZ DNA Methylation-Direct™ Kit technologies are patent pending.

Use of Methylation Specific PCR (MSP) is protected by US Patents 5,786,146 & 6,017,704 & 6,200,756 & 6,265,171 and International Patent WO 97/46705. No license under these patents to use the MSP process is conveyed expressly or by implication to the purchaser by the purchase of this product.

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