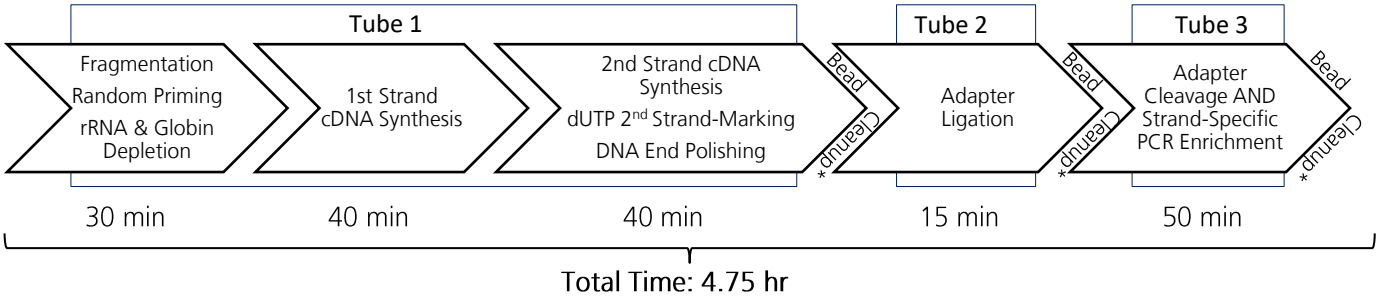
sparQ RNA-Seq HMR Kit for Singular Genomics G4™ Sequencing Platform

Cat. No.	95216-008 (Sample size only)	Size	8 reactions	For storage temperature upon receipt, refer to 'Storage and Stability'
	95216-024		24 reactions	
	95216-096		96 reactions	

Description

The sparQ RNA-Seq HMR Kit provides reagents essential for construction of stranded RNA libraries with rRNA and globin mRNA depletion for Human/Mouse/Rat (HMR) samples. The kit uses proprietary, highly optimized enzymes in a streamlined workflow with integrated ribosomal depletion for RNA library. High quality whole transcriptome libraries can be prepared from either intact or degraded RNA samples at varying input quantities (from 1 ng of high-quality RNA and 10 ng of degraded RNA up to 1000 ng RNA input). The protocol is simplified into 3 reaction tubes and 10 steps to minimize hands-on and total workflow time to prepare sequencer-ready libraries in a single day.

Workflow Overview



* Each bead cleanup takes approximately 25 minutes and is included in the total time.

Instrument Compatibility: This product is compatible with Illumina, Element and Singular sequencing insturments. This IFU is for use with Singular Genomics sequencing instruments.

Components

				Volume		
	Component Description	Cap Color		8 reactions	24 reactions	96 reactions
Box 1 Store at -25°C to -15° C	Frag Prime RG Depletion Mix	●	Blue	1 x 32 µl	1 x 96 µl	1 x 384 µl
	1st Strand Enzyme Mix	●	Brown	1 x 32 µl	1 x 96 µl	1 x 384 µl
	2nd Strand Buffer	●	Green	1 x 160 µl	1 x 480 µl	2 x 960 µl
	2nd Strand Enzyme Mix	●	Green	1 x 80 µl	1 x 240 µl	1 x 960 µl
	Rapid Ligation Buffer (5X)	●	Orange	1 x 160 µl	1 x 480 µl	2 x 960 µl
	T4 DNA Ligase	●	Orange	1 x 80 µl	1 x 240 µl	1 x 960 µl
	HiFi Plus Master Mix (2X)	○	White	1 x 200 µl	1 x 600 µl	2 x 1.2 ml
	Primer Mix	○	White	1 x 12 µl	1 x 36 µl	1 x 144 µl
Bag 2 Store at 2°C to 8°C	Bead Booster		Clear	1 x 48 µl	1 x 144 µl	1 x 576 µl
	UDI Dilution Buffer		Clear	1 x 480 µl	1 x 1.44 ml	1 x 5.76 ml

Storage and Stability

Store 'Bead Booster' 'UDI Dilution Buffer' in a constant temperature refrigerator at -2 - 8°C upon receipt.
Store the remaining components in a constant temperature freezer at -25°C to -15°C upon receipt.
For lot specific expiry date, refer to package label, Certificate of Analysis or Product Specification Form.

Additional reagents that are not supplied

- Elution Buffer: 10 mM Tris-HCl pH 8.0
- Nuclease-free water
- 80% freshly prepared ethanol
- Purification Beads for post-second strand, post-ligation and post-amplification reaction cleanups are not included with the kit and must be purchased separately. We recommend using sparQ PureMag Beads from Quantabio.

Part Number	Description	Kit Size
95196-005	sparQ PureMag Beads	5 ml
95196-060	sparQ PureMag Beads	60 ml
95196-450	sparQ PureMag Beads	450 ml

- Adapters are not included with the kit and must be purchased separately. The sparQ RNA-Seq HMR Kit with Singular Genomics G4 Sequencing Platform, the compatible adapter and UDI primers **must be purchased from Singular Genomics (SG)**.

Part Number	Description	Kit Size
700,110	SG Universal Library Prep Adapter + UDI Primers [1-96]	96
700,111	SG Universal Library Prep Adapter + UDI Primers Set A [1-24]	24
700,112	SG Universal Library Prep Adapter + UDI Primers Set B [25-48]	24

- To check NGS library size and quality, we recommend using automated electrophoresis systems, such as Agilent 2100 Bioanalyzer®, Agilent 2200 TapeStation® or QIAxcel® Connect from QIAGEN®.

General Guidelines

- Use good laboratory practice to minimize cross-contamination of nucleic acid products.
- Always use PCR tubes, microfuge tubes, and pipette tips that are certified sterile, DNase- and RNase-free.
- For consistent library amplification, ensure the thermal cycler used in this protocol is in good working order and has been calibrated to within the manufacturer's specifications.
- Briefly centrifuge tubes prior to opening to avoid loss of material.
- Read the entire protocol before beginning. Take note of stopping points and plan your workflow accordingly.

Point in protocol where procedure can be stopped, stored at appropriate conditions outlined, and continued within 24 hours

Take note of recommendations in protocol

Use caution when performing protocol to obtain the best results

Before You Begin

- **Prepare beads by mixing 300 µl of magnetic cleanup beads (sparQ PureMag Beads) with 6 µl Bead Booster for each library preparation. This mixture generates enough beads to take 1 sample through the entire library preparation process and should be kept at room temperature during preparation process.**
- Prepare a fresh solution of 80% ethanol, store at room temperature.
- Prepare 10 mM Tris-HCl pH 8.0, store at room temperature.
- Wipe down work areas and pipettes with an RNase and DNA cleaning product.
- Thaw reagents on ice. Once thawed, finger flick (do not vortex) the tubes containing 1st Strand Enzyme Mix, 2nd Strand Enzyme Mix, T4 DNA Ligase, Cleave Enzyme and HiFi Plus Master Mix (2X). Briefly centrifuge to ensure even distribution of contents. Other tubes can be briefly vortexed and centrifuged to ensure mixing.
- Determine the amount of input RNA using standard methods.
- High-quality RNA is recommended for successful library preparation and sequencing.
    - High quality RNA has an A260:A280 ratio of 1.9–2.1 in 10 mM Tris-HCl, pH 7.5.
    - RNA integrity: Total RNA extracts from cells and tissues can be checked for quality using an automated analysis system (such as the Agilent TapeStation or QIAxcel Connect from QIAGEN) that provides an RNA integrity number (RIN). Ideally, RIN value ≥ 9 is recommended for low input, such as 1 ng. For higher amounts, RIN ≥ 7 is recommended. However, sparQ RNA-Seq HMR Kit shows a high rate of successful libraries with samples with lower RIN values. For optimal performance with degraded and/or FFPE RNA samples, a minimum of 10 ng total RNA is recommended.

Protocol

RNA Fragmentation and Ribo-Globin Depletion

This step combines RNA fragmentation, random priming and ribo-globin depletion into one step.

1. Thaw RNA on ice. Gently mix, briefly centrifuge and return to ice.
2. Thaw Frag Prime RG Depletion Mix on ice.
3. Prepare the fragmentation and depletion reaction on ice per Table 1.

***Note**: The master mix combining 'Frag Prime RG Depletion Mix' and 'nuclease-free water' can be prepared for multiple reactions and stored on ice until use.*

The master mix should be mixed by medium speed vortexing for at least 5 seconds, then purified RNA added and mixed by vortexing or pipetting.

Pre-program a thermal cycler according to Table 2 with heated lid set to 105°C. Start program to pre-heat block to 94°C and pause.

Table 1

	Component	Per Reaction (µl)
	Purified RNA (1 - 1000 ng)	x
●	Frag Prime RG Depletion Mix	4
	Nuclease-free water	6 - x
	Total	**10**

***Note:** Recommended input volume for Purified RNA is between 2 µl and 6 µl depending on RNA amount and concentration.*

4. Mix well by pipetting up and down 5 times and briefly centrifuge.
5. Load the reactions into the pre-programmed cycler, resume the incubation at 94°C followed by cycling according to Table 2.

Table 2

Step	Temperature (lid 105°C)	Incubation Time		
		High Quality Input RNA (RIN > 7)	Partially Degraded Input RNA (RIN < 7)	FFPE RNA (10 - 1000 ng)
1	94°C	3 min for 500 - 1000 ng 2 min for < 500 ng	2 min	none
2	75°C	2 min		
3	70°C	2 min		
4	65°C	2 min		
5	60°C	2 min		
6	55°C	2 min		
7	37°C	5 min		
8	25°C	5 min		
9	4°C	Hold		

6. After fragmentation, briefly centrifuge the fragmentation reactions and proceed immediately to First Strand Synthesis.

First Strand Synthesis

7. Remove 1st Strand Enzyme Mix from the freezer just before use. Thaw on ice.
8. Prepare First Strand Synthesis Master Mix on ice according to Table 3.

Note: *First Strand Synthesis Master Mix can be prepared for multiple reactions and stored on ice until use.*

Pre-program a thermal cycler according to Table 4 with heated lid set to 75°C. Start program to pre-heat block to 25°C and pause.

Table 3: First Strand Synthesis Master Mix

	Component	Per Reaction (µl)
●	1st Strand Enzyme Mix	4
	Nuclease-free water	6
	Total	**10**

9. Add **10 µl** of the First Strand Synthesis Master Mix to the **10 µl** of fragmented, depleted, primed RNA from step 6 for a total reaction volume of 20 µl.
10. Mix well by pipetting up and down 5 times and briefly centrifuge. Incubate as described in Table 4.

Table 4

Step	Temperature (lid 75°C)	Incubation Time
1	25°C	10 min
2	42°C	15 min
3	70°C	15 min
4	4°C	Hold

11. After First Strand Synthesis, briefly centrifuge the reaction and place on ice. Proceed immediately to Second Strand Synthesis and End Polishing.

Second Strand Synthesis and End Polishing

12. Thaw 2nd Strand Buffer on ice. Remove 2nd Strand Enzyme Mix from the freezer just before use.
13. Prepare Second Strand Synthesis Master Mix on ice according to Table 5.

Note: *Second Strand Synthesis Master Mix can be prepared for multiple reactions and stored on ice until use.*

Pre-program a thermal cycler according to Table 6 with heated lid set to 75°C. Start program to pre-set block to 16°C and pause.

Table 5: Second Strand Synthesis Master Mix

	Components	Per Reaction (µl)
●	2nd Strand Buffer	20
●	2nd Strand Enzyme Mix	10
	Total	**30**

14. Add **30 µl** of the Second Strand Synthesis Master Mix to the **20 µl** of First Strand Synthesis product from step 11 for a total reaction volume of 50 µl.

15. Mix well by pipetting up and down 5 times and briefly centrifuge. Incubate as described in Table 6.

Table 6

Step	Temperature (lid 75°C)	Incubation Time
1	16°C	30 min
2	62°C	10 min
3	4°C	Hold

16. Briefly centrifuge and proceed to Post-Second Strand Cleanup.

Post-Second Strand Cleanup

Post-Second Strand Cleanup can be carried out in the PCR tube if a suitable magnetic stand is available. Alternatively, the full reaction volume can be transferred to low-bind 1.5 ml tubes for cleanup. If proceeding to adapter ligation following cleanup, the final elution must be collected into a thin-walled PCR tube.

17. Ensure that the sparQ PureMag Beads and Bead Booster mixture has been kept at room temperature (RT) for at least 20 min before use.
18. Thoroughly vortex the sparQ PureMag Beads slurry and add **90 µl** (1.8X) to the Second Strand Synthesis product from step 16. Mix well by pipetting.
19. Incubate the mixture for 5 min at RT. Pellet the beads on a magnetic stand and carefully remove and discard the supernatant.
20. Keeping the tube on the magnetic stand, add 200 µl of freshly-prepared 80% ethanol to wash the beads. Pellet the beads on the magnetic stand for 30 s then carefully remove and discard the supernatant. Repeat the wash once.
21. Air-dry the beads on the magnetic stand for 3 - 5 min or until the beads appear matte and no longer shiny. Over-drying of beads may result in lower cDNA recovery.
22. Remove the tube from the magnetic stand, resuspend the dried beads by adding 68 µl of 10 mM Tris-HCl pH 8.0, and mix well by pipetting up and down at least 5 times. Incubate the beads at RT for 2-3 min. Pellet the beads on the magnetic stand. Carefully transfer **65 µl** of supernatant into a new thin-walled PCR tube and proceed to Adaptor Ligation. Alternatively, the sample can be stored at 4ºC for up to 24 hours.

Adapter Ligation

SG Universal Library Prep Adapters may need to be diluted using UDI Dilution Buffer before use. To achieve optimal adapter ligation efficiency for various input RNA amounts, it is recommended to adjust insert/adapter molar ratio accordingly. Table 7 and the Appendix A provide general guidance on recommended SG Universal Library Prep Adapter concentrations.

Table 7

Total RNA Input	Adapter Dilution*
501 – 1000 ng	1:10
51 – 500 ng	1:50
1 – 50 ng	1:200

* Serial dilution of the adapters is recommended.
For further guidance, refer to Appendix B.

23. Thaw Rapid Ligation Buffer (5X) on ice. Remove T4 DNA Ligase from the freezer just before use.
24. Prepare Ligation Master Mix on ice according to Table 8.

Note: *Ligation Master Mix can be prepared for multiple reactions and stored on ice until use.*

Pre-program a thermal cycler according to Table 9 with heated lid off. Start program to pre-set block to 20°C and pause.

Table 8: Ligation Master Mix

	Components	Per Reaction (µl)
●	Rapid Ligation Buffer (5X)	20
●	T4 DNA Ligase	10
	Total	**30**

Use caution when pipetting adapters. Avoid touching the tube with any part of your pipette other than pipette tips to minimize potential cross contamination.

25. Add **5 µl** of diluted SG Universal Library Prep Adapter to each sample from step 22.
26. Transfer **30 µl** of Ligation Master Mix to each PCR tube containing sample plus adapter from step 25.
27. Mix well by pipetting up and down 5 times, gently vortexing if necessary, then briefly centrifuge. Incubate as described in Table 9.

Note: *Heated lid must be OFF and < 30°C.*

Table 9

Step	Temperature (lid off)	Incubation Time
1	20°C	15 min
2	4°C	hold

28. Briefly centrifuge and proceed to Post-Ligation Cleanup.

Post-Ligation Cleanup, Adapter Cleavage and Library Amplification

29. Thaw 4X Cleave Reaction Buffer, HiFi Plus Master Mix (2X) and UDI Primer on ice. Remove Cleave Enzyme from the freezer just before use.
30. Prepare the Adapter Cleavage/Amplification Reaction Mix according to Table 10.

Note: Adapter Cleavage/Amplification Reaction Mix can be prepared for multiple reactions and stored on ice until use.

Pre-program a thermal cycler according to Table 11 with heated lid set to 105°C. Start program to pre-heat block to 37°C and pause.

Table 10: Adapter Cleavage/Amplification Reaction Mix

Components	Per Reaction (µl)
4X Cleave Reaction Buffer	12.5
Cleave Enzyme	2
HiFi Plus Master Mix (2X)	25
Nuclease-free water	5.5
Total	**45**

31. Ensure that the sparQ PureMag Beads and Bead Booster mixture has been kept at room temperature (RT) for at least 20 min before use.
32. Thoroughly vortex the sparQ PureMag Beads slurry and add **70 µl** (0.7X) to the ligation sample from step 27. Mix well by pipetting.
33. Incubate the mixture for 5 min at RT. Pellet the beads on a magnetic stand and carefully remove and discard the supernatant.
34. Keeping the tube on the magnetic stand, gently pipette 200 µl of freshly-prepared 80% ethanol over the bead pellet to wash. Pellet the beads on the magnetic stand for 30 s then carefully remove and discard the supernatant. Repeat step 34 for a total of two washes.
35. Air-dry the beads on the magnetic stand for 3 – 5 min or until the bead pellet appears matte and no longer shiny. Over-drying of beads may result in lower DNA recovery.
36. Remove the tube from the magnetic stand, resuspend the dried beads by adding 45 µl of Adapter Cleavage/Amplification Reaction Mix.
37. Add individual 5 µl UDI Primer mix to each sample avoiding cross contamination for a total reaction volume of 50 µl.

38. Mix well by pipetting up and down 5 times. Incubate as described in Table 11.

Table 11

Step	Temperature (lid 105°C)	Incubation Time	Cycles
1	37°C	10 min	1
2	98°C	45 sec	1
3	98°C	20 sec	10-16*
	60°C	30 sec	
	72°C	30 sec	
4	72°C	1 min	1
5	4°C	Hold	1

* Refer to Table 12 for recommended number of PCR cycles.

Table 12

Total RNA Input Amount (ng)	Number of Cycles
800 – 1000	10
400 – 799	13
100 – 399	14
1 – 99	16

39. Briefly centrifuge and proceed to Post-Amplification Cleanup. Alternatively, the sample can be stored at 4°C for up to 24 hours.

Post-Amplification Cleanup

40. Ensure that the sparQ PureMag Beads and Bead Booster mixture has been kept at room temperature (RT) for at least 20 min before use.
41. Thoroughly vortex the sparQ PureMag Beads slurry and add **45 µl** (0.9X) to the sample from step 39. Mix well by pipetting.
42. Incubate the mixture for 5 min at RT. Pellet the beads on a magnetic stand and carefully remove and discard the supernatant.
43. Keeping the tube on the magnetic stand, add 200 µl of freshly-prepared 80% ethanol to wash the beads. Pellet the beads on the magnetic stand for 30 s then carefully remove and discard the supernatant. Repeat the wash once.

44. Air-dry the beads on the magnetic stand for about 3 - 5 min or until the beads appear matte and no longer shiny. Over-drying of beads may result in lower DNA recovery.
45. Remove the tube from the magnetic stand, resuspend the dried beads by adding 22 µl of 10 mM Tris-HCl, pH 8.0. and mix well by pipetting up and down at least 5 times. Incubate the beads at RT for 2-3 min. Pellet the beads on the magnetic stand. Carefully transfer **20 µl** of supernatant into a new thin-walled PCR tube. The sample can be stored at 4°C for up to 24 hours or at -20°C for longer term storage.

Library Validation and Quantification

RNA-Seq libraries constructed using this protocol should be validated and quantified to ensure optimal input for sequencing reactions.

Average fragment length can be measured using automated electrophoresis system such as the Agilent 2100 Bioanalyzer or Agilent 2200 TapeStation or QIAxcel Connect from QIAGEN per each manufacturer's instructions. Due to average product size, we recommend read lengths no longer than 100 bp. If a longer read length is used for sequencing, additional *in silico* adapter trimming may be necessary.

If an adapter-dimer peak at 120-150 bp is present at more than 25% of the library peak, an additional 0.9X bead clean up step should be performed according to steps **46-49** and **52** in the protocol before sequencing.

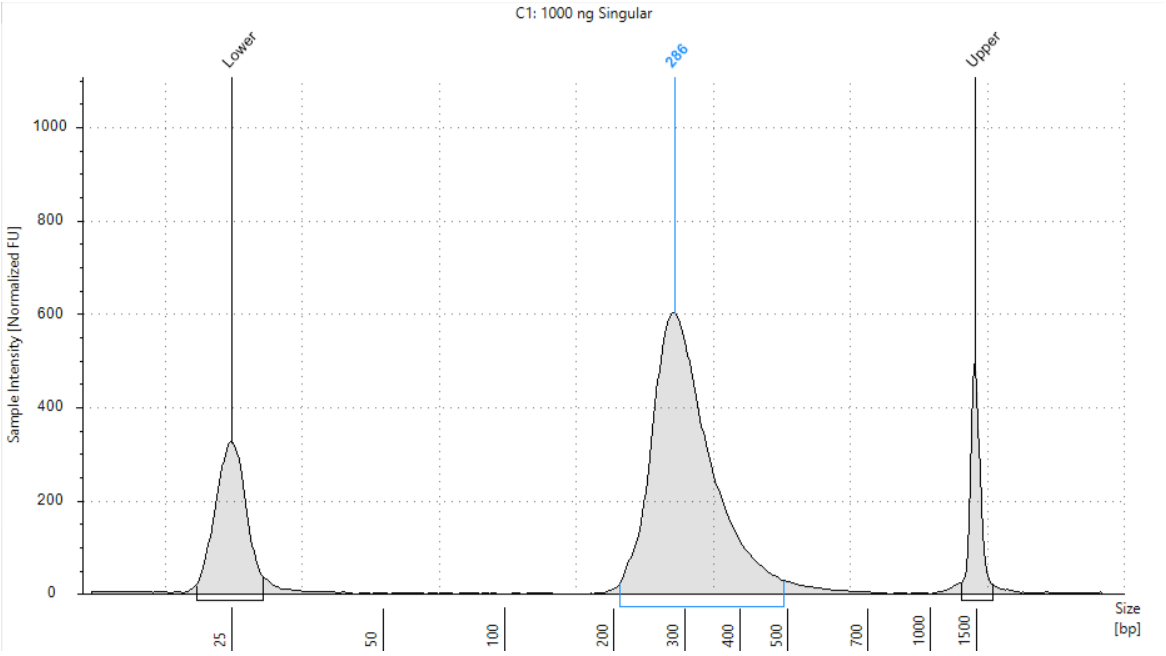


Figure 1: Example of cDNA library prepared using 1000 ng of Universal Human Reference (UHR) RNA. Analyzed using Agilent TapeStation 4200 instrument.

Quality Control

Contamination specifications: Kit enzyme components were tested prior to assembly and found free of contaminating endonucleases and exonucleases. Enzyme purity was >95% as determined by SDS-PAGE and negligible *E.coli* genomic DNA contamination was confirmed by qPCR.

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Appendix A: Guidance on Adapter Concentrations

Determining the correct concentration of adapters during ligation is critical for high quality sequencing data. A low concentration of adapters during ligation will impact overall library yields. A high concentration of adapters results in the dimerization of adapters as they ligate to one another. Presence of adapter dimers in the final library may negatively impact sequencing run data quality and lower sample data yield.

With the range of RNA input amounts and range of tunable insert sizes, it is recommended that the adapter concentration be adjusted accordingly. Generally, increasing the input amount of RNA and/or tuning to smaller insert sizes, increase the concentration of adapters required. Both result in an increase concentration of cDNA available for ligation. Decreasing the input amount of RNA and/or tuning to larger insert sizes will require lower adapter concentration in comparison.

Titration of adapters is recommended to find the optimal adapter concentration to your specified insert size and input amount. UDI Dilution Buffer or 10 mM Tris-HCl pH 8.0, 50 mM NaCl, 0.5 mM EDTA can be used for adapter dilution.

Libraries with adapters added at low concentrations in the ligation step will report lower than expected yields in qPCR when compared to yields estimated from trace analysis and Qubit™. Sequencing of these libraries may result in lower than expected coverage, depth of coverage and uniformity.

Libraries with adapters added with high concentrations in ligation step are characterized with defined peak at 120-150 bp in fragment analysis traces. Sequencing of libraries with adapter dimers present may negatively impact sequencing run data accuracy, or even cause an instrument error, terminating the run. Completed run data sets will see an over-representation of the adapter reads, a smaller DNA fragments preferentially cluster to the flow cell.

If additional adapter dimers remain, another round of 0.9X (beads to sample) bead cleanup with sparQ PureMag Beads is recommended.

For more information regarding adapter and index sequences, please refer to the product documents corresponding to the adapters and primers purchased from Singular Genomics (https://techwriting.singulargenomics.com/G4-Documentation/G4-Universal-Adapter-UDI-Overview-600014.pdf)

Appendix B: Preparation of Adapter Dilution for Adapter Ligation

It is recommended to perform serial dilutions of the adapters. Dilutions may be scaled up proportionally to prepare sufficient adapter for the number of reactions required.

Optimal dilutions may vary depending on the nature of your samples and NGS sequencing operations. Use the following example showing preparation of 1:10, 1:50 and 1:200 dilutions as a general guideline.

Prepare a **1:10 dilution** by adding 2 µl of the adapters (stock) to 18 µl of UDI Dilution Buffer.

Prepare a **1:50 dilution** by adding 4 µl of the diluted (1:10) adapters to 16 µl of UDI Dilution Buffer.

Prepare a **1:200 dilution** by adding 2 µl of the diluted (1:10) adapters to 38 µl of UDI Dilution Buffer.

NGS Products Used in Protocol Sold Separately

sparQ PureMag Beads

Cat. No.	95196-005	Size:	5 ml
	95196-060		60 ml
	95196-450		450 ml

The sparQ PureMag Beads is a fast and reliable nucleic acid purification system for reaction cleanup and size selection in NGS workflows. It can be used to quickly remove primers, primer-dimers, unincorporated nucleotides, salts, adapters and adapter-dimers from NGS library prep reactions to improve downstream sequencing performance.