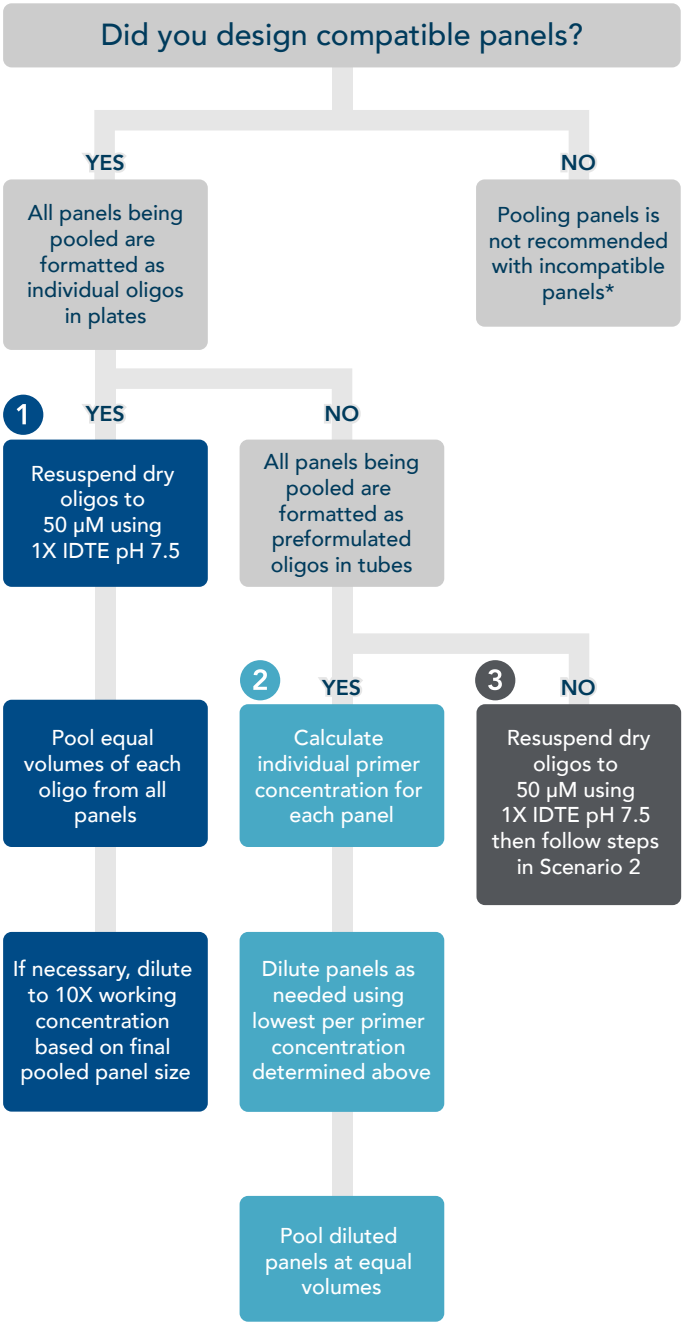
Pooling multiple rhAmpSeq panels

When pooling multiple rhAmpSeq panels, first determine which of the following scenarios applies to you:

1. All panels include individual oligos in plates.
2. All panels include preformulated oligos in tubes.
3. Panels include both preformulated tubes and individual oligos in plates.

Figure 1. Overview of scenarios when pooling multiple rhAmpSeq panels.



* You must redesign your panel using the 'Make compatible with a pre-existing panel' option in the rhAmpSeq Design Tool.

Scenario 1: Combining panels with individual oligos in plates

1. Resuspend each oligo (rhAmp primer) to 50 µM, then make separate forward and reverse pools by combining equal volumes of each primer from all panels.

   a. 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.

   b. Centrifuge all rhAmp primer plates before resuspension.

   c. Resuspend each rhAmp primer to 50 µM using the volume of IDTE, pH 7.5 determined in step a.

   d. Seal the plates, vortex to fully resuspend, then briefly centrifuge.

2. Combine equal volumes of each 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 Targeted rhAmpSeq PCR 1 in the rhAmpSeq library preparation standard protocol [1], or the rhAmpSeq high-throughput library preparation protocol [2].

3. Calculate the total number of primers in each pool.

   a. If there are <500 primers, refer to Table 1 and dilute each pool to the appropriate concentration using IDTE, pH 7.5 to create a 10X concentration.

Table 1. 10X primer pool calculations.

Panel size	10X rhAmp primer concentration
≥500-plex	50 µM (total)
101-plex through 499-plex	100 nM (each primer)
≤100-plex	250 nM (each primer)

b. If there are ≥500 primers, no dilution is needed because your pools are already at a 10X concentration.

4. Proceed directly to "Perform Targeted rhAmpSeq PCR 1" in your protocol [1] or [2].

Example 1

Panel 1: 250-plex, forward (FWD) and reverse (REV) pools ordered at 0.4 nmol scale in plates

Panel 2: 20-plex, FWD and REV pools ordered at 0.4 nmol scale in plates

Final pooled panel size: 270 primers in each forward and reverse pool

Final 10X rhAmp primer concentration (see Table 1): 100 nM each primer

a. Resuspend dried down oligos to 50 µM:
Add 8 µL of IDTE, pH 7.5 buffer to each well.

b. Create a single forward pool and separate reverse pool:

i. Combine 2 µL of forward primers (from Panel 1 and Panel 2) to create the forward pool.

ii. Combine 2 µL of reverse primers (from Panel 1 and Panel 2) to create the reverse pool.

Each pool has a total volume of 540 µL.

Note: If the pooled panel had been ≥500-plex, no further dilution of the panel would be needed; therefore, skip to step e.

c. Calculate individual primer concentration:
50 µM/270 primers = 185 nM per primer.

d. Dilute each pool to 100 nM per primer:
Combine 100 µL of stock pool (185 nM) with 85 µL of IDTE, pH 7.5.

The total volume of each forward and reverse pool should be 185 µL (100 nM per primer).

e. Proceed directly to "Perform Targeted rhAmpSeq PCR 1" in your protocol [1] or [2].

Example 2

Panel 1: 80-plex, FWD and REV pools ordered at 4 nmol scale in plates

Panel 2: 50-plex, FWD and REV pools ordered at 0.4 nmol scale in plates

Final pooled panel size: 130 primers in each forward and reverse pool

Final 10X rhAmp primer concentration (see Table 1): 100 nM each primer

a. Resuspend dried down oligos to 50 µM:

i. Panel 1: Add 80 µL of IDTE, pH 7.5 buffer to each well.

ii. Panel 2: Add 8 µL of IDTE, pH 7.5 buffer to each well.

b. Create a single forward pool and separate reverse pool:

i. Combine 2 µL of forward primers (from Panel 1 and Panel 2) to create the forward pool.

ii. Combine 2 µL of reverse primers (from Panel 1 and Panel 2) to create the reverse pool.

Each pool has a total volume of 260 µL.

Note: If the pooled panel had been ≥500-plex, no further dilution of the panel would be needed; therefore, skip to step e.

c. Calculate individual primer concentration:
50 µM/130 primers = 385 nM per primer.

d. Dilute each pool to 100 nM per primer:
Combine 100 µL of stock pool (385 nM) with 285 µL of IDTE, pH 7.5.

The total volume of each forward and reverse pool should be 385 µL (100 nM per primer).

e. Proceed directly to "Perform Targeted rhAmpSeq PCR 1" in your protocol [1] or [2].

Scenario 2: Combining preformulated oligos in tubes

1. Calculate the individual primer concentration in each panel.
   a. ≥500-plex panels: Divide 50 µM by the number of primers in the pool (i.e., 50 µM/1000 primers = 50 nM/primer).
   b. ≤499-plex panels: Use Table 1 to determine that individual primer concentration in each tube.
2. Determine which of the panels has the lower per primer concentration, then dilute other panels to that concentration.
3. Combine equal volumes of each panel to create separate forward and reverse pooled panels, then proceed directly to "Perform Targeted rhAmpSeq PCR 1" in your protocol [1] or [2].

Note: Resulting per primer concentration will be lower than amount recommended in Table 1. Reaction and thermal cycling parameters may require optimization for optimal assay performance.

Example 1

Panel 1: 250-plex, forward (FWD) and reverse (REV) pools provided as preformulated oligos in tubes

Panel 2: 20-plex, FWD and REV pools provided as preformulated oligos in tubes

Final pooled panel size: 270 primers in each forward and reverse pool

a. Determine the individual primer concentrations needed for each panel (See Table 1or pools with <500 primers):
   i. Panel 1: 100 nM per primer.
   ii. Panel 2: 250 nM per primer.
b. Dilute Panel 2 to 100 nM per primer:
   Dilute stock by combining 50 µL of Panel 2 with 75 µL IDTE, pH 7.5.
c. Combine equal volumes for each FWD and REV pool (final panel = 50 nM/primer):
   i. Combine 50 µL Panel 1 FWD + 50 µL diluted Panel 2 FWD.
   ii. Combine 50 µL Panel 1 REV + 50 µL diluted Panel 2 REV.
d. Proceed directly to "Perform Targeted rhAmpSeq PCR 1" in your protocol [1] or [2].

Example 2

Panel 1: 1000-plex, FWD and REV pools provided at 10X concentration

Panel 2: 2500-plex, FWD and REV pools provided at 10X concentration

Final pooled panel size: 3500 primers in each forward and reverse pools

a. Calculate individual primer concentration for each panel:
   i. Panel 1: 50 µM/1000 primers = 50 nM per primer.
   ii. Panel 2: 50 µM/2500 primers = 20 nM per primer.
b. Dilute Panel 1 to 20 nM per primer:
   Dilute stock by combining 50 µL of Pool 1 with 75 µL of IDTE, pH7.5.
c. Combine equal volumes for each FWD and REV pool (final panel = 10 nM/primer):
   i. Combine 50 µL diluted Panel 1 FWD + 50 µL Panel 2 FWD.
   ii. Combine 50 µL diluted Pool 1 REV + 50 µL Panel 2 REV.
d. Proceed directly to "Perform Targeted rhAmpSeq PCR 1" in your protocol [1] or [2].

Example 3

Panel 1: 1000-plex, FWD and REV pools provided at 10X concentration

Panel 2: 200-plex, FWD and REV pools provided at 10X concentration

Final pooled panel size: 1200 primers in each forward and reverse pool

a. Determine the individual primer concentrations needed for each panel (See Table 1or pools with <500 primers):
   i. Panel 1: 50 µM/1000 primers = 50 nM per primer.
   ii. Panel 2: 100 nM per primer.
b. Dilute Panel 2 to 50 nM per primer:
   Dilute stock by combining 50 µL of Panel 2 with 50 µL of IDTE, pH 7.5.
c. Combine equal volumes for each FWD and REV pool (final pooled panel = 25 nM/primer):
   i. Combine 50 µL Panel 1 FWD + 50 µL diluted Panel 2 FWD.
   ii. Combine 50 µL Panel 1 REV + 50 µL diluted Panel 2 REV.
d. Proceed directly to "Perform Targeted rhAmpSeq PCR 1" in your protocol [1] or [2].

Scenario 3: Combining preformulated oligos in tubes and individual oligos in plates

1. For the panel shipped in plates, resuspend rhAmpSeq oligos to 50 µM (see Scenario 1, steps 1 and 2), then make separate forward and reverse pools by combining equal volumes of each primer from all panels.
2. Calculate the individual per primer concentration in each panel.

   For preformulated oligos in tubes

   a. ≥500-plex panels: divide 50 µM by the number of primers in the pool (i.e., 50 µM/1000 primers = 50 nM/primer).

   b. ≤499-plex panels: use Table 1 to determine 10X per primer concentration of each panel.

   For 50 µM oligos in plates

   a. Divide 50 µM by the number of primers in the pool (i.e., 50 µM/1000 primers = 50 µM/primer).

3. Determine which of the panels has the lower per primer concentration, and dilute other panels to that concentration.
4. Combine equal volumes of each panel to create a single pooled panel, then proceed directly to "Perform Targeted rhAmpSeq PCR 1" in your protocol [1] or [2].

   Note: Resulting per primer concentration will be lower than amount recommended in Table 1; reaction and thermal cycling parameters may require optimization for optimal assay performance.

Example 1

Panel 1: 80-plex, 0.4 nmol scale shipped dried down in plates

Panel 2: 1000-plex, forward (FWD) and reverse (REV) pools provided lab ready at 10X concentration in tubes

Final pooled panel size: 1080 primers in each forward and reverse pool

a. For Panel 1, resuspend each primer to 50 µM and create separate forward and reverse pools:

   i. Add 8 µL of IDTE, pH 7.5 buffer to each well.

   ii. Combine 2 µL of each forward primer in the FWD pool and 2 µL of each reverse primer in the REV pool (total of 160 µL per pool).

b. Calculate individual primer concentration for each panel.

   i. Panel 1: 50 µM/80 primers = 625 nM per primer.

   ii. Panel 2: 50 µM/1000 primers = 50 nM per primer.

c. Dilute Panel 1 to 50 nM per primer:
   Dilute stock by combining 50 µL of Panel 1 with 575 µL of IDTE, pH 7.5.

d. Combine equal volumes for each FWD and REV pool (final panel = 25 nM/primer):

   i. Combine 50 µL diluted Panel 1 FWD + 50 µL Panel 2 FWD.

   ii. Combine 50 µL diluted Panel 1 REV + 50 µL Panel 2 REV.

e. Proceed directly to "Perform Targeted rhAmpSeq PCR 1" in your protocol [1] or [2].

References

1. Integrated DNA Technologies. (2019) Protocol: rhAmpSeq Library Preparation for Targeted Amplicon Sequencing. [Online] Coralville, IA, Integrated DNA Technologies, Inc. [Accessed 21 April, 2020]

2. Integrated DNA Technologies. (2019) Protocol: High-throughput rhAmpSeq Library Preparation for Targeted Amplicon Sequencing. [Online] Coralville, IA, Integrated DNA Technologies, Inc. [Accessed 21 April, 2020]

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