

High-throughput 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):


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


For example:

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

 **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 \times # of primers (FWD or REV pool)
≤ 100 -plex	250 nM (each primer)	250 nM \times # 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 1 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).

 **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 – 4.55 ng/μL using IDTE, pH 8.0.
4. Add 11 μL of diluted gDNA to each reaction well in a 96-well plate (10–50 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)	11 μL
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	14	95	15 sec
		61	8 min
Deactivate enzyme	1	99.5	15 min
Hold	1	4	∞ Hold

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

Dilute PCR 1 product


Make a 1:20 dilution of the rhAmp PCR 1 product

1. Briefly vortex the PCR 1 product, then centrifuge.
2. Add 95 μL of Nuclease-Free Water to each reaction well of a new 96-well plate.
3. Transfer 5 μL of the PCR 1 product to the 95 μL Nuclease-Free Water (1:20 dilution).
4. Seal the plate, thoroughly vortex and centrifuge, then go 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. At this time, remove the Agencourt AMPure XP beads from refrigerated storage and bring to room temperature.

 **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 a new 96-well plate, as shown:

Reagent	Volume per reaction
4X rhAmpSeq Library Mix 2	5 µL
Indexing PCR Primer i5 (1 µM)	2 µL
Indexing PCR Primer i7 (1 µM)	2 µL
rhAmpSeq PCR 1—1:20 dilution	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
		95	15 sec
Amplify	24	60	30 sec
		72	30 sec
Final extension	1	72	1 min
Hold	1	4	∞


2. Remove the indexing PCR 2 plate upon completion and go immediately to [Pool libraries](#).

Pool libraries


Prepare the rhAmpSeq library pool

Make the library pool by combining equal volumes of all your indexed libraries.

1. With a multichannel pipette, transfer 5 µL of each indexed library into an 8- or 12-well strip tube (use 10 µL if the total number of libraries is less than 20).

 **Tip:** Minimize sample-to-sample variability with precise pipetting.

2. Cap the strip tube, briefly vortex, then quickly centrifuge.
3. Combine all libraries from the strip tube into a single 1.5 mL reaction tube.


 **Important!** Pool the complete volume from each of the wells of the 8-well strip tube for even sample coverage.

4. Store the remaining individual indexed libraries at -20°C then 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 a loss in assay performance.

3. Transfer 100 µL of the rhAmpSeq library pool to a new 1.5 mL tube.
4. Add 100 µL of AMPure XP beads (1X) to the library pool.
5. Thoroughly pipette mix the contents of the tube.
6. Incubate for 10 minutes at room temperature.
7. Briefly centrifuge the tube then place it on a DynaMag™-2 Magnet, or equivalent, for 5 minutes, or until the solution is clear.
8. With the tube on the magnet, do the following:
 - a) Aspirate, then discard the supernatant.
 - b) Add 1000 µL of 80% ethanol to the tube.
 - c) Incubate at room temperature for 30 seconds.
 - d) Aspirate, then discard the supernatant.

- e) Repeat the 80% ethanol wash one more time for a total of 2 washes (Steps 8b–d).
 - f) Use a fresh pipette tip to remove all traces of ethanol from the tube.
 - g) Allow the beads to dry for 3 minutes at room temperature.
9. Remove the 1.5 mL tube from the magnet.
 10. Add 22 μ L of IDTE, pH 8.0 to elute rhAmpSeq library pool.
 11. Thoroughly vortex to fully resuspend the beads, then briefly centrifuge the tube.
 12. Incubate at room temperature for 3 minutes.
 13. Place the tube on the magnet to collect the beads for 1 minute, or until the solution is clear.
 14. With the tube on the magnet, transfer 20 μ L of the final rhAmpSeq library pool elution into a new 1.5 mL tube.
 15. Proceed to quantify, then sequence your rhAmpSeq library pool. Follow the instructions for your specific Illumina platform.
 16. Store any remaining library pool at -20°C for up to 3 weeks.

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