



ZYMO RESEARCH

The Beauty of Science is to Make Things Simple

INSTRUCTION MANUAL

EZ RNA Methylation[™] Kit

Catalog Nos. **R5001 & R5002**

Highlights

- Fast and reliable bisulfite conversion of RNA for methylation analysis.
- Specifically optimized for complete conversion of non-methylated cytosine in RNA.
- Ideal for all RNA inputs.

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Product Contents:

EZ RNA Methylation™ Kit	R5001 50 rxns.	R5002 200 rxns.	Storage Temperature
RNA Conversion Reagent	5 tubes	20 tubes	Room Temp.
RNA Binding Buffer	25 ml	100 ml	Room Temp.
RNA Wash Buffer¹ (concentrate)	12 ml	48 ml	Room Temp.
RNA Desulphonation Buffer	10 ml	40 ml	Room Temp.
DNase/RNase-Free Water	1 ml	4 ml	Room Temp.
Zymo-Spin™ IC Columns	50 columns	200 columns	Room Temp.
Collection Tubes	50 tubes	200 tubes	Room Temp.
Instruction Manual	1	1	–

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

¹ Add 48 ml 100% ethanol (52 ml of 95% ethanol) to the 12 ml **RNA Wash Buffer** concentrate (R5001) or 192 ml 100% ethanol (208 ml of 95% ethanol) to the 48 ml **RNA Wash Buffer** concentrate (R5002) before use.

Specifications:

- **RNA Input:** Samples containing 32 ng - 3 µg of DNA-free RNA. For optimal results, the amount of input RNA should be between 0.5 - 1 µg.
- **Conversion Efficiency:** > 99% of non-methylated C residues are converted to U with > 99% protection of 5-methylcytosine.
- **RNA Recovery:** > 80%

Note: For purification of high-quality DNA-free RNA, we recommend the **Quick-RNA™** (R1050) or **Direct-zol™** (R2050) purification kits, see page 9 for details. DNase I treatment of RNA samples is recommended.

Note: m4mC is also partially resistant to conversion with bisulfite, however, in comparison with 5-mC, m4mC may be more easily converted to U during the procedure.

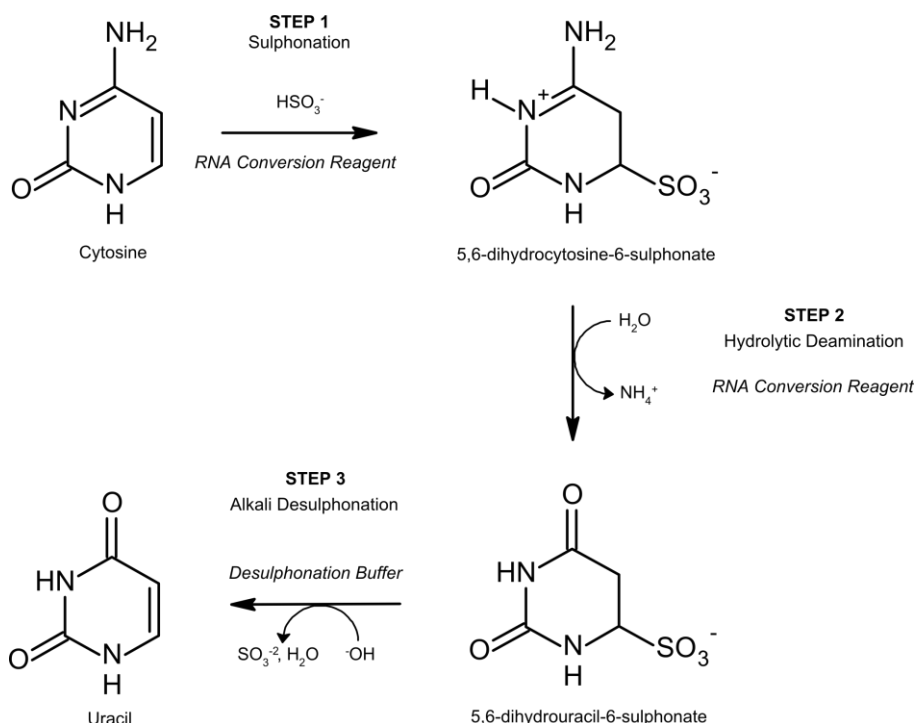
Use of Methylation Specific PCR (MSP) is protected by US Patents 5,786,146 & 6,017,704 & 6,200,756 & 6,265,171 and International Patent WO 97/46705. No license under these patents to use the MSP process is conveyed expressly or by implication to the purchaser by the purchase of this product.

Note - ™ Trademarks of Zymo Research Corporation. This product is for research use only and should only be used by trained professionals. It is not intended 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.

Introduction to RNA Methylation:

Although the majority of nucleic acid modification research involves 5-methylcytosine in DNA, RNA is also extensively modified. In fact, there exist over a hundred modifications to RNA (1). 5-methylcytosine (5-mC) is present in RNA, and methylation is a common and naturally-occurring event in the RNA from both prokaryotic and eukaryotic organisms (2, 3). However, the function of RNA methylation remains unknown. Some reports describe a role for RNA methylation in translational regulation (4, 5), while others support a role for methylation in regulating RNA stability (6, 7) or the facilitation of RNA structure formation (8, 9).

The ability to detect and quantify 5-methylcytosine in RNA efficiently and accurately has been troublesome due to the inability of RNA to withstand the pH and temperatures used in the standard workflow for bisulfite conversion of DNA. Zymo Research offers a solution to these problems with the **EZ RNA Methylation™ Kit** that has been optimized and validated for bisulfite conversion of RNA. This technique involves treating RNA with a unique bisulfite conversion reagent, which converts non-methylated cytosines into uracil while preserving the integrity of the RNA. Methylated cytosines remain unchanged during the treatment. After performing bisulfite treatment, the methylation profile of the RNA can be determined using techniques like RT-PCR followed by DNA sequencing (see figure on page 3).



The Chemistry of Bisulfite Conversion

References:

1. Cantara WA, *et al.* Nucleic Acids Res. 2011; 39: D195-201.
2. Motorin Y, Helm M. Nucleic Acids Res. 2010; 38(5): 1415-1430.
3. Squires JE, Preiss T. Epigenomics. 2010; 2(5): 709-715.
4. Chow CS, *et al.* ACS Chem Biol. 2007; 2(9): 610-619.
5. Baudin-Baillieu A, *et al.* Nucleic Acids Res. 2009; 37(22): 7665-7677.
6. Alexandrov A, *et al.* Mol Cell. 2006; 21(1): 87-96.
7. Schaefer M, *et al.* Genes Dev. 2010; 24(15): 1590-1595.
8. Helm M. Nucleic Acids Res. 2006; 34(2): 721-733.
9. Motorin Y, Helm M. Biochemistry. 2010; 49(24): 4934-4944.

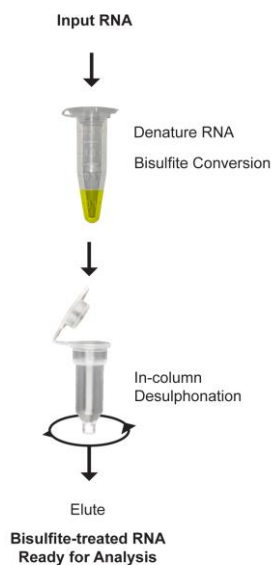
Selected EZ RNA Methylation™ Kit Citation:

Amort T, *et al.* RNA Biol. 2013; 10(6): PMID 23595112.

Product Description:

For bisulfite treatment of DNA for methylation analysis, see the comprehensive line of **EZ DNA Methylation™** products from Zymo Research. (D5001-D5047)

The **EZ RNA Methylation™ Kit** features rapid and reliable bisulfite treatment and conversion of cytosines in RNA for methylation analysis. The kit streamlines the three-step process for complete conversion of cytosine into uracil. RNA denaturation and bisulfite conversion processes are combined into a single step. No buffer preparation is necessary. The **RNA Conversion Reagent** is provided ready-to-use: simply add the reagent to an RNA sample and incubate as indicated. Also, innovative in-column desulphonation technology eliminates messy precipitation steps, ensuring researchers obtain consistent results. The product has been designed to minimize template degradation, loss of RNA during treatment and clean-up, and to provide complete conversion of cytosine for accurate methylation analysis. Recovered RNA is ideal for RT-PCR, sequencing, library preparation and Next-Gen sequencing.



Original RNA with Methylated CpG:

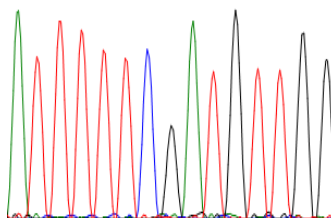
AUCCUUC^mGAUGUCGG

Converted RNA:

AUUUUU^mGAUGUUGG

cDNA Sequence:

ATTTTC^mGATGTTGG



Sequencing results following bisulfite treatment. RNA with methylated C (5-mC) at nucleotide position #7 was processed using the **EZ RNA Methylation™ Kit**. The recovered RNA was amplified by RT-PCR and then cloned and sequenced. The methylated cytosine at position #7 remained intact while the non-methylated cytosines at positions #3, 4, and 13 were completely converted into uracil (post-bisulfite treatment) and detected as thymine following RT-PCR and sequencing.

Reagent Preparation:

- **Preparation of RNA Wash Buffer:** Add 48 ml of 100% ethanol (52 ml of 95% ethanol) to the 12 ml **RNA Wash Buffer** concentrate or 192 ml of 100% ethanol (208 ml of 95% ethanol) to the 48 ml **RNA-Wash Buffer** concentrate before use.

Protocol for Bisulfite Conversion of RNA:

1. Add 130 µl of **RNA Conversion Reagent** to 20 µl of RNA sample in a PCR tube. Mix the sample by flicking the tube or pipetting up and down, then centrifuge briefly to ensure there are no droplets in the cap or sides of the tube.

Note: If the sample volume is less than 20 µl, compensate with DNase/RNase-Free Water.

2. Place the PCR tube(s) in a thermal cycler and perform the following steps:

1. 70°C for 5 minutes
2. 54°C for 45 minutes
3. 4°C up to 20 hours

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

3. Place a **Zymo-Spin™ IC Column** into a **Collection Tube** and add 250 µl of **RNA Binding Buffer** to the column.
4. Load the sample (from Step 2) into the **Zymo-Spin™ IC Column** containing the **RNA Binding Buffer** and mix by pipetting up and down.
5. Add 400 µl of 95-100% ethanol to the sample-**RNA Binding Buffer** mixture in the column. Close the cap and immediately mix by inverting the column several times.
6. Centrifuge at full speed ($\geq 10,000 \times g$) for 30 seconds. Discard the flow-through.
7. Add 200 µl **RNA Wash Buffer** to the column and centrifuge at full speed for 30 seconds.
8. Add 200 µl of **RNA Desulphonation Buffer** to the column and let stand at room temperature (20°C – 30°C) for 30 minutes. After the incubation, centrifuge at full speed for 30 seconds. Discard the flow-through.
9. Add 400 µl **RNA Wash Buffer** to the column and centrifuge at full speed for 30 seconds. Repeat the wash step with an additional 400 µl **RNA Wash Buffer**. Discard the flow-through.
10. Centrifuge the **Zymo-Spin™ IC Column** in the emptied **Collection Tube** at full speed for 2 minutes. Remove the **Zymo-Spin™ IC Column** carefully from the **Collection Tube** and transfer it into an RNase-free Tube.
11. Add ≥ 10 µl of **DNase/RNase-Free Water** directly to the column matrix and let stand for 1 minute at room temperature. Centrifuge at full speed for 30 seconds. The eluted RNA can be used immediately or stored at -20°C for up to 3 months. For long-term storage, keep at or below -70°C.

Note: The elution volume can be > 10 µl depending on the requirements of your experiments.

Appendix I: Using 28S rRNA as a Positive Control

We recommend using 28S ribosomal RNA (*H. sapiens*) as a positive control for RNA methylation analysis, as the C at position 4447 (GenBank accession # NR_003287) is generally 100% methylated. Total RNA from cells or tissues (i.e. HeLa, HCT116, keratinocytes, brain tissue, liver tissue, etc.) can be used directly for the bisulfite conversion. The following sequence is the 28S rRNA region amplified (post-conversion) using the primer set indicated below.

Original (non-converted):

```
4321 -----ggg gccucacgau ccuucugacc uuuuggguuu uaagcaggag gugucagaaa aguuaaccaca
4391 gggaauaacug gcuuguggcg gccaacgguu cauagcgacg ucgcuuuuug auccuuCgau gucgggcucuu
4461 ccuaucuuug ugaagcagaa uucaccaagc guuggauugu ucacccacua auagggaacg ugagcugg--
```

Bisulfite-Converted:

```
4321 -----ggg guuuuau gau uuuuuugauu uuuuggguuu uaaguaggag guguuagaaa aguuaauaua
4391 gggaauauug guuugugugug guuaaguguu uauagugaug uuguuuuuug auuuuuCgau guuggguuuu
4461 uuuuuuauug ugaaguagaa uuuaauaagu guuggauugu uuauuuaua auagggaauug ugaguugg--
```

H 28SF primer: 5' -GGGGTTTTAYGATTTTTTTGATTTTTTTGGG-3'

H 28SR primer: 5' -CCAACTCACRTTCCCTATTAATAAATAAAC-3'

Representative sequencing data obtained using 28S rRNA: The RT-PCR sequencing results of 10 clones (below) were obtained using bisulfite-converted total RNA extracted from HeLa cells. Underlined C represents 5-mC, highlighted **C** represents non-converted cytosine, *italics* are primer regions. The original, non-converted RNA sequence with non-methylated **C** highlighted is shown below the converted cDNA sequencing results for comparison.

Conversion Efficiency (C to T): C: 99.5%

```

1
HeLa01 - GGGGTTTTATGATTTTTTTGATTTTTTTGGGTTTAAAGTAGGAGGTGTAGAAAAGTTATTATAGGGATAATTGGTTTGTGGTGGTTAAGTGTTTATAGTGA
HeLa02 - GGGGTTTTATGATTTTTTTGATTTTTTTGGGTTTAAAGTAGGAGGTGTAGAAAAGTTATTATAGGGATAATTGGTTTGTGGTGGTTAAGTGTTTATAGTGA
HeLa03 - GGGGTTTTACGATTTTTTTGATTTTTTTGGGTTTAAAGTAGGAGGTGTAGAAAAGTTATTATAGGGATAATTGGTTTGTGGTGGTTAAGTGTTTATAGTGA
HeLa04 - GGGGTTTTACGATTTTTTTGATTTTTTTGGGTTTAAAGTAGGAGGTGTAGAAAAGTTATTATAGGGATAATTGGTTTGTGGTGGTTAAGTGTTTATAGTGA
HeLa05 - GGGGTTTTACGATTTTTTTGATTTTTTTGGGTTTAAAGTAGGAGGTGTAGAAAAGTTATTATAGGGATAATTGGTTTGTGGTGGTTAAGTGTTTATAGTGA
HeLa06 - GGGGTTTTACGATTTTTTTGATTTTTTTGGGTTTAAAGTAGGAGGTGTAGAAAAGTTATTATAGGGATAATTGGTTTGTGGTGGTTAAGTGTTTATAGTGA
HeLa07 - GGGGTTTTACGATTTTTTTGATTTTTTTGGGTTTAAAGTAGGAGGTGTAGAAAAGTTATTATAGGGATAATTGGTTTGTGGTGGTTAAGTGTTTATAGTGA
HeLa08 - GGGGTTTTACGATTTTTTTGATTTTTTTGGGTTTAAAGTAGGAGGTGTAGAAAAGTTATTATAGGGATAATTGGTTTGTGGTGGTTAAGTGTTTATAGTGA
HeLa09 - GGGGTTTTATGATTTTTTTGATTTTTTTGGGTTTAAAGTAGGAGGTGTAGAAAAGTTATTATAGGGATAATTGGTTTGTGGTGGTTAAGTGTTTATAGTGA
HeLa10 - GGGGTTTTATGATTTTTTTGATTTTTTTGGGTTTAAAGTAGGAGGTGTAGAAAAGTTATTATAGGGATAATTGGTTTGTGGTGGTTAAGTGTTTATAGTGA

Orig. - GGGGCCUCACGAUCCUUCGACCUUUGGGUUUUAAGCAGGAGGUGUCGAAAAGUUAACCAGAGGGAUAAUGGCUUGUGGGGCCAGGUUCAUAGGA

102
HeLa01 - TGTGTTTTTTGATTTTTCGATGTTGGTTTTTTTTATTATTGTGAAGTAGAATTTATTAAGTGTTGGATTGTTTATTTATTAATAGGGAATGTGAGTTGG
HeLa02 - TGTGTTTTTTGATTTTTCGATGTTGGTTTTTTTTATTATTGTGAAGTAGAATTTATTAAGTGTTGGATTGTTTATTTATTAATAGGGAATGTGAGTTGG
HeLa03 - TGTGTTTTTTGATTTTTCGATGTTGGTTTTTTTTATTATTGTGAAGTAGAATTTATTAAGTGTTGGATTGTTTATTTATTAATAGGGAATGTGAGTTGG
HeLa04 - TGTGTTTTTTGATTTTTCGATGTTGGTTTTTTTTATTATTGTGAAGTAGAATTTATTAAGTGTTGGATTGTTTATTTATTAATAGGGAATGTGAGTTGG
HeLa05 - TGTGTTTTTTGATTTTTCGATGTTGGTTTTTTTTATTATTGTGAAGTAGAATTTATTAAGTGTTGGATTGTTTATTTATTAATAGGGAATGTGAGTTGG
HeLa06 - TGTGTTTTTTGATTTTTCGATGTTGGTTTTTTTTATTATTGTGAAGTAGAATTTATTAAGTGTTGGATTGTTTATTTATTAATAGGGAATGTGAGTTGG
HeLa07 - TGTGTTTTTTGATTTTTCGATGTTGGTTTTTTTTATTATTGTGAAGTAGAATTTATTAAGTGTTGGATTGTTTATTTATTAATAGGGAATGTGAGTTGG
HeLa08 - TGTGTTTTTTGATTTTTCGATGTTGGTTTTTTTTATTATTGTGAAGTAGAATTTATTAAGTGTTGGATTGTTTATTTATTAATAGGGAATGTGAGTTGG
HeLa09 - TGTGTTTTTTGATTTTTCGATGTTGGTTTTTTTTATTATTGTGAAGTAGAATTTATTAAGTGTTGGATTGTTTATTTATTAATAGGGAATGTGAGTTGG
HeLa10 - TGTGTTTTTTGATTTTTCGATGTTGGTTTTTTTTATTATTGTGAAGTAGAATTTATTAAGTGTTGGATTGTTTATTTATTAATAGGGAATGTGAGTTGG

Orig. - CUCCUUUUUGAUCCUUCGAUGUCGGUUUUCCUAUCAUUGUGAAGCGAAAUUCCCAGGUUGGAUUGUCACCCAUAAUAGGGAACGUGACUGG
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Frequently Asked Questions:

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

A: *Water is recommended.*

Q: Should the RNA be DNase I treated?

A: *Yes, it is recommended to treat RNA samples with DNase I (e.g., E1009) prior to the bisulfite conversion.*

(For clean-up of DNase I-treated RNA use the RNA Clean & Concentrator™, R1015)

Q: At what temperature and for how long can converted RNA be stored?

A: *The sample should be stored at $\leq -20^{\circ}\text{C}$ whenever possible and freeze-thaw cycles should be minimized. The quality of the RNA should remain relatively unchanged for up to 3 months. For long term storage samples should be kept at $\leq -70^{\circ}\text{C}$.*

Q: Which Taq polymerase(s) do you recommend for PCR amplification of cDNA generated from bisulfite-converted RNA?

A: *We recommend a “hot start” DNA polymerase (e.g., ZymoTaq™, E2001).*

Q: What RNA purification methods do you recommend?

A: *For RNA purification from cells or soft tissues use Quick-RNA™ kits (R1054). For samples in Tri-Reagent® or similar, the Direct-zol™ kits (D2050, D2051) are recommended. Both technologies allow for total RNA recovery (including small RNAs) and facilitate on-column DNase I treatment.*

Ordering Information:

Product Description	Catalog No.	Kit Size
EZ RNA Methylation™ Kit	R5001	50 rxns.
EZ RNA Methylation™ Kit	R5002	200 rxns.

For Individual Sale	Catalog No.	Amount(s)
RNA Conversion Reagent	R5001-1-1	1 tube
RNA Binding Buffer	R1013-2-25	25 ml
	R1013-2-100	100 ml
RNA Wash Buffer (concentrate)	R1003-3-6	6 ml
	R1003-3-12	12 ml
	R1003-3-24	24 ml
	R1003-3-48	48 ml
RNA Desulphonation Buffer	R5001-3-10	10 ml
	R5001-3-40	40 ml
DNase/RNase-Free Water	W1001-1	1 ml
	W1001-4	4 ml
	W1001-6	6 ml
	W1001-10	10 ml
Zymo-Spin™ IC Columns (capped)	C1004-50	50 columns
	C1004-250	250 columns
Collection Tubes	C1001-50	50 tubes
	C1001-500	500 tubes
	C1001-1000	1,000 tubes

Popular RNA Products from Zymo Research

Product	Description	Prep/Format	Catalog	
RNA Clean-Up				
RNA Clean & Concentrator™ -5	Cleanup and concentration of modified, labeled, impure, diluted, DNase treated RNA (≥ 17nt) and purification of RNA from aqueous phase of organic extracts. (DNase I included with R1013 and R1014.)	50/column 200/column	R1015 R1016	
RNA Clean & Concentrator™ -25		50/column 100/column	R1017 R1018	
RNA Clean & Concentrator™ -100		25/column	R1019	
ZR-96 RNA Clean & Concentrator™		2x 96/plate	R1080	
DNA-Free RNA Kit™		50/column 200/column	R1013 R1014	
Oligo Clean & Concentrator™	Cleanup and concentration of RNA and/or DNA oligos. Good for clean-up of miRNAs and siRNAs.	50/column 200/column	D4060 D4061	
ZR-96 Oligo Clean & Concentrator™		2x 96/plate 4x 96/plate	D4062 D4063	
ssDNA/RNA Clean & Concentrator™	Separation of short ssRNA and ssDNA (up to 200 nt) from double stranded species.	20/column 50/column	D7010 D7011	
Zymoclean™ Gel RNA Recovery Kit	Recovery of RNA from agarose gels.	50/column	R1011	
ZR small-RNA™ PAGE Recovery Kit	Small RNA (> 17nt) from polyacrylamide gels.	20/column	R1070	
OneStep™ PCR Inhibitor Removal Kit	Removal of polyphenolics, humic/fulvic acids, tannins, melanin etc. from RNA.	50/column	D6030	
OneStep™ -96 PCR Inhibitor Removal Kit		2x 96/plate	D6035	
RNA from Samples in Trizol®, TRI Reagent®, etc.				
Direct-zol™ RNA MiniPrep	RNA (>17 nt) from TRI Reagent®, TRIzol®, and all other acid-guanidinium-phenol based reagents without phase separation. DNase I included.	50/column 200/column	R2050 R2052	
Direct-zol™ RNA MiniPrep w/ TRI Reagent®		50/column w/ (50 ml) 200/column w/ (100 ml)	R2051 R2053	
Direct-zol™ -96 RNA		2x 96/plate 4x 96/plate	R2054 R2056	
Direct-zol™ -96 RNA w/ TRI Reagent®		2x 96/plate w/ (200 ml) 4x 96/plate w/ (400 ml)	R2055 R2057	
Direct-zol™ -96 MagBead RNA	RNA (>17 nt) from TRI Reagent®, TRIzol®, and all other acid-guanidinium-phenol based reagents without phase separation. These kits are in a magnetic bead format that is adaptable for high-throughput and automated protocols. DNase I included.	2x 96/plate 4x 96/plate 8x 96/plate	R2100 R2102 R2104	
Direct-zol™ -96 MagBead RNA w/ TRI Reagent®		2x 96/plate w/ (200 ml) 4x 96/plate w/ (400 ml) 8x 96/plate w/ (800 ml)	R2101 R2103 R2105	
RNA from Cells and Tissue				
Quick-RNA™ MicroPrep		Total RNA from cells and tissue. DNase I included.	50/column 200/column	R1050 R1051
Quick-RNA™ MiniPrep	50/column 200/column		R1054 R1055	
Quick-RNA™ MidiPrep	25/column		R1056	
ZR-96 Quick-RNA™	2x 96/plate 4x 96/plate		R1052 R1053	
ZR-Duet™ DNA/RNA MiniPrep	Parallel purification of DNA/RNA from cells.	50/column	D7001	
Pinpoint™ Slide RNA Isolation System Kit I	RNA from fresh/frozen tissue sections.	50/column	R1003	
Pinpoint™ Slide RNA Isolation System Kit II	RNA from paraffin-embedded (FFPE) tissue.	50/column	R1007	
RNA from Biological Liquids				
ZR Viral RNA Kit™	RNA (DNA) from body fluids (plasma, serum, CSF, urine).	50/column 200/column	R1034 R1035	
ZR-96 Viral RNA Kit™		2x 96/plate 4x 96/plate	R1040 R1041	
ZR Viral DNA/RNA Kit™		25/column 100/column	D7020 D7021	
ZR Whole-Blood RNA MiniPrep™	RNA from whole blood or partitioned blood.	50/column 100/column	R1020 R1021	
ZR Urine RNA Isolation Kit™	Cellular and endosomal RNA from urine.	20/column 50/column	R1038 R1039	
RNA from Tough-to-Lyse Samples				
ZR Fungal/Bacterial RNA MicroPrep™	RNA from bacteria, yeast, fungi; BashingBead™ lysis.	50/column	R2010	
ZR Fungal/Bacterial RNA MiniPrep™		50/column	R2014	
ZR Plant RNA MiniPrep™	RNA from leaves, stems, buds, flowers, fruits, seeds, etc; BashingBead™ lysis, RT/PCR inhibitor removal.	50/column	R2024	
ZR Tissue & Insect RNA MicroPrep™	RNA from insect, arthropod specimen and small tissue samples; BashingBead™ lysis.	50/column	R2030	
ZR Soil/Fecal RNA MicroPrep™	RNA from soil, sludge, sediment, feces.	50/column	R2040	
YeaStar RNA Kit™	RNA from yeast strains susceptible to Zymolyase.	50/column	R1002	
DNA and RNA Sample Preservation and Storage				
DNA/RNA Shield™	Cells, biological liquid, tissue storage and DNA/RNA purification.	50 ml 250 ml	R1100-50 R1100-250	
DNA/RNA Shield™ w/ Quick-RNA™ MiniPrep		50/column w/ (50 ml)	R1100	
Enzymes and Markers				
DNase I w/ 10X Reaction Buffer	Lyophilized	250 U	E1009	
ZR small-RNA™ Ladder	ssRNA (17, 21, 25, 29 nt)	10 ug	R1090	

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