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Globin-Zero™ Gold Kit

Cat. No. GZG1206 – 6 Reactions

(Contains 1 box of Cat. No. GZRR1306 and 1 box of Cat. No. MRZ116C)

Cat. No. GZG1224 – 24 Reactions

(Contains 1 box of Cat. No. GZRR1324 and 1 box of Cat. No. MRZ11124C)

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Quick Protocol for Globin-Zero™ Gold Kit

For experienced users only! The standard protocol begins on page 4.

Cat. No. GZRR1306/MRZ116C or GZRR1324/MRZ11124C

Step	Procedure	Pages
Prepare Magnetic Beads	Add 225 µl Magnetic Beads to RNase-free tube. Place in magnetic stand for 1 min at RT. Wash with 225 µl RNase-Free Water. Place in magnetic stand, repeat wash step. Resuspend in 65 µl Resuspension Solution Optional: Add 1 µl RiboGuard RNase Inhibitor	4
Treat sample with Globin-Zero Removal Solution	Mix in 40 µl total volume: 1 µg - 5 µg DNA-free RNA total RNA 10 µl - 12.5 µl Globin-Zero Gold Removal Solution 4 µl Reaction Buffer Incubate 10 min @ 68˚C, then 5 min @ RT.	5
Remove globin mRNA/ rRNA	Mix previously prepared Magnetic Beads. Add RNA mixture, mix well by pipetting, vortex well. Incubate 5 min @ RT, vortex well. Incubate 5 min @ 50˚C. Place in magnetic stand, transfer supernatant (globin mRNA/rRNA-depleted sample) to RNase-free tube.	6
Purify Globin-Zero treated RNA	Ethanol precipitation or alternative method	6

1. Kit Contents

Globin-Zero™ Gold Reagents (Cat. No. GZRR1306/GZRR1324)

Component Name	Tube Label	Volume		Cap Color
		6 rxn	24 rxn	
RiboGuard™ RNase Inhibitor (100 U/µl)	RiboGuard™ RNase Inhibitor	10 µl	30 µl	Blue
Globin-Zero™ Gold Removal Solution	Globin-Zero™ Removal Solution	80 µl	320 µl	
Globin-Zero™ Gold Reaction Buffer	Globin-Zero™ Reaction Buffer	50 µl	110 µl	
RNase-Free Water	RNase-Free Water	2 x 1 ml	2 x 1.8 ml	Clear
Baseline-ZERO™ DNase	Baseline-ZERO™ DNase	30 µl	130 µl	Green
Baseline-ZERO™ 10x Reaction Buffer	Baseline-ZERO™ Buffer	60 µl	275 µl	
Glycogen 10 mg/ml	Glycogen 10 mg/ml	20 µl	60 µl	Clear
Sodium Acetate 3M	Sodium Acetate 3M	150 µl	500 µl	

Storage: Store this kit box and its contents at –70°C to –80°C.

Magnetic Core Kit (Cat. No. MRZ116C, MRZ11124C)

Component Name	Tube Label	Volume		Cap Color
		6 rxn	24 rxn	
Magnetic Beads	Magnetic Beads	1.4 ml	5.4 ml	Clear
Magnetic Bead Resuspension Solution	Magnetic Bead Resuspension Solution	500 µl	2 ml	Yellow
RNase-Free Water	RNase-Free Water	2 x 1.5 ml	11 ml	Clear

Storage: Store this kit box and its contents at +4°C (Do Not Freeze!).

Additional Required Reagents and Equipment:

Magnetic rack or stand

Vortex mixer

0.2-ml or 0.5-ml microcentrifuge tubes (RNase-free)

Thermocycler or other temperature control device for 0.2-ml or 0.5-ml tubes

Water bath, heating block, or other temperature control device for 1.5-ml tubes

Microcentrifuge

RNA purification kits or reagents (see Part 3.A)

Performance Specifications and Quality Control:

A Globin-Zero™ Gold Kit reaction removes >99% of cytoplasmic 28S, 18S, 5.8S rRNA, >95% of cytoplasmic 5S rRNA, >99% of 16S and 12S mitochondrial rRNA and >98% of globin (α1, α2, β and γ) mRNA from an RNA mixture containing 5 µg of Human Reference RNA + 100 ng of globin RNA as assessed by qRT-PCR.

2. RNA Sample Considerations

A kit reaction uses 1µg - 5 µg of input total blood-derived RNA. Total RNA isolated from blood samples are often contaminated with DNA and inhibitors of downstream enzymatic reactions. The DNA and enzymatic inhibitors must be removed from the RNA sample prior to treatment with the Globin-Zero Gold Kit.

For total RNA sample containing genomic DNA contamination, a DNase I protocol and reagents for removal of the contaminating DNA is provided in Part 3A. Agarose gel electrophoresis is the most convenient and commonly used method for detecting DNA stained with either ethidium bromide or SYBR Gold. For DNA-free RNA, proceed directly to Part 3.B.

Note: *It is important to quantify the amount of DNA-free total RNA in the sample as accurately as possible in order to use the appropriate amount of Globin-Zero Gold Removal Solution in Part 3.C.*

3. Globin-Zero Kit Procedure

Required in Part 3.A for treating total RNA samples containing genomic DNA contamination.

Note: *For DNA-free RNA, proceed directly to Part 3.B.*

Component Name	Tube Label	Cap Color
Baseline-ZERO 10X Reaction Buffer	Baseline-ZERO™ Buffer	Green
Baseline-ZERO™ DNase	Baseline-ZERO™ DNase	
RNase-Free Water	RNase-Free Water	Clear

Additionally required for each reaction (provided by the user):

Two RNeasy™ MinElute Cleanup Kit columns (Qiagen; cat no. 74204)

OR

Two RNA Clean & Concentrator™ Columns, (Zymo Research; cat. no. R1015, R1016)

0.5 ml or 1.5 ml microcentrifuge tube (RNase-free)

3.A. Baseline-ZERO™ DNase treatment of blood-derived RNA containing genomic DNA

DNA contamination must be removed from the RNA sample prior to Globin-Zero Gold Kit treatment.

Note: *Due to the presence of enzymatic inhibitors, total RNA isolated using the PAXgene Blood RNA kits must be repurified prior to treatment with DNase I.*

1. Purify the total RNA isolated using the PAXgene Blood RNA Kit or PAXgene Blood miRNA Kit with either the modified RNeasy™ MinElute® Cleanup Kit procedure described in Section 3.E.1 or the RNA Clean & Concentrator™ column, General Procedure, (Zymo Research; cat. no. R1015, R1016) as described by the manufacturer. For either procedure, elute the RNA in 12 µl of RNAse-free water and proceed with Step 3.A.2.
2. Assemble the Baseline-ZERO DNase reaction:

   Combine on ice:

   10 µl Baseline-ZERO 10X Reaction Buffer
   10 µl Purified RNA (from Step 1)
   75 µl RNase-free water
   5 µl Baseline-ZERO DNase
   100 µl Total volume
3. Incubate at 37°C for 15 minutes
4. Purify the Baseline-ZERO treated RNA using either the modified RNeasy™ MinElute® Cleanup Kit procedure described in Section 3.E.1 or the RNA Clean & Concentrator™ column, General Procedure, (Zymo Research; cat. no. R1015, R1016) as described by the manufacturers. For either procedure, elute the RNA in 12 µl of RNase-Free Water.
5. Measure the concentration of the RNA and proceed to Part 3.B. It is important to quantify the yield of Baseline-ZERO treated RNA as accurately as possible in order to use the appropriate amount of the Globin-Zero Gold Removal Solution in Part 3.C.

3.B. Preparation of the Magnetic Beads

Required in Part 3.B

Component Name	Tube Label	Cap Color
Magnetic Beads	Magnetic Beads	Clear
RNase-Free Water	RNase-Free Water	
Magnetic Bead Resuspension Solution	Magnetic Bead Resuspension Solution	
RiboGuard RNase Inhibitor (100 U/µl)	RiboGuard RNase Inhibitor	Blue

Remove the Globin-Zero Gold Reagents from –70°C to –80°C storage, thaw the tubes, and place them on ice. Wash the magnetic beads by using either the batch washing or individual washing procedure.

Important! *Allow the Magnetic Core Kit components to equilibrate to room temperature.*

Additionally required (provided by the user):
Magnetic rack or stand
Vortex mixer

Batch Washing Procedure

1. For each Globin-Zero Gold reaction, 225 µl of the Magnetic Beads is required.

   Important! *Resuspend the Magnetic Beads well by pipetting or gentle vortexing.*

2. Determine the amount of Magnetic Beads required for the total number of reactions and dispense a maximum of 1,350 µl into a 1.5-ml RNase-free microcentrifuge tube (sufficient for 6 reactions). Pipet the Magnetic Bead suspension slowly to avoid air bubbles and to ensure pipetting of the correct volume. Store the unused Magnetic Beads at +4°C.
3. Place the 1.5-ml microcentrifuge tube containing the Magnetics Beads on the magnetic stand for at least 1 minute until the solution appears clear.
4. With the 1.5-ml microcentrifuge tube still on the stand, remove and discard the supernatant.

   Caution: *The supernatant contains 0.1% sodium azide.*

5. Remove the 1.5-ml microcentrifuge tube from the stand and add a volume of RNase-Free Water equal to the original volume of Magnetic Beads. Mix well by repeated pipetting or by vortexing at medium speed.
6. Repeat steps 3, 4 and 5 (i.e. wash the beads a total of 2 times with RNase-Free water).
7. Remove the 1.5-ml microcentrifuge tube from the magnetic stand. Add a volume of Magnetic Bead Resuspension Solution equal to the number of reactions x 60 µl. For example, for 6 reactions, add 6 x 60 µl = 360 µl of Globin-Zero Magnetic Bead Resuspension Solution. Mix well by repeated pipetting or by vortexing at medium speed.

   Note: *The volumes of the beads and Resuspension Solution are additive. Although the washed beads are resuspended in 60 µl per reaction, each reaction uses 65 µl of resuspended beads.*

8. Aliquot 65 µl of the washed Magnetic Beads into each new 1.5-ml RNase-free microcentrifuge tube (corresponding to the number of Globin-Zero reactions).

   Note*: There should be essentially little or no beads remaining otherwise, dispense equally into each aliquot.*

9. Optional: Add 1.0 µl of RiboGuard RNase Inhibitor to each tube of resuspended Magnetic Beads, and mix briefly by vortexing.
10. Store the microcentrifuge tubes at room temperature until required in Part 3.D.

Individual Washing Procedure

Important! *Resuspend the Magnetic Beads well by pipetting or gentle vortexing.*

1. For each reaction, pipet 225 µl of Magnetic Beads into a 1.5-ml RNase-free microcentrifuge tube. Pipet the Magnetic Bead suspension slowly to avoid air bubbles and to ensure pipetting of the correct volume. Store unused Magnetic Beads at 4°C.
2. Place each 1.5-ml microcentrifuge tube on the magnetic stand for at least 1 minute until the solution appears clear.
3. With the 1.5-ml microcentrifuge tube still on the stand, remove and discard the supernatant.

   Caution: *The supernatant contains 0.1% sodium azide.*

4. Remove the 1.5-ml microcentrifuge tube from the magnetic stand and add 225 µl of RNase-Free Water to each tube. Mix well by repeated pipetting or vortexing at medium speed.
5. Repeat steps 2, 3 and 4 (i.e. wash the beads a total of 2 times with RNase-Free water).
6. Remove the 1.5-ml microcentrifuge tube from the magnetic stand. Add 65 µl of Magnetic Bead Resuspension Solution to each tube. Mix well by repeated pipetting or vortexing at medium speed.
7. Optional: Add 1.0 µl of RiboGuard RNase Inhibitor to each tube of resuspended Magnetic Beads, and mix briefly by vortexing.
8. Store the microcentrifuge tubes at room temperature until required in Part 3.D.

3.C. Treatment of the Total RNA Sample with Globin-Zero Gold Removal Solution

Required in Part 3.C

Component Name	Tube Label	Cap Color
Globin-Zero Gold Reaction Buffer	Globin-Zero Reaction Buffer	Blue
Globin-Zero Gold Removal Solution	Globin-Zero Removal Solution	
RNase-Free Water	RNase-Free Water	Clear

Additionally required for each reaction (provided by user):
Magnetic stand or rack
0.2-ml or 0.5-ml microcentrifuge tube (RNase-free)
Vortex mixer

Important! *The maximum volume of the RNA sample and the volume of the Globin-Zero Gold Removal Solution used per reaction is dependent on the amount of total RNA in the sample (see Table 1).*

Table 1. Volumes of Globin-Zero™ Gold Removal Solution.

Amount of Input Total RNA	Maximum Volume of Total RNA That Can Be Added to each reaction	Volume of Globin-Zero Gold Removal Solution Used per Reaction
1 µg - 2.5 µg	26 µl	10 µl
2.5 µg - 5 µg	23.5 µl	12.5 µl

1. In a 0.2-ml or 0.5-ml RNase-free microcentrifuge tube, combine in the order given:

x µl	RNase-Free Water
4 µl	Globin-Zero Gold Reaction Buffer
1 µg to 5 µg	DNA-Free RNA sample (see Table 1)
y µl	Globin-Zero Gold Removal Solution (see Table 1)
40 µl	Total volume

2. Gently mix the reaction(s) by pipetting and incubate at 68°C for 10 minutes. During the incubation, return the remaining Globin-Zero Gold Removal Solution and Globin-Zero Reaction Gold Buffer at –70°C to –80°C.
3. Remove the reaction tube(s) and incubate each at room temperature for 5 minutes.

3.D. Magnetic Bead Reaction, and Globin mRNA and rRNA Removal

Required in Part 3.D: 50°C water bath or heating block for 2.0-ml tubes.

Important! *Always add the treated RNA sample to the washed Magnetic Beads and immediately mix by pipetting. Never add the Magnetic Beads to the treated RNA sample. Immediate and thorough mixing prevents the beads from forming clumps that can significantly impact the efficiency of the globin mRNA and rRNA removal.*

1. Using a pipette, add the treated RNA from Part 3.C, Step 3 to the 1.5-ml microcentrifuge tube containing the washed, room temperature Magnetic Beads and without changing the pipet tip ***immediately and thoroughly mix the contents of the tube by pipetting at least 10 times. Then, vortex the tube immediately at medium setting for 10 seconds and place at room temperature.*** Repeat this process for each sample.
2. Incubate the 1.5 ml microcentrifuge tube at room temperature for 5 minutes.
3. Following the room temperature incubation, ***mix the reactions by vortexing at medium speed for at least 10 seconds*** and then place at 50°C for 5 minutes in an appropriate water bath or heating block. Avoid any significant condensation during this incubation step.
4. After the 5-minute incubation at 50°C, remove the microcentrifuge tubes and immediately place them on a magnetic stand for at least 1 minute until the solution appears clear.
5. Carefully remove each supernatant (85-90 µl) containing the RNA and transfer to a labeled 1.5-ml RNase-free microcentrifuge tube.

   Important! *The supernatant contains the Globin-Zero-treated RNA.*

   Caution: *If a small amount of Magnetic Beads is still visible in the supernatant, place the collected supernatant onto the magnetic stand for 1 minute. Remove the supernatant containing the Globin-Zero treated RNA and transfer to a new 1.5-ml RNase-free microcentrifuge tube.*
6. Place the supernatant (RNA solution) on ice and immediately proceed to Part 3.E. Alternatively, the supernatant may be stored at –70°C to –80°C before completing Part 3.E.

3.E. Purification of the Globin-Zero-Treated Sample

The Globin-Zero treated samples can be purified by three methods: ethanol precipitation, AMPure® beads, or spin columns. Ethanol precipitation and the modified RNeasy MinElute procedure provide optimal recovery of small RNAs that may be lost with other purification methods; however, ethanol precipitation can be challenging for inexperienced users. We also provide an alternative protocol using AMPure beads (provided by the user) for ease of automation but this will not quantitatively recover small RNAs.

3.E.1 Modified Qiagen RNeasy™ MinElute® Cleanup Kit Procedure (Cat. No. 74204)

Note: *RNA purification kits from other suppliers may also be used; however, performance may vary.*

1. Adjust the sample to a volume of 100 µl with RNase-Free Water. Add 350 µl of Buffer RLT and mix well.
2. Add 550 µl of 96%-100% ethanol to the sample, and mix well by pipetting. Do not centrifuge. Proceed immediately to Step 3.E.1.3.
3. Transfer half of the sample (~500 µl) to an RNeasy MinElute spin column placed in a 2-ml collection tube (supplied in the Qiagen kit). Close the lid gently, and centrifuge for 15 seconds at 8,000 x g (~10,000 rpm). Discard the flow-through. Reuse the collection tube for Step 4.

4. Transfer the remaining sample and repeat the centrifugation. Discard the flow-through and collection tube.
5. Prepare the RPE Buffer by adding ethanol as described in the Qiagen procedure "Things to do before starting". Place the RNeasy MinElute spin column in a new 2-ml collection tube (supplied in the Qiagen kit). Add 500 µl Buffer RPE to the spin column. Close the lid gently, and centrifuge for 15 seconds at 8,000 x g (~10,000 rpm) to wash the spin-column membrane. Discard the flow-through.
6. Add 500 µl of 80% ethanol to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 2 minutes at 8,000 x g (~10,000 rpm) to wash the spin-column membrane. After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur. Discard the flow-through and collection tube.
7. Place the RNeasy MinElute spin column in a new 2-ml collection tube (supplied in the Qiagen kit). Open the lid of the spin column. To avoid damage to the spin-column lids, place the spin columns into the centrifuge with at least one empty position between columns. Orient the lids so that they point in a direction opposite to the rotation of the rotor (i.e., if the rotor rotates clockwise, orient the lids counterclockwise). Centrifuge at full speed for 5 minutes. Centrifugation with the lids open ensures that no ethanol is carried over during RNA elution Discard the flow-through and collection tube.
8. Place the RNeasy MinElute spin column in a new 1.5-ml collection tube (supplied in the Qiagen kit). Add 12 µl of RNase-Free Water directly to the center of the spin-column membrane. As little as 10 µl of RNase-Free Water can be used for elution if a higher RNA concentration is required, for down-stream applications, but the yield will be reduced by approximately 20%. Do not elute with less than 10 µl of RNase-Free Water, as the spin-column membrane will not be sufficiently hydrated. Close the lid of the spin column gently, and centrifuge for 1 minute at full speed to elute the Globin-Zero treated RNA.

3.E.2 Purification using Agencourt RNAClean™ XP Kit (Cat. No. A63987)

Note: *A fresh 80% ethanol solution is required for Steps 5 and 7 below.*

1. Vortex the AMPure® RNAClean XP Beads until they are well dispersed. Then add 160 µl of the mixed AMPure RNAClean XP Beads to each 1.5-ml microcentrifuge tube containing 85-90 µl of the Globin-Zero treated RNA from Part 3.D, Step 6. Mix thoroughly by gently pipetting the entire volume 10 times.
2. Incubate the tube(s) at room temperature for 15 minutes. During incubation, prepare 80% ethanol solution required for Steps 5 and 7.
3. Place the tube(s) on the magnetic stand at room temperature for at least 5 minutes until the liquid appears clear.
4. Remove and discard the supernatant from each tube. Take care not to disturb the beads.
5. With the tube(s) still on the magnetic stand, add 200 µl of freshly prepared 80% ethanol to each tube, without disturbing the beads.
6. Incubate at room temperature for at least 30 seconds while still on the magnetic stand. Then remove and discard all of the supernatant from each tube. Take care not to disturb the beads.
7. Repeat Steps 5 and 6 one time.
8. Allow the tubes to air dry on the magnetic stand at room temperature for 15 minutes.
9. Add a desired elution volume of RNase-Free water to each tube.

   Note: *For Globin-Zero-treated RNA that will be used directly in a ScriptSeq™ v2 RNA-Seq Library Preparation Kit (available separately from Epicentre; see Related Products) reaction, we recommend eluting with 11 µl of RNase-Free Water in order to yield a final eluted volume of 9 µl.*
10. Remove the tube(s) from the magnetic stand and thoroughly resuspend the beads by gently pipetting 10 times.
11. Incubate the tubes at room temperature for 2 minutes and then place the tubes back onto the magnetic stand at room temperature for at least 5 minutes until the liquid appears clear.
12. Transfer the clear supernatant from each tube to an appropriate collection tube, always leaving at least 1 µl of the supernatant behind to avoid carryover of magnetic particles. Store on ice for immediate use or store at –70°C or –80°C until required.

3.E.3 Ethanol Precipitation of the Globin-Zero-Treated RNA

Important! *The RNA pellet from an ethanol precipitation is virtually impossible to see. Use extreme care to avoid loss of the RNA.*

1. Adjust the volume of each Globin-Zero treated sample to 180 µl using RNase-Free Water.
2. Add 18 µl of 3 M Sodium Acetate to each tube.
3 Add 2 µl of RNase-free Glycogen (10 mg/ml) to each tube and mix by gentle vortexing.
4. Add three volumes (600 µl) of ice-cold 100% ethanol to each tube and mix thoroughly by gentle vortexing.
5. Place the tubes at –20°C for at least 1 hour.
6. Centrifuge the tubes at >10,000 x g in a microcentrifuge for 30 minutes. Carefully remove and discard the supernatant.
7. Wash the pellet with ice-cold 70% ethanol and centrifuge at >10,000 x g for 5 minutes. Carefully remove and discard the supernatant.
8. Repeat Step 7 above one more time.
9. Centrifuge briefly to collect any residual supernatant. Carefully remove and discard the supernatant and allow the pellet to air dry at room temperature for 5 minutes.
10. Dissolve the pellet in the desired volume of RNase-Free Water or buffer. The Globin-Zero-treated RNA can be used immediately or stored at –70°C to –80°C.

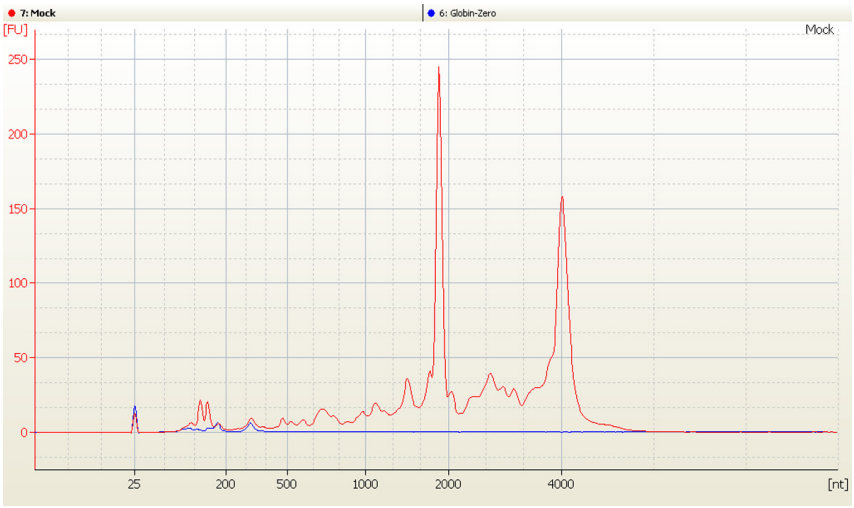

Figure 4. A 5 ug aliquot of total RNA isolated from whole blood was treated with the Globin-Zero Gold kit as described in the protocol. A corresponding control mock treated (no probe) sample was also prepared. An aliquot of the Globin-Zero (blue line) and mock treated (red line) samples corresponding to 5 ng total RNA input was analyzed using a 2100 RNA Pico chip (Agilent).

4. Quantifying the Yield and Assessing the Quality of the Globin-Zero-Treated RNA

If assessing the quality of the Globin-Zero-treated RNA using an Agilent 2100 Bioanalyzer, use the Agilent RNA6000 Pico Chip and load 2-4 ng of the Globin-Zero treated RNA. The Agilent RNA Nano Chip does not provide sufficient sensitivity.

When purifying the Globin-Zero treated RNA by ethanol precipitation, small RNAs such as miRNA and tRNA are recovered along with the mRNAs and large noncoding RNAs. Therefore, the presence of a high proportion of small RNA in the Globin-Zero treated sample should not be interpreted as degradation of the RNA.

Appendix 1: Profile of Globin-Zero Gold Kit treated RNA.

Fig. 4 shows representative 2100 Bioanalyzer (Agilent) profiles of Globin-Zero™ Gold treated blood RNA.

5. Related Products

Cat. #	Quantity
ScriptSeq™ v2 RNA-Seq Library Preparation Kit	
SSV21106	6 Reactions
SSV21124	24 Reactions
ScriptSeq™ Complete Gold Kit (Blood)–Low Input	
SCL6GBL	6 Reactions
SCL24GBL	24 Reactions
ScriptSeq™ Complete Gold Kit (Blood)	
BGGB1306	6 Reactions
BGGB1324	24 Reactions
Ribo-Zero™ Magnetic Kit (Epidemiology)	
MRZE706	6 Reactions
MRZE724	24 Reactions
Ribo-Zero™ Magnetic Gold Kit (Human/Mouse/Rat)	
MRZG126	6 Reactions
MRZG12324	24 Reactions
ScriptSeq™ Complete Gold Kit (Human/Mouse/Rat)–Low Input	
SCL6G	6 Reactions
SCL24G	24 Reactions
ScriptSeq™ Complete Gold Kit (Human/Mouse/Rat)	
BG1206	6 Reactions
BG1224	24 Reactions
ScriptMiner™ Small RNA-Seq Library Preparation Kit	
SMMP101212	12 Reactions

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