STANDARD OPERATING PROTOCOL · EnGeL-MOLB-EXOSAP

# PCR product clean-up with ExoSAP and preparation of samples for Sanger sequencing

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|---|---|
| **Protocol ID** | EnGeL-MOLB-EXOSAP |
| **Version** | 1.1.1 |
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| **Status** | draft |
| **Area** | Molecular biology (MOLB) |
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| **Licence** | Text: CC BY 4.0 · Code: MIT |
| **Main hazards** | No hazardous reagents identified in the sources [TO CONFIRM against the ExoSAP-IT and enzyme SDS] |

## 1 Purpose and scope

This protocol removes the leftover primers and nucleotides from PCR products and prepares the cleaned products, with their sequencing primer, for Sanger sequencing by an external provider. It follows the barcode PCR and precedes the analysis of the chromatograms (EnGeL-BIOINF-SANGER) in the single-specimen barcoding workflow. The barcode PCR itself is a separate protocol [TO CONFIRM: to be written].

Three clean-up options are in use; ExoSAP-IT Express is the usual one.

## 2 Principle

After PCR, the reaction still contains unused primers and dNTPs, which would take part in the sequencing reaction and spoil the read. Exonuclease I digests single-stranded DNA (the leftover primers) and shrimp alkaline phosphatase removes the phosphates of the dNTPs. Both enzymes are then inactivated by heat, so the product can go straight into the sequencing reaction without a column or bead clean-up. The template only works if the PCR gave a single, sharp band: other bands or smears give mixed signals in the chromatogram.

# 3 Before you start

| Item | Details |
|---|---|
| Input | PCR products checked on a gel |
| Hands-on time | [TO CONFIRM] |
| Total time | About 10 min with ExoSAP-IT Express; about 30 min with ExoSAP-IT; about 1 h with the home-made mix (incubation), plus sample preparation |
| Prepare in advance | Sequencing primers diluted (Calculations); order placed with the provider by Filipa Reis or Luís Cunha, and the provider's current sample instructions at hand; samples entered in your sheet of the Sanger recording file |

**CRITICAL** Check every PCR product on a gel first. Only products with one clear, sharp band of the expected size are sent. Products with several bands or smears give unreadable chromatograms whatever the clean-up.

# 4 Materials

## 4.1 Equipment

- Thermocycler (programmable incubation at 37 °C and 80 °C)
- Microcentrifuge or plate centrifuge
- Gel electrophoresis and imaging (to judge the PCR band before clean-up)

## 4.2 Consumables

- PCR tubes or plates
- Tubes or plates as required by the sequencing provider (1.5 mL tubes in the source)
- Plastic zip-lock bags (drop-off services)
- Filter tips

## 4.3 Reagents and kits

- ExoSAP-IT Express (Thermo Fisher Scientific), the lab's usual reagent [TO CONFIRM: catalogue number]
- ExoSAP-IT (Thermo Fisher Scientific) [TO CONFIRM: catalogue number]
- Home-made mix: Exonuclease I (New England Biolabs M0293S, 20 U/µL) and Shrimp Alkaline Phosphatase (1 U/µL; USB/Amersham E70092X in the source)
- Sequencing primer (forward or reverse), diluted so that 5 µL contain 10 pmol (2 µM)
- Nuclease-free water

# 5 Reagent preparation

- **ExoSAP-IT Express and ExoSAP-IT:** store at −20 °C (non-frost-free freezer); keep on ice while in use and return to the freezer straight away.
- **Sequencing primer:** dilute the stock so that 5 µL contain 10 pmol (2 µM; see Calculations). Prepare enough for all samples of the order.

# 6 Procedure

## 6.1 Before starting

- Clean the working space and material with bleach and water and leave the UV light on for at least 15 min.
- Use filter tips at all steps.
- Clean forceps and spatulas with 3% sodium hypochlorite and 96% ethanol between samples.

## 6.2 1. Judge the PCR products

1. Look at the gel image of each PCR product. Mark it as **strong** (clear, bright band) or **faint** (visible but weak band). Products without a single band of the expected size are not sent.

## 6.3 2. Clean up the PCR products

Choose one option and use it for the whole order.

**Option A: ExoSAP-IT Express (usual)**

2. Mix 5 µL of PCR product with 2 µL of ExoSAP-IT Express on ice.
3. In the thermocycler: 37 °C for 4 min, 80 °C for 1 min, then hold at 4 °C.

**Option B: ExoSAP-IT**

4. Mix 5 µL of PCR product with 2 µL of ExoSAP-IT on ice.
5. Run the ExoSAP-IT programme (about 30 min in total) [TO CONFIRM: temperatures and times from the ExoSAP-IT user guide].

**Option C: home-made Exonuclease I and SAP mix**

6. Per sample, mix 1 µL SAP (1 U/µL), 0.25 µL Exonuclease I (20 U/µL) and 3.75 µL nuclease-free water, and add 15 µL of PCR product (20 µL in total).
7. In the thermocycler, with the heated lid switched off: 37 °C for 40–45 min, 80 °C for 15 min, then hold at 4 °C.

**PAUSE POINT.** Cleaned products can be kept at −20 °C (ExoSAP-IT Express user guide).

## 6.4 3. Prepare the sequencing samples

8. For each product and each primer, label a tube (or plate well) with the sample name of the order, following the provider's instructions for tube type and labelling.
9. Pipette the cleaned product and water:

| Band | Cleaned product | Nuclease-free water |
|---|---|---|
| Strong | 1 µL | 4 µL |
| Faint | 2.5 µL | 2.5 µL |

10. Add 5 µL of diluted primer (10 pmol). Total volume: 10 µL.
11. If more volume is needed, scale all three volumes by 1.5 (total 15 µL) [TO CONFIRM: when the 1.5× volumes are used].
12. Close, spin briefly and keep at 4 °C (fridge) until shipping.

## 6.5 4. Record and send

13. Complete your sheet in the Sanger recording file (see Data and metadata) before the samples leave the lab.
14. Send the samples to the provider, following its current instructions for volumes, tubes and labels:

| Provider | How samples are sent |
|---|---|
| Eurofins | Drop-off box at UC-Biotech; samples in a closed plastic zip-lock bag |
| StabVida | Drop-off box at UC-Biotech; samples in a closed plastic zip-lock bag |
| Macrogen | Pick-up service; a shipping label is generated for the shipment when needed |

Orders are placed by Filipa Reis or Luís Cunha.

## 6.6 After finishing

- Clean the bench with 70% ethanol.
- Empty the small bench bin into the right waste bin.
- Wash your hands.

- Turn off equipment and lights.
- Lock doors, fridge and freezer.

# 7 Calculations

**Primer dilution.** The sample receives 10 pmol of primer in 5 µL, i.e. a concentration of

$$c = \frac{n}{V} = \frac{10\ \text{pmol}}{5\ \mu\text{L}} = 2\ \text{pmol}/\mu\text{L} = 2\ \mu\text{M}$$

To prepare it from a stock, $V_{\text{stock}} = c \times V_{\text{final}} / c_{\text{stock}}$, where $c$ is the target concentration (µM), $V_{\text{final}}$ the volume needed (µL) and $c_{\text{stock}}$ the stock concentration (µM).

**Worked example.** For 20 samples (100 µL of diluted primer, with excess 110 µL) from a 10 µM stock: $V_{\text{stock}} = 2 \times 110/10 = 22$ µL of stock plus 88 µL of nuclease-free water.

# 8 Quality control

| Control | Purpose | Acceptance criterion |
|---|---|---|
| PCR band on the gel | Template quality | One clear, sharp band of the expected size; strong or faint decides the volume (step 3) |
| Clean-up | Primers and dNTPs removed | Programme run to the end, including the 80 °C inactivation |
| Sequence quality | Overall result | Read quality and identification checked in EnGeL-BIOINF-SANGER |

# 9 Data and metadata

Each person records their samples in their own sheet of the Sanger recording file, named with their full name and built from the template sheet. Only the curator (Luís Cunha) merges the personal sheets into the “All” sheet, after checking them; nobody else edits “All”. The columns follow the template, which is also the sample sheet read by the Sanger pipeline (EnGeL-BIOINF-SANGER): plate or order code, well or tube code, primer pair, specimen ID and putative species and family.

**Minimum to record**

- Specimen ID (EnGeL label) and DNA extract; PCR date and primer pair
- Band strength (strong or faint) and clean-up option used
- Primer sent with each sample; provider, order number, plate or tube code and position
- Date sent; operator

# 10 Safety and waste

Follow the manufacturer’s instructions and safety data sheets for each reagent. Biological samples are potentially hazardous and are handled following the facility’s procedures. EnGeL lab rules:

- Before you enter: wear your lab coat, use appropriate gloves, closed shoes only, tie back long hair.
- Not allowed: eating or drinking; touching phones or laptops with contaminated gloves; bags or coats on benches.
- Label all reagents (name, concentration, date) and treat all chemicals as hazardous.

**Waste**

EnGeL waste streams (choose the right bin):

- **Biological solid waste** (black bin, white bag): contaminated gloves, contaminated disposable materials, solid biological laboratory waste, contaminated PPE. No liquids or sharps.
- **Specific hazardous waste** (red bin, red bag): chemicals and pharmaceuticals designated for this waste stream, specific hazardous laboratory waste, anatomical material; sharps only in a rigid sharps container. No loose sharps.
- **Biological liquid waste** (jerrican, UN 3291): liquid biological waste and contaminated biological liquids only. Use a funnel; rinse the original container and the funnel with $dH_2O$ and discard the rinse water in the same jerrican.

For this protocol: tubes, plates and tips go to biological solid waste (black bin); expired reagents to the red bin.

# 11 Troubleshooting

| Problem | Likely cause | Solution |
|---|---|---|
| Noisy chromatogram from the start | Leftover primers or dNTPs | Check that the clean-up programme ran to the end; check the reagent volumes and the reagent's storage. |
| Mixed peaks along the read | Several PCR products or contamination | Send only single, sharp bands; repeat the PCR. |
| Weak or short read | Too little template | Use the faint-band volumes (2.5 µL), or repeat the PCR. |
| Sample missing in the provider's results | Sample name or position differs from the order | Check names against the order and your recording sheet. |
| Sample rejected by the provider | Volume, tube or label not as required | Check the provider's current instructions before sending. |

# 12 Remarks

ExoSAP-IT Express is the lab's usual reagent; ExoSAP-IT and the home-made mix are also in use. Do not mix options within one order, so that results can be compared. The home-made mix uses less enzyme than the manufacturers' protocols (which give 10 U Exonuclease I and 1 U SAP per 5 µL of product, 15 min at 37 °C; New England Biolabs) and a longer incubation; it is kept as the lab uses it. Plate orders and tube orders give differently named files, which the Sanger pipeline handles with separate scripts (EnGeL-BIOINF-SANGER).

# 13 References

Thermo Fisher Scientific. ExoSAP-IT Express PCR Product Cleanup Reagent, user guide [TO CONFIRM: document version] (kept with the lab's kit manuals).

New England Biolabs. Enzymatic PCR cleanup protocol using Exonuclease I (NEB #M0293) and Shrimp Alkaline Phosphatase (NEB #M0371). https://international.neb.com/protocols/2017/07/10/enzymatic-pcr-cleanup-protocol-neb-m0371

# 14 Version history and citation

| Version | Date | Change | By |
|---|---|---|---|
| 1.0.0 | 2026-11-20 | First EnGeL-UC version, from Luísa Fraga's bench protocol "Protocolo EXOSAP + EUROFINS" (2023), the lab's older protocol "PCR product cleaning with | Luísa Fraga, Luís Cunha |

| Version | Date | Change | By |
|---|---|---|---|
| | | ExoSAP", the ExoSAP-IT Express user guide and the lab's guidance for the Sanger recording sheet. | |
| 1.1.0 | 2026-11-20 | Ordering and shipping confirmed by Luís Cunha: orders by Filipa Reis or Luís Cunha; Eurofins and StabVida via drop-off boxes at UC-Biotech (samples in a zip-lock bag), Macrogen via pick-up with a shipping label; provider instructions to be followed. Older ExoSAP protocol by Luís Cunha. | Luís Cunha |
| 1.1.1 | 2026-11-20 | Home-made Exonuclease I/SAP recipe kept as the lab uses it (confirmed by Luís Cunha); comparison with the manufacturer protocols kept in Remarks. | Luís Cunha |

**How to cite**

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# 15 Open questions

1. Catalogue numbers of ExoSAP-IT Express and ExoSAP-IT; ExoSAP-IT programme (temperatures and times).
2. When the 1.5× volumes (total 15 µL) are used.
3. Barcode PCR protocol (primers, master mix, cycling) for the workflow.
4. Hands-on time; hazards from the SDS; DOI.