

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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For Research Use Only Ver. 1.0.4

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 Buffer ¹ (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.

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.

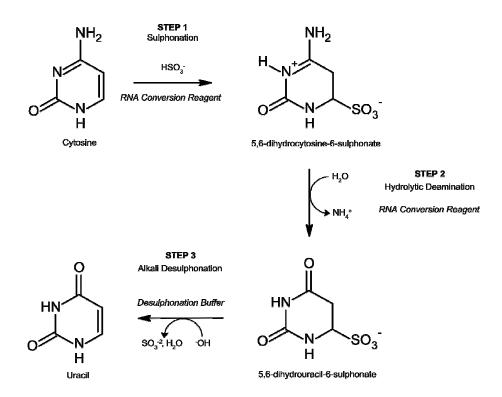
Note - [™] Trademarks of Zymo Research Corporation. This product is for research use only and should only be used by trained professionals. It is not intended for use in diagnostic procedures. Some reagents included with this kit are irritants. Wear protective gloves and eye protection. Follow the safety guidelines and rules enacted by your research institution or facility.

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.

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.

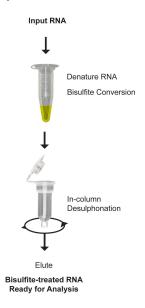
Selected EZ RNA Methylation[™] Kit Citation:

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

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

Product Description:

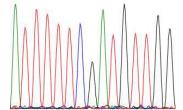
The EZ RNA Methylation™ Kit features rapid and reliable bisulfite treatment and conversion of cytosines in RNA for methylation analysis. The kit streamlines the three-step process for complete conversion of cytosine in into uracil. RNA denaturation and bisulfite conversion processes are combined into 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: Converted RNA:

cDNA Sequence:

AUCCUUC^mGAUGUCGG AUUUUUC^mGAUGUUGG 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 μ I, compensate with DNase/RNase-Free Water.

- 2. Place the PCR tube(s) in a thermal cycler and perform the following steps:
 - 1. 70℃ for 5 minutes
 - 2. 54℃ for 45 minutes
 - 3. 4℃ up to 20 hours

Note: The 4℃ 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.
- 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 μ l 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℃ for up to 3 months. For long-term storage, keep at or below -70℃.

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 aguuaccaca 4391 gggauaacug gcuuguggcg gccaagcguu cauagcgacg ucgcuuuuug auccuucgau gucggcucuu 4461 ccuaucauug ugaagcagaa uucaccaagc guuggauugu ucacccacua auagggaacg ugaqcugg--
```

Bisulfite-Converted:

H 28SF primer: 5'-GGGGTTTTAYGATTTTTTGGG-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 <u>C</u> represents 5-mC, highlighted <u>C</u> represents non-converted cytosine, *italics* are primer regions. The original, non-converted RNA sequence with non-methylated <u>C</u> highlighted is shown below the converted cDNA sequencing results for comparison.

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

```
101
Orig. - GGGGCCUCACGAUCCUUCUGACCUUUUGGGUUUUAAGCAGGAGGUGUCAGAAAAGUUACCACAGGGAUAACUGGCUUGUGGGGGCCAAGCGUUCAUAGCGA
\mathtt{HeLa04} - \mathtt{TGTTGTTTTTGATTTTT}{\overline{\mathtt{G}}} GATGTTGGTTTTTTTATTATTGTGAAGTAGAATTTGTTAAGTGTTGGATT\mathtt{GTTATTTATTATTATTAGGGAATGTGAGTTGG}
Orig. - CGUCGCUUUUUGAUCCUUCGAUGUGGGCUCUUCCUAUCAUUGAAGCAGAAUUCACCAAGCGUUGGAUUGUUCACCCACUAAUAGGGAAAGCGUAGGAAAUUCACCAAGCAAAGCGUUGGAUUGUUCACCCACUAAUAGGGAAACGUGAGCUAG
```

Appendix II: Bisulfite Conversion and PCR Optimization

1. Bisulfite Conversion of Double-Stranded DNA Templates. The following illustrates what occurs to a DNA template during bisulfite conversion. The same principles apply to secondary structure or double-stranded RNA.

```
Template:

A: 5'-GACCGTTCCAGGTCCAGCAGTGCGCT-3'
B: 3'-CTGGCAAGGTCCAGGTCGTCACGCGA-5'

Bisulfite-Converted:

A: 5'-GATCGTTTTAGGTTTAGTAGTGCGTT-3'

B: 3'-TTGGCAAGGTTTAGGTTGTTATGCGA-5'
```

2. PCR Primer Design. Generally, primers 26 to 32 bases are required for amplification of bisulfite-converted samples. All Cs should be treated as Ts for primer design purposes, unless they are in a CpG context. When using specific primers for the reverse transcription reaction to perform RT-PCR, it is important to use the "reverse" primer, as the "forward" primer will not hybridize to the template. See example below.

```
Bisulfite-Converted: A: 5'-GATCGTTTAGGTTTAGTAGTGCGTT-3'
Primers: Reverse: 3'-ATCATCACRCAA-5' R= G/A
Forward: 5'-GATYGTTTTAGGT-3' Y= C/T
```

Zymo Research provides primer design assistance with its <u>Bisulfite Primer Seeker Program</u>, available at: <u>www.zymoresearch.com/tools/bisulfite-primer-seeker</u>. Please feel free to contact us at tech@zymoresearch.com for additional help.

- 3. Amount of RNA Required for Bisulfite Conversion. The minimal amount of human RNA required for bisulfite-treatment and subsequent PCR amplification is 32 ng. The optimal amount of RNA per bisulfite treatment is 0.5 to 1 µg. Although, up to 3 µg of RNA can also be processed, it should be noted that high input levels of RNA may result in incomplete bisulfite conversion for some GC-rich regions.
- **4. PCR Conditions.** We recommend using 1 4 μl of eluted RNA for each RT-PCR. However, up to 10 μl can be used if necessary. Usually, 35 to 45 cycles are required for successful PCR amplification of bisulfite-converted RNA. Optimal amplicon size should be between 100 − 200 bp; however larger amplicons can be generated with optimization of the PCR conditions. We have found that annealing temperatures between 55 60℃ typically work well. As most non-methylated cytosine residues are converted into uracil, the bisulfite-treated RNA usually is AU-rich and has low GC composition. Thus, it may be necessary to reduce the annealing temperature accordingly.

Non-specific PCR amplification is relatively common with bisulfite-treated RNA due to its AU-rich nature. PCR using "hot start" polymerases is strongly recommended for the amplification of bisulfite-treated RNA.

5. Quantifying RNA. For the absorption coefficient at 260 nm, use a value of 40 μ g/ml for Ab₂₆₀ = 1.0 when determining the concentration of both normal and bisulfite-treated RNA.

Note: Methylated "C" is underlined in the examples.

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

Note: Only one strand (A) is amplified by a given primer set. Only the reverse primer binds to the converted RNA, the forward primer will bind the strand generated during the reverse transcription.

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.

Zymo Taq[™] is a "hot start" DNA polymerase <u>specifically</u> <u>designed</u> for the amplification of bisulfite treated DNA. (E2001)

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 ≤ -20°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 ≤ -70°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., ZymoTag[™], 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 R1013-2-100	25 ml 100 ml
RNA Wash Buffer (concentrate)	R1003-3-6 R1003-3-12 R1003-3-24 R1003-3-48	
RNA Desulphonation Buffer	R5001-3-10 R5001-3-40	10 ml 40 ml
DNase/RNase-Free Water	W1001-1 W1001-4 W1001-6 W1001-10	1 ml 4 ml 6 ml 10 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



Popular RNA Products from Zymo Research

Product	Description	Prep/Format	Catalog
	RNA Clean-Up	F0/221: 122.2	D4045
RNA Clean & Concentrator [™] -5		50/column 200/column	R1015 R1016
TM	Cleanup and concentration of modified, labeled, impure, diluted, DNase treated RNA	50/column	R1017
RNA Clean & Concentrator [™] -25	(≥ 17nt) and purification of RNA from aqueous phase of organic extracts.	100/column	R1018
RNA Clean & Concentrator [™] -100		25/column	R1019
ZR-96 RNA Clean & Concentrator™	(DNase I included with R1013 and R1014.)	2x 96/plate	R1080
DNA-Free RNA Kit [™]		50/column	R1013
DIA 1100 MATM		200/column	R1014
Oligo Clean & Concentrator [™]	Olever and the control of DNA and the DNA allows One of the classic of the DNA	50/column	D4060
	Cleanup and concentration of RNA and/or DNA oligos. Good for clean-up of miRNAs and siRNAs.	200/column	D4061 D4062
ZR-96 Oligo Clean & Concentrator [™]	and sinnas.	2x 96/plate 4x 96/plate	D4062 D4063
TM.		20/column	D7010
ssDNA/RNA Clean & Concentrator™	Separation of short ssRNA and ssDNA (up to 200 nt) from double stranded species.	50/column	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		50/column	R2050
· · · · · · · · · · · · · · · · · · ·	_	200/column	R2052
Direct-zol [™] RNA MiniPrep w/ TRI Reagent [®]	RNA (>17 nt) from TRI Reagent®, TRIzol®, and all other acid-guanidinium-phenol	50/column w/ (50 ml) 200/column w/ (100 ml)	R2051 R2053
TM	based reagents without phase separation. DNase I included.	2x 96/plate	R2053
Direct-zol [™] -96 RNA	Date to agone mare a priace coparation. Driace timolasca.	4x 96/plate	R2056
Direct-zol [™] -96 RNA w/ TRI Reagent [®]		2x 96/plate w/ (200 ml)	R2055
Direct-201 -96 RNA W/ TRI Reagent		4x 96/plate w/ (400 ml)	R2057
71		2x 96/plate	R2100
Direct-zol [™] -96 MagBead RNA	RNA (>17 nt) from TRI Reagent®, TRIzol®, and all other acid-quanidinium-phenol	4x 96/plate	R2102
	based reagents without phase separation. These kits are in a magnetic bead format	8x 96/plate	R2104
Direct-zol [™] -96 MagBead RNA w/ TRI Reagent [®]	that is adaptable for high-throughput and automated protocols. DNase I included.	2x 96/plate w/ (200 ml)	R2101
Direct-201 -96 Magbeau KNA W/ TKI Keagerit		4x 96/plate w/ (400 ml) 8x 96/plate w/ (800 ml)	R2103 R2105
	RNA from Cells and Tissue	8x 90/plate w/ (000 IIII)	112103
○ / / □ · / □ · · · · □		50/column	R1050
<i>Quick-RNA</i> [™] MicroPrep		200/column	R1051
<i>Quick-RNA</i> ™ MiniPrep		50/column	R1054
•	Total RNA from cells and tissue. DNase I included.	200/column	R1055
<i>Quick-RNA</i> [™] MidiPrep	_	25/column	R1056
ZR-96 Quick-RNA [™]		2x 96/plate	R1052 R1053
ZR-Duet [™] DNA/RNA MiniPrep	Parallel purification of DNA/RNA from cells.	4x 96/plate 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 [™]		50/column	R1034
ZK VIIdi KIVA KIL		200/column	R1035
ZR-96 Viral RNA Kit [™]	RNA (DNA) from body fluids (plasma, serum, CSF, urine).	2x 96/plate	R1040
	— · · · · · · · · · · · · · · · · · · ·	4x 96/plate 25/column	R1041
ZR Viral DNA/RNA Kit [™]		25/column 100/column	D7020 D7021
	<u> </u>	50/column	R1020
ZR Whole-Blood RNA MiniPrep [™]	RNA from whole blood or partitioned blood.	100/column	R1020
7D Uring DNA location VitTM	Collular and and completion IRMA from uring	20/column	R1038
ZR Urine RNA Isolation Kit [™]	Cellular and endosomal RNA from urine.	50/column	R1039
10	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,	50/column	R2024
ZR Tissue & Insect RNA MicroPrep™	RT/PCR inhibitor removal. RNA from insect, arthropod specimen and small tissue samples; BashingBead™ lysis.	50/column	R2030
ZR Soil/Fecal RNA MicroPrep™	RNA from insect, arthropod specimen and small tissue samples; BashingBead im lysis. RNA from soil, sludge, sediment, feces.	50/column	R2030 R2040
YeaStar RNA Kit [™]	RNA from yeast strains susceptible to Zymolyase.	50/column	R1002
	DNA and RNA Sample Preservation and Storage	20,00141111	
DNA/RNA Shield [™]		50 ml	R1100-50
DINAKNA SNIBIO	Colla historical liquid ticque eterogo and DNA/DNA avvillantia	250 ml	R1100-250
DNA/RNA Shield [™] w/ <i>Quick-RNA</i> [™] MiniPrep	Cells, biological liquid, tissue storage and DNA/RNA purification.	50/column w/ (50 ml)	R1100
Distriction of Quick-tony million tep		Jo/Column W/ (JO IIII)	131100
	Enzymes and Markers		
DNase I w/ 10X Reaction Buffer ZR small-RNA [™] Ladder	Lyophilized	250 U	E1009
	ssRNA (17, 21, 25, 29 nt)	10 μg	R1090