Experiments:

Experiment 1

| Rxn ID | Strain: SIMD# | Primer A | Primer A TM | Primer B | Primer B TM | Expected Product Size in bp |
|---|---|---|---|---|---|---|
| 0 | 40 | 40F | 55.76 | 40R | 58.42 | 909 |
| 2 | 42 | 42F | 55.62 | 42R | 59.83 | 861 |
| 5 | 45 | 45F | 55.53 | 45R | 58.33 | 846 |
| 7 | 47 | 47F | 55.29 | 47R | 60.97 | 930 |
| 8 | 48 | 48F | 55.02 | 48R | 58.6 | 966 |

Experiment 2

| Rxn ID | Strain: SIMD# | Primer A | Primer A TM | Primer B | Primer B TM | Expected Product Size in bp |
|---|---|---|---|---|---|---|
| 58 | 58 | 45F | 55.29 | ExoR | 57.1 | 1545 |
| 59 | 59 | 47F | 55.62 | ExoR | 57.1 | 1611 |

Experiment 3

| Rxn ID | Strain: SIMD# | Primer A | Primer A TM | Primer B | Primer B TM | Expected Product Size in bp |
|---|---|---|---|---|---|---|
| 3 | 43 | 43F | 56.43 | 43R | 58.73 | 783 |
| 4 | 44 | 44F | 57.21 | 44R | 58.91 | 969 |
| 9 | 49 | 49F | 57.24 | 49R | 61.07 | 855 |
| 10 | 50 | 50F | 56.95 | 50R | 58.8 | 831 |

Experiment 4

| Rxn ID | Strain: SIMD# | Primer A | Primer A TM | Primer B | Primer B TM | Expected Product Size in bp |
|---|---|---|---|---|---|---|
| 57 | 57 | 44F | 57.21 | ExoR | 57.1 | 1650 |
| 60 | 60 | 49F | 57.24 | ExoR | 57.1 | 1536 |
| 61 | 61 | 50F | 56.95 | ExoR | 57.1 | 1512 |

Experiment 5

| Rxn ID | Strain: SIMD# | Primer A | Primer A TM | Primer B | Primer B TM | Expected Product Size in bp |
|---|---|---|---|---|---|---|
| 1 | 41 | 41F | 58.35 | 41R | 59.97 | 1107 |
| 6 | 46 | 46F | 57.67 | 46R | 61.33 | 966 |

Protocols from Kappa:

KAPA HiFi reactions must be **set up on ice**, according to the following recommendations:

| Component | Final concentration | Volume in a 25 µl reaction[1] |
|---|---|---|
| PCR grade water | – | Up to 25.0 µl |
| 5X KAPA HiFi Fidelity or GC Buffer[2] **(contains 2.0 mM $Mg^{2+}$ at 1X)** | 1X | 5.0 µl |
| KAPA dNTP Mix (10 mM each dNTP)[3] | 0.3 mM each dNTP | 0.75 µl |
| Forward primer (10 µM) | 0.3 µM | 0.75 µl |
| Reverse primer (10 µM) | 0.3 µM | 0.75 µl |
| DMSO (100%) **(for amplicons with a GC content >70%)** | 5% | 1.25 µl |
| Template DNA[4] | As needed | 10 – 100 ng for genomic DNA<br>1 – 10 ng for less complex DNA |
| KAPA HiFi DNA Polymerase (1 U/µl) | 0.5 U/25 µl rxn | 0.50 µl |

[1] For smaller reaction volumes, scale all volumes down proportionately. Reaction volumes >25 µl are not recommended.

[2] Use the Fidelity Buffer for most reactions. The GC Buffer is specifically formulated for amplicons/templates with a high GC content and/or stable secondary structure, and is recommended when the Fidelity Buffer produces a low yield. The additives in the GC Buffer results in a two-fold decrease in fidelity (as compared to the Fidelity Buffer).

[3] The performance of all high-fidelity DNA Polymerases, including KAPA HiFi, is highly dependent on the quality of dNTPs used; the presence of even small amounts of dUTP has a dramatic impact. Only use the highest quality KAPA dNTP Mix supplied in your kit.

[4] Use 10 ng genomic DNA or 1 ng less complex DNA per 25 µl reaction as a starting point.

| Step | Temperature | Time | Number of cycles |
|---|---|---|---|
| Initial denaturation[1] | 95 °C | 2 – 5 min | 1 |
| **Denaturation[1]** | **98 °C** | **20 sec** | 15 - 35[4] |
| Primer annealing[2] | 60 – 75 °C | 15 sec | |
| Extension[3] | 72 °C | 15 – 60 sec/kb | |
| Final extension | 72 °C | 1 – 5 min | 1 |
| Cooling | 4 – 10 °C | HOLD | 1 |

[1] KAPA HiFi Buffers have a higher salt concentration than conventional PCR buffers. Since buffer composition affects DNA melting, it is important to denature complex templates and targets with a high GC content sufficiently. **The above denaturation parameters must therefore be strictly adhered to.**

[2] Due to the high salt concentration of KAPA HiFi Buffers, the optimal annealing temperature for a specific primer pair is likely to be different than when used in a conventional PCR buffer. An annealing temperature of 65 °C is recommended as a starting point. Two-step cycling protocols, with a combined annealing/extension temperature in the range of 68 – 75 °C and a combined annealing/extension time of 30 sec/kb may also be used.

[3] Use 15 sec per cycle for amplicons ≤1 kb and 30 – 60 sec/kb per cycle for long amplicons or to improve yields.

[4] ≤25 cycles are recommended for most high-fidelity applications. In cases where very low template concentrations or low reaction efficiency results in low yields, 30 or 35 cycles may be performed to produce sufficient product for downstream analysis or cloning.

**Experiment #1**:

| Rxn ID | Strain: SIMD# | Primer A | Primer A TM | Primer B | Primer B TM | Expected Product Size in bp |
|---|---|---|---|---|---|---|
| 0 | 40 | 40F | 55.76 | 40R | 58.42 | 909 |
| 2 | 42 | 42F | 55.62 | 42R | 59.83 | 861 |
| 5 | 45 | 45F | 55.53 | 45R | 58.33 | 846 |
| 7 | 47 | 47F | 55.29 | 47R | 60.97 | 930 |
| 8 | 48 | 48F | 55.02 | 48R | 58.6 | 966 |

1. Dissolve colonies 40, 42, 45, 47, 48 in 25 ul nuclease-free water separately (1 colony sample in 25ul) in 1.5ml eppendorf tubes. Heat the samples for 5 min at 95C. Centrifuge at 13,000 RPM for 3 min.

2. Make master mix for: 6 x 25ul Reactions:
   Combine the following in a 500ul tube: (22ul x 6 = 132ul)
   - 97.5 ul nuclease-free H2O
   - 30ul HiFi Buffer (5x)
   - 4.5ul dNTP Mix

3. Aliqot 22 ul of master mix from previous step into 5 labeled PCR tubes (0, 2, 5, 7, 8)

4. Add .75ul 10uM Primers A and B each and 1ul template into corresponding tubes:

| Tube ID | Template (SIMD#) | Primer A | Primer B |
|---|---|---|---|
| **0** | 40 | 40F | 40R |
| **2** | 42 | 42F | 42R |
| **5** | 45 | 45F | 45R |
| **7** | 47 | 47F | 47R |
| **8** | 48 | 48F | 48R |

5. Add .5ul HiFi DNA Polymerase to each tube

6. Thermocycle as follows:

Initial denaturation: 4min 30sec at 95C
35 cycles of:
- Denaturation: 20sec at 98C
- Annealing: 15sec at 55
- Extension: 30sec at 72C

Final Extension: 2min at 72C

**Experiment #2**:

| Rxn ID | Strain: SIMD# | Primer A | Primer A TM | Primer B | Primer B TM | Expected Product Size in bp |
|---|---|---|---|---|---|---|
| 58 | 58 | 45F | 55.29 | ExoR | 57.1 | 1545 |
| 59 | 59 | 47F | 55.62 | ExoR | 57.1 | 1611 |

1. Follow same directions as in Experiment 1 for colonies 58 and 59 but make 66ul of master mix:
   Combine the following in a 500ul tube: (2 x 22ul)
   - 48.75ul nuclease-free H2O
   - 15ul HiFi Buffer (5x)
   - 2.25ul dNTP Mix

3. Aliqot 22 ul of master mix from previous step into 2 labeled PCR tubes (58, 59)

4. Add .75ul 10uM Primers A and B each and 1ul template into corresponding tubes:

5. Add .5ul HiFi DNA Polymerase to each tube

6. Thermocycle as follows:

Initial denaturation: 4min 30sec at 95C
35 cycles of:
Denaturation: 20sec at 98C
Annealing: 15sec at 55
Extension: 1min 20sec at 72C
Final Extension: 3min at 72C

**Experiment #3**:

| Rxn ID | Strain: SIMD# | Primer A | Primer A TM | Primer B | Primer B TM | Expected Product Size in bp |
|---|---|---|---|---|---|---|
| 3 | 43 | 43F | 56.43 | 43R | 58.73 | 783 |
| 4 | 44 | 44F | 57.21 | 44R | 58.91 | 969 |
| 9 | 49 | 49F | 57.24 | 49R | 61.07 | 855 |
| 10 | 50 | 50F | 56.95 | 50R | 58.8 | 831 |

2. Dissolve colonies 43, 44, 49, and 50 in 25 ul nuclease-free water separately (1 colony sample in 25ul) in 1.5ml eppendorf tubes. Heat the samples for 5 min at 95C. Centrifuge at 13,000 RPM for 3 min.

2. Make master mix for: 5 x 25ul Reactions:
Combine the following in a 500ul tube: (22ul x 5 = 110ul)
81.25ul nuclease-free H2O
25ul HiFi Buffer (5x)
3.75ul dNTP Mix

3. Aliqot 22 ul of master mix from previous step into 5 labeled PCR tubes (3,4,9,10)

4. Add .75ul 10uM Primers A and B each and 1ul template into corresponding tubes:

5. Add .5ul HiFi DNA Polymerase to each tube

6. Thermocycle as follows:

Initial denaturation: 4min 30sec at 95C
35 cycles of:
Denaturation: 20sec at 98C
Annealing: 15sec at 56C
Extension: 30sec at 72C
Final Extension: 2min at 72C

**Experiment #4**:

| Rxn ID | Strain: SIMD# | Primer A | Primer A TM | Primer B | Primer B TM | Expected Product Size in bp |
|---|---|---|---|---|---|---|
| 57 | 57 | 44F | 57.21 | ExoR | 57.1 | 1650 |
| 60 | 60 | 49F | 57.24 | ExoR | 57.1 | 1536 |
| 61 | 61 | 50F | 56.95 | ExoR | 57.1 | 1512 |

**Experiment #5**:

| Rxn ID | Strain: SIMD# | Primer A | Primer A TM | Primer B | Primer B TM | Expected Product Size in bp |
|---|---|---|---|---|---|---|
| 1 | 41 | 41F | 58.35 | 41R | 59.97 | 1107 |
| 6 | 46 | 46F | 57.67 | 46R | 61.33 | 966 |

3. Dissolve colonies 41, 46, 57, 60, 61 in 25 ul nuclease-free water separately (1 colony sample in 25ul) in 1.5ml eppendorf tubes. Heat the samples for 5 min at 95C. Centrifuge at 13,000 RPM for 3 min.

2. Make master mix for: 6 x 25ul Reactions:
   Combine the following in a 500ul tube: (22ul x 6 = 132ul)
   - 97.5 ul nuclease-free H2O
   - 30ul HiFi Buffer (5x)
   - 4.5ul dNTP Mix

3. Aliqot 22 ul of master mix from previous step into 5 labeled PCR tubes (1, 6, 57, 60, 61)

4. Add .75ul 10uM Primers A and B each and 1ul template into corresponding tubes:

5. Add .5ul HiFi DNA Polymerase to each tube

6. Thermocycle as follows:

Experiment 4 samples:

Initial denaturation: 4min 30sec at 95C
35 cycles of:
- Denaturation: 20sec at 98C
- Annealing: 15sec at 56.5C
- Extension: 1min 20sec at 72C

Final Extension: 3min at 72C

Experiment 5 samples:

Initial denaturation: 4min 30sec at 95C
35 cycles of:
- Denaturation: 20sec at 98C
- Annealing: 15sec at 57C
- Extension: 1min at 72C

Final Extension: 2min 30sec at 72C