RealSeq-Dual simplified protocol
For more detailed instructions use RealSeq-Biofluids protocol as guideline

1. Dispense 5 uL of RB into each well of 96-plate
2. Add 10 uL of sample into each well
3. Dispense 1 uL of RealSeq-Adapter (dual) into each well
4. Vortex at 1500 rpm for 1 minute, spin down
5. Heat to 70°C for 2 minutes
6. Transfer to ice for at least 2 minutes
7. Dispense:
    a. 2 uL Ligation buffer (LB)
    b. 1 uL RNase Inhibitor (RI)
    c. 1 uL Ligase (L)
8. Vortex at 1500 rpm for 1 minute, spin down
9. Heat to 25°C for 1 hour
10. Heat to 65°C for 5 minutes
11. Dispense 1 uL of Blocking Agent (BA)
12. Vortex at 1500 rpm for 1 minute, spin down
13. Heat to 65°C for 5 minutes
14. Step down to 37°C (0.1°C/second)
15. Dispense 1 uL Blocking Enzyme (BE)
16. Vortex at 1500 rpm for 1 minute, spin down
17. Heat to 37°C for 1 hour
18. Heat to 65°C for 20 minutes
19. Dispense:
    a. 1 uL RealSeq Buffer (RSB)
    b. 1 uL RealSeq Enzyme (RSE)
20. Vortex at 1500 rpm for 1 minute, spin down
21. Heat to 37°C for 1 hour
22. Dispense 1 uL Dimer Removal Agent (DRA)
23. Vortex at 1500 rpm for 1 minute, spin down
24. Heat to 37°C for 10 minutes
    a. While incubating prepare RealSeq Beads
        i. 20 uL RealSeq Beads to each well
        ii. Expose to magnet and remove liquid
25. After incubation resuspend beads with 25 uL of sample and mix by pipetting up and down
26. Heat to 37°C for 10 minutes
27. Expose to magnet for 2 minutes
28. Transfer 21 uL of sample to new plate

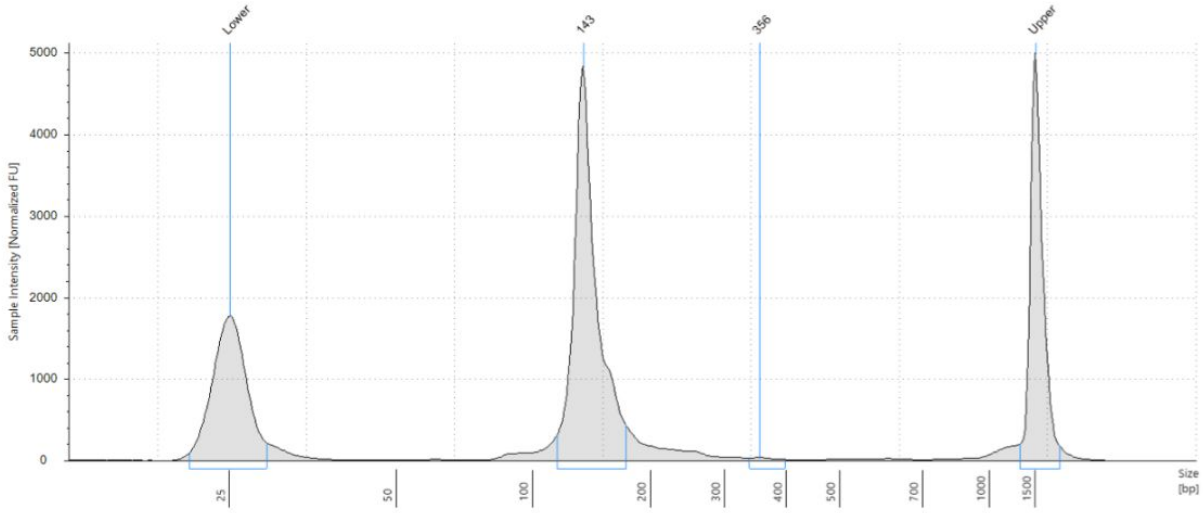
29. Dispense:
    a. 2 uL RT Primer (RTP)
    b. 2 uL dNTPs
30. Vortex at 1500 rpm for 1 minute, spin down
31. Heat to 65°C for 5 minutes
32. Transfer to ice for at least 2 minutes
33. Dispense:
    a. 4 uL RT Buffer (RTB)
    b. 8 uL RNase free water
    c. 1 uL RNase Inhibitor (RI)
    d. 2 uL RT Enzyme (RTE)
34. Vortex at 1500 rpm for 1 minute, spin down
35. Heat to 42°C for 1 hour
36. Heat to 65°C for 20 minutes
37. Dispense:
    a. 20 uL PCR Buffer (PB)
    b. 19 uL RNase free water
    c. 3 uL dNTPs
    d. 14 uL From same well of Barcodes plate (Well A1 of barcodes plate to Well A1 of sample plate)
    e. 4 uL PCR Polymerase (PP)
38. Vortex at 1500 rpm for 1 minute, spin down
39. Optional: Split sample in two 50 uL aliquots, PCR one of them save the other one at -20°C
40. PCR amplification:
    a. 94°C 30 seconds
    b. 20 cycles of:
        i. 94°C 15 seconds
        ii. 62°C 30 seconds
        iii. 70°C 15 seconds
    c. 70°C 5 minutes
41. SPRI beads Cleanup (Following page)

SPRI Cleanup protocol

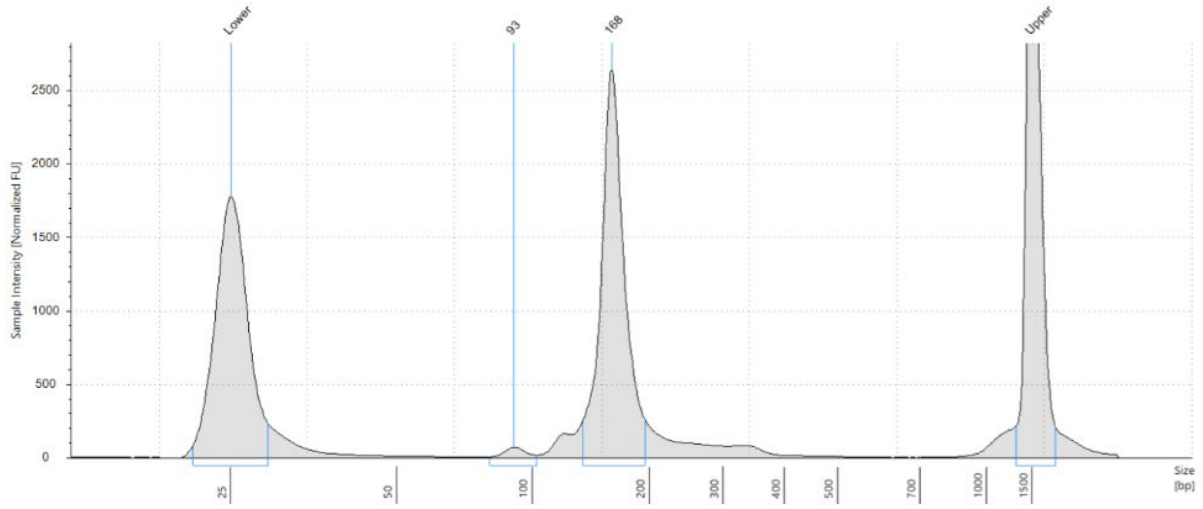
- Prepare 70% ethanol (500 µl per sample).
- Ensure SPRI Beads are at room temperature, and resuspend before use.
- Vortex and spin down each PCR reaction. Transfer 50 µl of sample to new PCR tubes.
- Add 70 µl of SPRISelect® Reagent to each sample. Mix reagent and PCR thoroughly by pipette mixing 10 times.
- Let the mixed samples incubate for 5 minutes at room temperature for maximum recovery.
- Place the samples on a magnet until all the beads separate from solution (wait for the solution to clear before proceeding to the next step). (~3-6 minutes)
- Carefully remove the cleared solution from the tube and discard. Take care to not disturb the beads in the process.
- Without removing tube from magnet, add 200 µl of freshly prepared 70% ethanol to each sample and incubate for 30 seconds at room temperature. Remove the ethanol and discard. Repeat for a total of two washes.
- Briefly spin the tubes (~2,000 g) to collect the remaining liquid at the bottom of each tube. Place the tubes on the magnetic separation device for 30 seconds, then remove all remaining liquid with a pipette.
- Let the sample tubes rest open on the magnet at room temperature until the pellet appears dry and is no longer shiny. (~3-6 minutes)
- Once the bead pellet has dried, remove the tubes from magnet and add 12.5 µl of RNase free water (Tube 18). Mix thoroughly by pipetting up and down to ensure complete bead dispersion.
- Incubate at room temperature for at least 5 minutes.
- Place the samples on a magnet for 3 minutes or longer, until the solution is completely clear.
- Transfer 10 µl of the clear supernatant containing purified PCR products from each tube to a new tube. Ensure that no beads follow the library during this step.
- Quantify library with Agilent Bioanalyzer®/TapeStation® and Qubit® Fluorometer

Example libraries with RealSeq-Dual adapter

Negative control (~147bp peak)



Positive control (~168bp peak)



Forward Primer	Sequence
FP1	**TGAACCTT**
FP2	**TGCTAAGT**
FP3	**TGTTCTCT**
FP4	**TAAGACAC**
FP5	**CTAATCGA**
FP6	**CTAGAACA**
FP7	**TAAGTTCC**
FP8	**TAGACCTA**