Feb 06, 2025

Universal (second) PCR of the Leeselab 2-step PCR protocol

DOI
https://dx.doi.org/10.17504/protocols.io.rm7vzkq34vx1/v1

Marie-Thérése Werner[1], Dominik Buchner[1]

[1]University of Duisburg-Essen, Aquatic Ecosystem Research

Aquatic Ecosystem Rese...

Dominik Buchner
University of Duisburg-Essen, Aquatic Ecosystem Research

OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.rm7vzkq34vx1/v1

Protocol Citation: Marie-Thérése Werner, Dominik Buchner 2025. Universal (second) PCR of the Leeselab 2-step PCR protocol. **protocols.io** https://dx.doi.org/10.17504/protocols.io.rm7vzkq34vx1/v1

Protocol status: Working
We use this protocol and it's working

Created: January 30, 2025

Last Modified: February 06, 2025

Protocol Integer ID: 119326

Keywords: pcr, second pcr step, two-step PCR, pcr of the leeselab, step pcr protocol this protocol, step pcr protocol, first pcr step, second pcr, universal second step of the leeselab, pcr, specific primer, universal second step, primer, fwhr2n, protocol this protocol, leeselab, protocol, second step, reaction, fwhf2

Abstract

This protocol describes how to perfom the universal second step of the Leeselab 2-step PCR protocol in 10 µL reactions. In the first PCR step, specific primers (e.g. fwhF2/fwhR2n) are used to tag each plate with an individual combination of tags (1-24) and a universal tail. In the second step of the 2-step-PCR, each well is tagged with an individual index sequence (1-96) enabling the identification of each sample (well) after library sequencing.

Guidelines

Follow general lab etiquette. Wear gloves to prevent contaminating the samples. Clean the workspace before starting with 80% EtOH.

Materials

Below all materials needed for the protocol are listed. Vendors and part numbers are listed but interchangeable.

Chemicals:

Multiplex Mastermix 5x Hot-Start Multiplex PCR-Mastermix **Bio&SELL Catalog #**BS91.522.1000MPP
Forward and reverse primers (universal), see Excel spreadsheet below
PCR-grade water
Invitrogen UltraPure DNase/RNase-Free Distilled Water **Fisher Scientific Catalog #**11538646

Labware:

Micro-Twist-Top-Tube Micro-Twist-Top-Tube **Sarstedt Catalog #**72.693
PCR plate Hard-Shell 96-well Hard-Shell 96-well PCR plate **Bio-Rad Laboratories Catalog #**HSP9601
PCR foil, DNase-/RNase-free PCR foil, DNase-/RNase-free **Sarstedt Catalog #**95.1993

Devices:

Vortexer
Centrifuge for SBS format microplates
Table centrifuge for tubes
Pipettes and Multichannel pipettes including pipette tips
Thermocycler compatible wth low profile PCR plates

Documents:

Second_step_primer_Leeselab.xlsx 16.4KB

Protocol materials

Micro-Twist-Top-Tube **Sarstedt Catalog #**72.693
5x Hot-Start Multiplex PCR-Mastermix **Bio&SELL Catalog #**BS91.522.1000MPP
UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #**10977023
Hard-Shell 96-well PCR plate **Bio-Rad Laboratories Catalog #**HSP9601
PCR foil, DNase-/RNase-free **Sarstedt Catalog #**95.1993
Invitrogen UltraPure DNase/RNase-Free Distilled Water **Fisher Scientific Catalog #**11538646

Safety warnings

Reagents are potentially damaging to the environment. Dispose waste responsibly.

Before start

Make sure all materials are available before starting. Make sure all materials and samples are unfrozen.

This protocol uses clean first step PCR product as input material, e.g. prepared according to the following protocol:

Protocol

NAME

PCR cleanup and size selection with magnetic beads

CREATED BY

Dominik Buchner

Preview

Note

The protocol described here is designed for the use of PCR plates, but can also be done in tubes, strips, or any sufficient reaction vessel. The recommended shaking speeds are adjusted to the plates mentioned in the materials.

Preparations

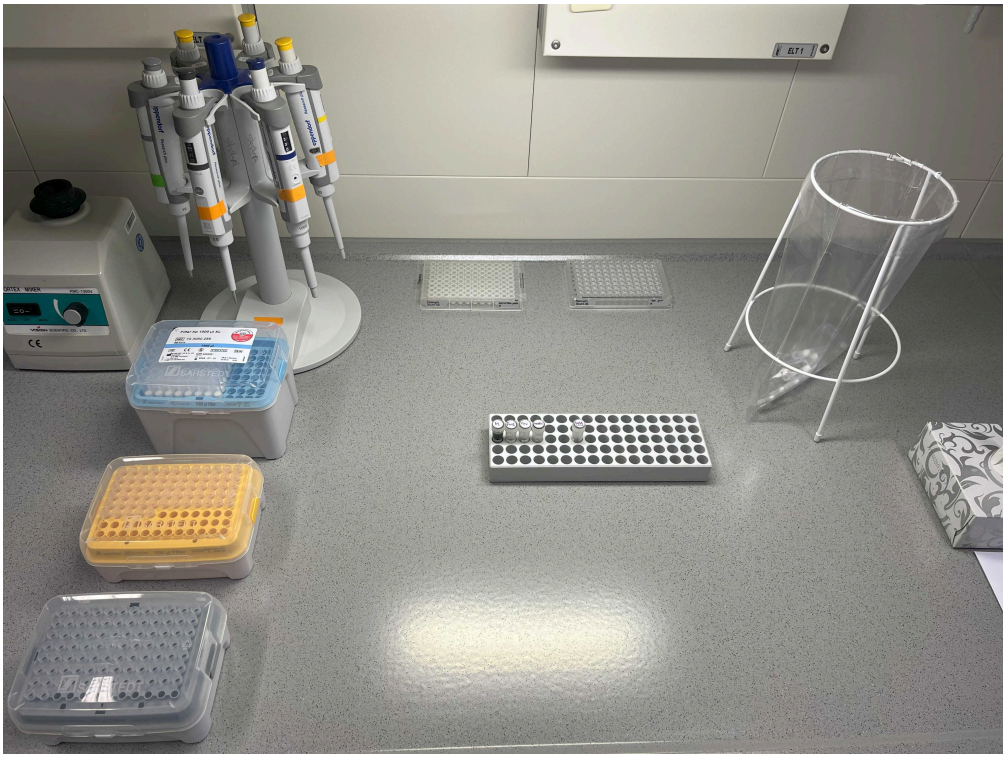
30m

1 Calculate the required volumes for the PCR Mastermix using the Excel spreadsheet. 1m

Note

You can download the Exel spreadsheet here:
Mastermix_calculation_2.xlsx

2 Prepare the workplace to ensure contamination-free handling. 2m



Workspace prepared for a right-handed person to work from left (pipette tips, samples) to right (waste). Clean PCR products and (unused) labware are placed in the back.

2.1 Label the Micro-Twist-Top-Tube **Sarstedt Catalog #**72.693 (e.g., "MM") and Hard-Shell 96-well PCR plate **Bio-Rad Laboratories Catalog #**HSP9601 (e.g. project name, date, "2nd_pcr", "A"). 2m

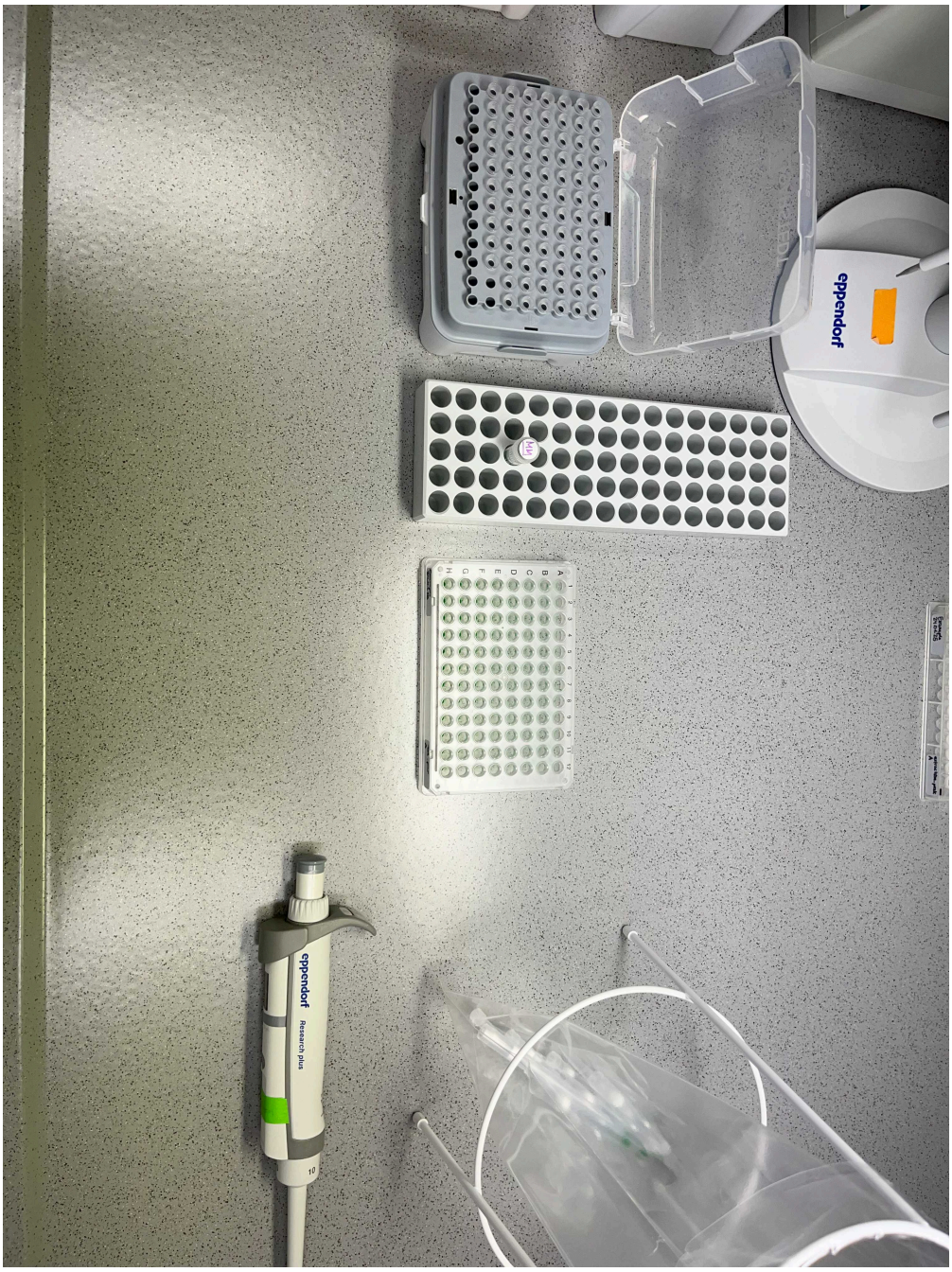
3 Briefly vortex and shortly spin down the clean PCR products and required chemicals. 2m

4 Pipette the PCR Mastermix in a Micro-Twist-Top-Tube **Sarstedt Catalog #**72.693 containing the calculated volumes of 5x Hot-Start Multiplex PCR-Mastermix **Bio&SELL Catalog #**BS91.522.1000MPP and UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #**10977023 . 3m

> Note
>
> Do not include the tagging primers in the master mix. Each well has to be tagged separately.

4.1 Briefly vortex and shortly spin down the prepared PCR Mastermix. 1m

5 Distribute 6 µL per sample in each well used of the Hard-Shell 96-well PCR plate **Bio-Rad Laboratories Catalog #**HSP9601 . To speed up this step, a Multidispenser can be used. 8m



Distribution of the PCR Mastermix (MM) in all required wells.

6 Pipette 2 µL primer mix [100 µM] per well.

4m

Note

To ensure correct assignment and minimize the contamination risk, it is best to prepare a tagging primer plate and add the primer mix with a Multichannel pipette.

7 Pipette 2 µL clean PCR product per well. This step has to be done with a Multichannel pipette to ensure correct assignment and minimize the contamination risk.

4m

Note

Always maintain the order of samples (e.g., top to bottom, left to right) for pipette tips, source plate and target plate (pictures available in protocol below).

Protocol

NAME

Specific (first) PCR of the Leeselab 2-step PCR protocol

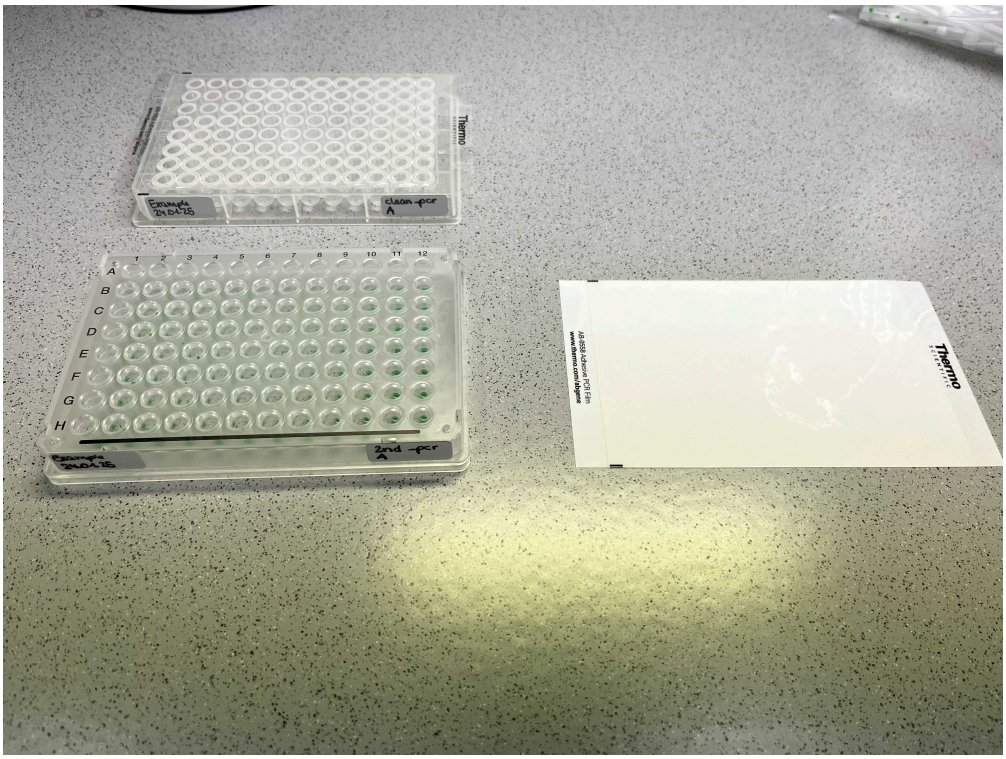
CREATED BY

Marie-Thérése Werner

Preview

8 Close the Hard-Shell 96-well PCR plate **Bio-Rad Laboratories Catalog #**HSP9601 with PCR foil, DNase-/RNase-free **Sarstedt Catalog #**95.1993. Make sure that each well is properly sealed.

1m



Sealed clean PCR products plate (back) and 2nd PCR plate with foil (front).

8.1 Close the clean PCR product plate with PCR foil. Make sure each well is properly sealed. 1m

9 Briefly vortex and shortly spin down the sealed Hard-Shell 96-well PCR plate **Bio-Rad Laboratories Catalog #**HSP9601. 1m

PCR 1h 32m

10 Place the sealed Hard-Shell 96-well PCR plate **Bio-Rad Laboratories Catalog #**HSP9601 in a Thermocycler and close it properly. 1m

10.1 Depending on the Thermocycler, a lid or pad should be placed on the PCR plate before closing the Thermocycler. 1m

11 Enter/select the correct program (depending on used primers and chemicals) and start the polymerase chain reaction. 1h 30m

Note

PCR program for universal Illumina primers:

Step 1: 95°C for 5 minutes (initial denaturation)
Step 2: 95°C for 30 seconds (denaturation)
Step 3: 61°C for 90 seconds (annealing)
Step 4: 72°C for 30 seconds (elongation)
--- repeat for 25 cycles ---
Step 5: 68°C for 10 minutes (final elongation)

Storage

1m

12 Vortex and spin down the Hard-Shell 96-well PCR plate **Bio-Rad Laboratories Catalog #**HSP9601 to use it for further processing, e.g. gel electrophoresis, and/or store it at 4°C (short-term) or -20°C (long term).

1m