rhAmpSeq library preparation protocol

Prepare reagents

Make a 10X rhAmp Primer Pool

If you have pooled and ready-to-use rhAmp primers, begin the protocol at Prepare the rhAmpSeq Index Primers section.

If you have individually plated (dried down) rhAmp primers, begin by resuspending the primers, as described here.

1. Use the following formula to determine the amount of IDTE, pH 7.5 needed to resuspend each to a final concentration of 50 µM (0.05 nmol/µL):

$$\text{Amount of IDTE, pH 7.5} = \frac{\text{nmol of rhAmp primer}}{0.05\ \text{nmol/µL}}$$

For example:

$$\text{Amount of IDTE, pH 7.5} = \frac{0.4\ \text{nmol of rhAmp primer}}{0.05\ \text{nmol/µL}} = 8\ \text{µL}$$

Important! Use IDTE, pH 7.5 (10 mM Tris; 0.1 mM EDTA, pH 7.5), or equivalent. Do not resuspend in water or TE buffer.

2. Centrifuge all rhAmp primer plates before resuspension.
3. Resuspend each rhAmp primer to 50 µM using the volume of IDTE (pH 7.5) determined in Step 1.
4. Seal the plates, vortex to fully resuspend, then briefly centrifuge.
5. Combine equal volumes of each individual 50 µM stock rhAmp primer to create stock forward or reverse primer pools.

Note: Forward primers are combined to make a forward rhAmp Primer Pool, and reverse primers are combined to make a reverse rhAmp Primer Pool.

Important! Do not combine forward and reverse rhAmp primers until you are ready to perform the Targeted rhAmp PCR 1.

6. Create a 10X rhAmp Primer Pool for your rhAmpSeq assay. Use this table to determine the appropriate 10X concentration based on the plexity of your primer pool.

Panel size	10X rhAmp primer concentration	Calculate 10X rhAmp Primer Pool working concentration
≥500-plex	50 µM (total)	50 µM (FWD and REV pool)
101-plex < X <499 plex	100 nM (each primer)	100 nM × # of primers (FWD or REV pool)
≤100-plex	250 nM (each primer)	250 nM × # of primers (FWD or REV pool)

7. If necessary, dilute the 50 µM forward or reverse rhAmp Primer Pool to the appropriate 10X working concentration using IDTE, pH 7.5.

Important! If the rhAmp Primer Pool needs to be diluted, use IDTE, pH 7.5 (10 mM Tris; 0.1 mM EDTA, pH 7.5), or equivalent.

8. Store the stock rhAmp primer plates and forward and reverse rhAmp Primer Pools at –20°C.

Note: Do not combine forward and reverse rhAmp primers for long-term storage.

Prepare the rhAmpSeq Index Primers

rhAmpSeq Index Primers (i5 or i7) are supplied as individual dried down primers (6 nmol) and need to be resuspended.

1. Resuspend the rhAmpSeq Index Primer to 100 µM by adding 60 µL of IDTE, pH 8.0. Vortex to resuspend, then centrifuge.

Important! Use IDTE, pH 8.0 (10 mM Tris; 0.1 mM EDTA, pH 8.0), or equivalent. Do not resuspend in water or TE buffer.

2. Dilute rhAmpSeq Index Primer to 5 µM using IDTE, pH 8.0.
3. Make aliquots to minimize freeze–thaws.
4. Store the dilutions at –20°C.

Perform Targeted rhAmp PCR 1

Set up Targeted rhAmp PCR 1

1. Completely thaw rhAmpSeq forward and reverse pools and 4X rhAmpSeq Library Mix 1 to room temperature (15–20°C). Also, bring AMPure XP beads to room temperature.

   Note: This protocol demonstrates using 96-well PCR plates; however, strip tubes can be used instead.

2. After thawing, briefly vortex the following reagents, then centrifuge:

   - 10X rhAmp PCR Panel—Forward Pool
   - 10X rhAmp PCR Panel—Reverse Pool
   - 4X rhAmpSeq Library Mix 1

3. Dilute gDNA to 0.91 ng/µL – 9.1 ng/µL using IDTE, pH 8.0.

4. Add 11 µL of diluted gDNA to each reaction well in a 96-well plate (10–100 ng total input).

   Tip: For quantification of DNA, use a Qubit dsDNA Quantitation Assay Kit, or equivalent.

   Important! Not all DNA quantitation methods produce equivalent results.

5. Include the following in each reaction well:

Reagent	Volume per reaction
4X rhAmpSeq Library Mix 1	5 µL
10X rhAmp PCR Panel—Forward Pool	2 µL
10X rhAmp PCR Panel—Reverse Pool	2 µL
Diluted gDNA sample (from step 4)	11µL
Total volume	**20 µL**

6. Seal the targeted rhAmp PCR 1 plate, then briefly vortex and centrifuge.

Run the Targeted rhAmp PCR 1 program

1. Place the Targeted rhAmp PCR 1 plate in a thermal cycler and run the Targeted rhAmp PCR 1 thermal cycler program as described, with a heated lid set to 105°C:

Targeted rhAmp PCR 1 program			
Step	**Cycle**	**Temperature (°C)**	**Duration**
Activate enzyme	1	95	10 min
Amplify	10	95	15 sec
		61	4 min
Deactivate enzyme	1	99.5	15 min
Hold	1	4	∞

2. Remove the targeted rhAmp PCR 1 plate from the thermal cycler when the program completes and proceed immediately to Clean up the rhAmp PCR 1 product.

Clean up the rhAmp PCR 1 product

Purify amplicons from PCR 1

1. With the Agencourt AMPure XP beads at room temperature, vortex thoroughly before use.

2. Prepare an 80% ethanol solution by combining 1 part molecular-grade water and 4 parts molecular-grade ethanol (200 proof).

   Important! Use fresh 80% ethanol to avoid a loss in assay performance.

3. Add 30 µL of beads into each PCR 1 well and pipet up and down to mix.

4. Incubate for 10 minutes at room temperature.

5. Collect the beads on a magnetic plate for 5 minutes, or until the solution is clear.

6. With the plate on the magnet, do the following:

   a) Aspirate and discard the supernatant.

   b) Add 200 µL of 80% ethanol to each well, then incubate at room temperature for 1 minute.

   c) Aspirate and discard the supernatant.

   d) Perform a second 80% ethanol wash (repeat Step 6 b–c).

   e) Use a fresh pipette tip to remove all traces of ethanol without disturbing the beads.

   f) Allow the beads to dry for 3 minutes at room temperature.

7. Remove the PCR plate from the magnet.
8. Add 15 µL of IDTE, pH 8.0 to each well, seal the plate, then thoroughly vortex to fully resuspend the beads. Briefly centrifuge the plate.
9. Incubate for 3 minutes at room temperature to elute sample from beads.
10. Place the PCR plate on the magnet to collect the beads for 3 minutes, or until clear.
11. Transfer 11 µL of each sample elution into a new PCR plate well, ensuring no beads are carried over.

Important! Proceed immediately to Perform Indexing PCR 2.

Perform Indexing PCR 2

Set up Indexing PCR 2

Use room temperature 4X rhAmpSeq Library Mix 2 and Index Primers i5 and i7 for this section. Keep the Agencourt AMPure XP beads at room temperature in preparation for a second clean up step.

Note: Use a different combination of i5 and i7 index primers for each sample that is combined in a multiplex sequencing run.

1. Briefly vortex, then centrifuge the 4X rhAmpSeq Library Mix 2 and Index Primers i5 and i7.
2. Prepare Indexing PCR 2 in the 96-well plate containing PCR 1 sample elution (made during Step 11 in Clean up the PCR 1 product), by including the following in each reaction well:

Reagent	Volume per reaction
4X rhAmpSeq Library Mix 2	5 µL
Indexing PCR Primer i5 (5 µM)	2 µL
Indexing PCR Primer i7 (5 µM)	2 µL
rhAmpSeq PCR 1 elution (from step 11)	11 µL
Total volume	**20 µL**

3. Seal the Indexing PCR 2 plate, then briefly vortex and centrifuge.

Run the PCR 2 program

1. Place the indexing PCR 2 plate in a thermal cycler and run the Indexing PCR 2 thermal cycler program as described, with a heated lid set to 105°C:

Targeted rhAmp PCR 2 program			
Step	**Cycle**	**Temperature (°C)**	**Duration**
Activate enzyme	1	95	3 min
Amplify	18	95	15 sec
		60	30 sec
		72	30 sec
Final extension	1	72	1 min
Hold	1	4	∞

2. Remove the Indexing PCR 2 plate from the thermal cycler after the program completes and proceed immediately to Clean up library.

Clean up library

Purify the rhAmpSeq library

1. With the Agencourt AMPure XP beads at room temperature, vortex thoroughly before use.
2. Prepare an 80% ethanol solution by combining 1 part molecular-grade water and 4 parts molecular-grade ethanol (200 proof).

Important! Use fresh 80% ethanol to avoid loss in assay performance.

3. Add 20 µL of beads into each PCR 2 well, then pipet up and down to mix.
4. Incubate for 10 minutes at room temperature.
5. Place plate on a magnetic plate for 5 minutes, or until the solution is clear.
6. With the plate on the magnet, do the following
   a) Aspirate and discard the supernatant.
   b) Add 200 µL of 80% ethanol to each well, then incubate at room temperature for 1 minute.
   c) Aspirate and discard the supernatant.
   d) Perform a second 80% ethanol wash (repeat Steps 6 b–c).
   e) Use a fresh pipette tip to remove all traces of ethanol without disturbing the beads.
   f) Allow the beads to dry for 3 minutes at room temperature.

7. Remove the PCR plate from the magnet.
8. Add 22 µL of IDTE, pH 8.0 to each well, seal the plate, and thoroughly vortex to fully resuspend the beads. Briefly centrifuge the plate.
9. Incubate for 3 minutes at room temperature to elute sample from beads.
10. Place the PCR 2 plate on the magnet for 3 minutes, or until the solution is clear.
11. Keeping the PCR 2 plate on the magnet, transfer 20 µL of each sample elution into a new PCR plate well, ensuring no beads are carried over.
12. Proceed to quantify, normalize, and pool your rhAmpSeq libraries following the sequencing instructions from your specific Illumina platform.
13. Store the rhAmpSeq library at –20°C for up to 3 weeks.

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