ZYMO RESEARCH

Epigenetics
Made Simple™

EZ-96 DNA Methylation-Gold™ MagPrep

Simplified bisulfite conversion in a scalable, high-throughput format

Highlights

- Complete, high-throughput, bisulfite conversion of GC-rich DNA in less than 3 hours.
- A coupled heat denaturation/conversion reaction step streamlines the conversion of non-methylated cytosines into uracil.
- High throughput (96-well), automated desulphonation and recovery of bisulfite-treated DNA.
- Eluted, ultra-pure DNA is ideal for use in subsequent molecular-based analyses.

Catalog Numbers:
D5042, D5043

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

tech@zymoresearch.com www.zymoresearch.com Toll Free: (888) 882-9682

Table of Contents

Product Contents

EZ-96 DNA Methylation-Gold™ MagPrep	D5042 (4x96 rxns)	D5043 (8x96 rxns)	Storage Temperature
CT Conversion Reagent[1]	4 bottles	8 bottles	Room Temp.
M-Dilution Buffer	2 x 7 ml	4 x 7 ml	Room Temp.
M-Dissolving Buffer	2 x 1.2 ml	4 x 1.2 ml	Room Temp.
M-Binding Buffer	250 ml	2 x 250 ml	Room Temp.
M-Wash Buffer[2]	2 x 72 ml	4 x 72 ml	Room Temp.
M-Desulphonation Buffer	80 ml	2 x 80 ml	Room Temp.
M-Elution Buffer	2 x 8 ml	40 ml	Room Temp.
EZ-Methylation Magprep Beads	8 ml	16 ml	Room Temp.
Conversion Plates w/ Pierceable Cover Film	4 plates/films	8 plates/films	Room Temp.
Collection Plates[3]	6 plates	10 plates	Room Temp.
Elution Plates	4 plates	8 plates	-
Instruction Manual	1	1	

[1] 9 ml water, 500 µl **M-Dissolving Buffer**, and 3 ml **M-Dilution Buffer** must be added per bottle of **CT Conversion Reagent** prior to use.
[2] Add 288 mL of 95-100% ethanol to the 72 mL **M-Wash Buffer** concentrate prior to use.
[3] Two additional **Collection Plates** are provided as stands for the **Conversion Plates** during processing.

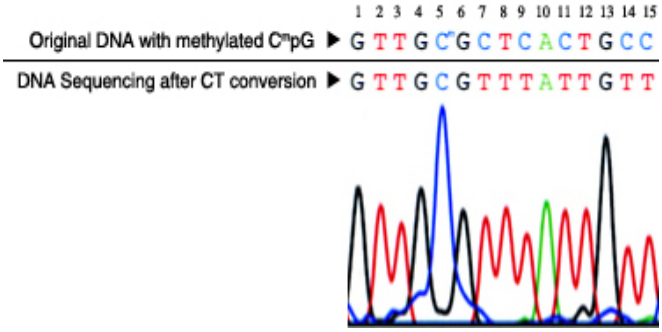
Introduction to DNA Methylation

Cytosine methylation is a naturally occurring base modification, in both prokaryotic and eukaryotic organisms, consisting of the addition of a methyl group to the fifth carbon position of the cytosine pyrimidine ring via a methyltransferase enzyme (1). In prokaryotes DNA methylation provides a way to protect host DNA from digestion by restriction endonucleases that are designed to eliminate foreign DNA. DNA methylation in higher eukaryotes functions in the regulation/control of gene expression (2).

The majority of DNA methylation in mammals occurs in 5′-CpG-3′ dinucleotides, although other patterns do exist. About 80 percent of all 5′-CpG-3′ dinucleotides in mammalian genomes are found to be methylated, and the majority of the twenty percent that remain unmethylated are within promoters or in the first exons of genes. 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 (3). DNA methylation has also been shown to play a central role in gene imprinting, embryonic development, X-chromosome gene silencing, and cell cycle regulation.

The ability to detect and quantify DNA methylation efficiently and accurately has become essential for the study of cancer, gene expression, genetic diseases, and 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 techniques used today still rely on bisulfite conversion (6).

Treating DNA with bisulfite chemically modifies non-methylated cytosines into uracil, methylated cytosines remain unchanged. Once converted, the methylation profile of the DNA can be determined using the desired downstream application. For single locus analysis, the region of interest is generally amplified following bisulfite conversion (i.e., bisulfite PCR) and then sequenced or processed for Pyrosequencing®. Recent advances in methylation detection also allow the investigation of genome-wide methylation using technologies including array-based methods, reduced representation bisulfite sequencing (RRBS), and whole genome bisulfite sequencing (7).



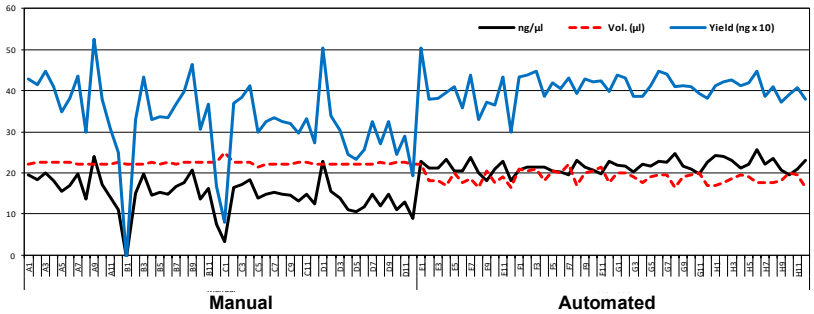
DNA sequencing results following bisulfite treatment. DNA with methylated C at nucleotide position #5 was processed using the **EZ DNA Methylation™ Kit**. The recovered DNA was amplified by PCR and then sequenced directly. The methylated cytosine at position #5 remains intact while the unmethylated cytosines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

References:

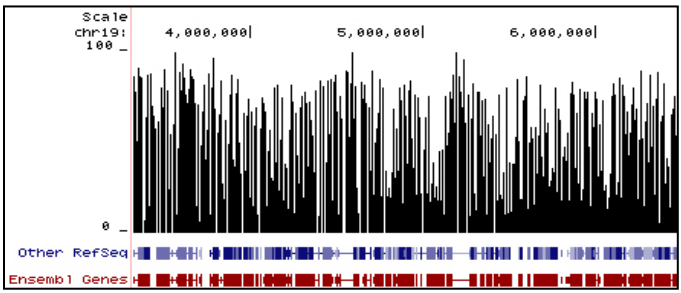
1. Adams RL. Bioessays. 1995; 17(2): 139-145.
2. Costello JF, Plass CJ. Med. Genet. 2001; 38(5): 285-303.
3. Stirzaker C. Cancer Res. 1997; 57(11): 2229-2237.
4. Fraga MF, et al. Electrophoresis. 2000; 21(14): 2990-2994.
5. Gonzalgo ML. Cancer Res. 1997; 57(4): 594-599.
6. Frommer M. Proc. Natl. Acad. Sci. USA. 1992; 89(5): 1827-1831.
7. Rakyan VK, et al. Nat. Rev. 2011, 12(8): 529-541.

Product Description

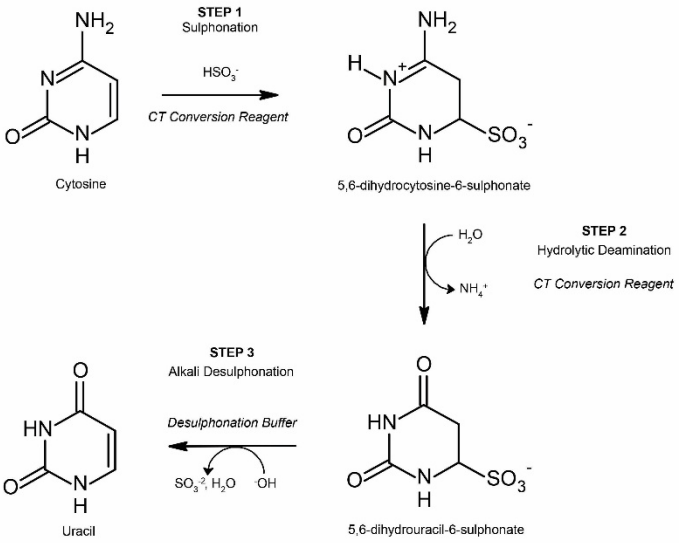
The **EZ-96 DNA Methylation-Gold™ MagPrep**[1] integrates DNA denaturation and bisulfite conversion processes into one-step coupled to a magnetic bead based clean-up for high-throughput methylation analysis. This is accomplished using temperature denaturation to replace chemical denaturation with sodium hydroxide in the previous protocols. Also, the kit has been streamlined for high yield recovery of DNA following DNA bisulfite conversion. Desulphonation and clean-up of the converted DNA is performed while bound to the **EZ-Methylation Magprep Beads**. 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 endonuclease digestion, sequencing, microarrays, etc.



Comparison of Manual vs. Automated Processing. Data show concentration, volume and total yield for DNA samples across a 96-well plate. Half of the samples (rows A-D) were processed manually. The other half of the samples "Automated" (rows E-H) were processed using the Tecan – Freedom EVO® platform and a dedicated script.



Methylation Plot From Reduced Representation Bisulfite Sequencing (RRBS). Data shows the relative percentage of methylation at individual CpG sites in mouse DNA. Methylation percentage is shown across a ~3 Mb region of mouse chromosome 19. Bisulfite sequencing libraries were prepared using mouse genomic DNA prepped with the **Genomic Clean & Concentrator™** (D4010, D4011 – Zymo Research) and bisulfite converted using **EZ DNA Methylation™** technology prior to Next-Gen sequencing.



Overview of Bisulfite Conversion. Steps 1 and 2 occur during bisulfite conversion, while Step 3 is performed as the DNA is bound to the column matrix. For the reaction to proceed to completion, it is essential the DNA be fully denatured.

Select 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.
6. Berman BP, et al. Nature Gen. 2012; 44: 40-46.
7. Leung DC, et al. Proc. Natl. Acad. Sci. USA. 2011; 108 (14): 5718-5723.
8. Hesselink AT, et al. Clin. Cancer Res. 2011; 17: 2459-2465.
9. Campan M, et al. PLoS ONE. 2011, 6 (12): e28141.

[1] Single spin-column and 96-Well spin-plate formats are available.

Specifications

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

- **Conversion Efficiency:** > 99% of non-methylated cytosine residues are converted to uracil; > 99% protection of methylated cytosines.

- **Required Additional Equipment:** Magnetic Stand[1], Heating element for 96-well plate.

[1] A strong-field magnetic stand is recommended (e.g., ZR-96 MagStand, Cat. No. P1005)

Protocol

Reagent Preparation

A. CT Conversion Reagent Preparation

✓ 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 9 ml water, 500 µl **M-Dissolving 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.

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.

B. M-Wash Buffer Preparation

✓ Add 288 ml of 100% ethanol to the 72 ml **M-Wash Buffer** concentrate before use.

Bisulfite Conversion

1. Add 130 µl of **CT Conversion Reagent** to 20 µl of a DNA sample in a **Conversion Plate**. Mix the samples by pipetting up and down.

Note: If the volume of DNA is less than 20 µl, compensate with water.

Note: For DNA volumes >20 µl, an adjustment needs to be made during the preparation of the **CT Conversion Reagent**. The amount of water is decreased 1 ml for each 10 µl increase in DNA sample volume. For example, for 40 µl DNA samples, 7 ml of water is added to make the **CT Conversion Reagent**. The volume of **CT Conversion Reagent** added to the sample must also be decreased by the same volume as the sample is increased, total reaction volume remains 150 µl. The maximum DNA sample volume to be used for each conversion reaction is 45 µl. Do not adjust the volumes of either the **M-Dissolving Buffer** or **M-Dilution Buffer**.

2. Seal the plate with the provided film. Transfer the **Conversion Plate** to a thermal cycler and perform the following steps:

    1. 98 °C for 10 minutes
    2. 64 °C for 2.5 hours
    3. 4 °C storage for up to 20 hours

    Note: The 4 °C storage step is *optional*. For some samples, alternative parameters may yield improved results (see Appendix). If you have been using this kit with good results using different reaction conditions than described above, you can continue using those same conditions.

3. Pre-heat a plate heating element to 55 °C.

    Note: Alternatively, depending on the time necessary for the element to reach temperature, this can be performed any time prior to step 10.

4. Add 600 µl of **M-Binding Buffer** and 10 µl of **EZ-Methylation Magprep Beads** to each well of a **Collection Plate**.

    Note: EZ-Methylation Magprep Beads settle very quickly, ensure that beads are kept suspended in the reservoir while adding to the plate.

5. Transfer the samples from the **Conversion Plate** into the **Collection Plate** containing the **M-Binding Buffer** and **EZ-Methylation Magprep Beads**. Mix by pipetting up and down 3-6 times and, if available, shaking the plate at 1,300-1,500 rpm for 30 seconds (e.g. Tecan - Te-Shake™).

    Note: Transfer may be accomplished by either piercing or removing the cover foil on the **Conversion Plate**. If using a **Collection Plate** as a stand for the **Conversion Plate** it may be necessary to secure the plates together using the tabs on the cover foil to prevent lifting of the **Conversion Plate**.

6. Let plate stand at room temperature for 5 minutes, then transfer plate to a magnetic stand for an additional 5 minutes or until beads pellet and supernatant is cleared. With the plate on the magnetic stand remove the supernatant and discard.

    Note: Some beads will adhere to the sides of the well. Remove supernatant slowly to allow these beads to be pulled to the magnet as the liquid level is lowered.

7. Remove the **Collection Plate** from the magnetic stand for this and each subsequent buffer addition. Add 400 µl of **M-Wash Buffer** to the beads. Re-suspend the beads by pipetting up and down or shaking the plate at 1,300-1,500 rpm for 30 seconds. Replace the plate on the magnetic stand for 3 minutes or until beads pellet. Remove and discard supernatant.

8. Add 200 µl of **M-Desulphonation Buffer** to the beads. Re-suspend the beads by pipetting up and down or shaking the plate for 30 seconds. Let plate stand at room temperature (20°C-30°C) for 15-20 minutes. After the incubation, replace the plate on the magnetic stand for 3 minutes or until beads pellet. Remove and discard supernatant.

   Note: Take time for handling/re-suspension into account for the total incubation time. Adjust time as necessary to ensure that no sample remains in the **M-Desulphonation Buffer** for more than 20-25 minutes.

9. Add 400 µl of **M-Wash Buffer** to the beads. Re-suspend the beads by pipetting up and down or shaking the plate for 30 seconds. Replace the plate on the magnetic stand for 3 minutes or until beads pellet. Remove and discard supernatant. Repeat this wash step.

   Note: Remove as much buffer as possible after final wash to aid in the drying of the beads.

10. Transfer the plate to a heating element at 55°C for 20-30 minutes to dry the beads and remove residual **M-Wash Buffer**.

    Note: Beads will change in appearance from glossy black when still wet to a dull brown when fully dry.

11. Add 25 µl of **M-Elution Buffer** directly to the dried beads and pipette or shake the plate for 30 seconds to re-suspend. Heat the elution at 55°C for 4 minutes then transfer the plate to the magnetic stand for 1 minute or until beads pellet. Remove the supernatant and transfer to a clean **Elution Plate**.

    Note: If beads are removed with the elution, slowly pipetting up and down one or two times will allow them to be pulled to the magnet.

    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 or below -70°C. We recommend using 1-4 µl of eluted DNA for each PCR, however, up to 25 µl can be used if necessary. The elution volume can be > 25 µl depending on the requirements of your experiments, but small elution volumes will yield higher DNA concentrations.

Automation Scripts:

Various automation scripts are available and can be obtained free of charge by contacting Zymo Research at tech@zymoresearch.com. Include "Automation Scripts" in the subject line and provide kit catalog number and the automation platform desired in the email.

Appendix

Appendix I: Bisulfite Conversion and PCR Optimization

1. Reaction Conditions.

The reaction conditions given in Step 2 of the Protocol will generate consistent results for both easy and difficult to convert template DNAs including those that are GC rich. However, the two protocols provided below (alternative 1 & 2) may yield better results in PCR amplification of longer DNA fragments. However, should the DNA template have >80% GC composition, then these conditions may result in incomplete template cytosine to uracil conversion.

Alternative 1:

1. 98 °C for 10 minutes
2. 53 °C for 30 minutes
3. 53 °C for 6 minutes
4. 37 °C for 30 minutes } 8 cycles
5. 4 °C storage

Alternative 2:

1. 98 °C for 10 minutes
2. 53 °C for 4 hours
3. 4 °C storage

2. Bisulfite Conversion of Double Stranded DNA Templates.

The following illustrates what occurs to a DNA template during bisulfite conversion.

Template:
A: 5′-GACCGTTCCAGGTCCAGCAGTGCGCT-3′
B: 3′-CTGGCAAGGTCCAGGTCGTCACGCGA-5′

Bisulfite Converted:
A: 5′-GATCGTTTTAGGTTTAGTAGTGCGTT-3′
B: 3′-TTGGCAAGGTTTAGGTTGTTATGCGA-5′

Note: Methylated “C” is underlined in the examples.

Note: Following bisulfite conversion, the strands are no longer complementary.

3. PCR Primer Design.

Generally, primers 26 to 32 bases are required for amplification of bisulfite converted DNA. In general, all Cs should be treated as Ts for primer design purposes, unless they are in a CpG context. See example below.

Bisulfite Converted: **A:** 5′-GATCGTTTTAGGTTTAGTAGTGCGTT-3′
Primers: Reverse: 3′-ATCATCACRCAA-5′
Forward: 5′-GATYGTTTTAGGT-3′

R= G/A
Y= C/T

Zymo Research provides primer design assistance with its Bisulfite Primer Seeker Program, available at: www.zymoresearch.com/tools/bisulfite-primer-seeker

Note: Only one strand (**A**) is amplified by a given primer set. Only the reverse primer binds to the converted DNA, the forward primer will bind the strand generated by the reverse primer. If the primer contains CpG dinucleotides with uncertain methylation status, then mixed bases with C and T (or G and A) can be used. Usually, there should be no more than one mixed position per primer, and it should be located toward the 5' end of the primer. It is not recommended to have mixed bases located at the 3' end of the primer.

4. Amount of DNA Required for Bisulfite Conversion

The minimal amount of human or mouse genomic DNA required for bisulfite treatment and subsequent PCR amplification is 100 pg. The optimal amount of DNA per bisulfite treatment is 200 to 500 ng. Although up to 2 µg of DNA can be processed, it should be noted that high input levels of DNA may result in incomplete bisulfite conversion for some GC-rich regions.

5. 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: **Zymo*Taq*™** is a "hot start" DNA polymerase specifically designed for the amplification of 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 are there two different catalog numbers for the EZ-96 DNA Methylation™ 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.*

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	**D5007**	**D5008**

Ordering Information

Product Description	Catalog No.	Size
EZ DNA Methylation-Gold™ Kit	D5005 D5006	50 rxns. 200 rxns.
EZ-96 DNA Methylation-Gold™ Kit (Shallow-Well)	D5007	2 x 96 rxns.
EZ-96 DNA Methylation-Gold™ Kit (Deep-Well)	D5008	2 x 96 rxns.
EZ-96 DNA Methylation-Gold™ MagPrep	D5042 D5043	4 x 96 rxns. 8 x 96 rxns.

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-Dissolving Buffer	D5005-6 D5006-6	500 µl 1.2 ml

Zymo-Spin™ IC Columns (capped)	C1004-50 C1004-250	50 columns 250 columns
Collection Tubes	C1001-50 C1001-500 C1001-1000	50 tubes 500 tubes 1,000 tubes
EZ-Methylation Magprep Beads	D4100-5-8 D4100-5-16	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
ZR-96 MagStand	P1005	1 stand

Notes

Notes

100% satisfaction guarantee on all Zymo Research products, or your money back.

Zymo Research is committed to simplifying your research with quality products and services. If you are dissatisfied with this product for any reason, please call 1(888) 882-9682.

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.

ZYMO RESEARCH

The **BEAUTY** *of* **SCIENCE** *is to Make Things* **SIMPLE**®

tech@zymoresearch.com www.zymoresearch.com Toll Free: (888) 882-9682