# Generating PureTarget™ libraries with PureTarget kit 96 – automation protocol

Procedure & checklist

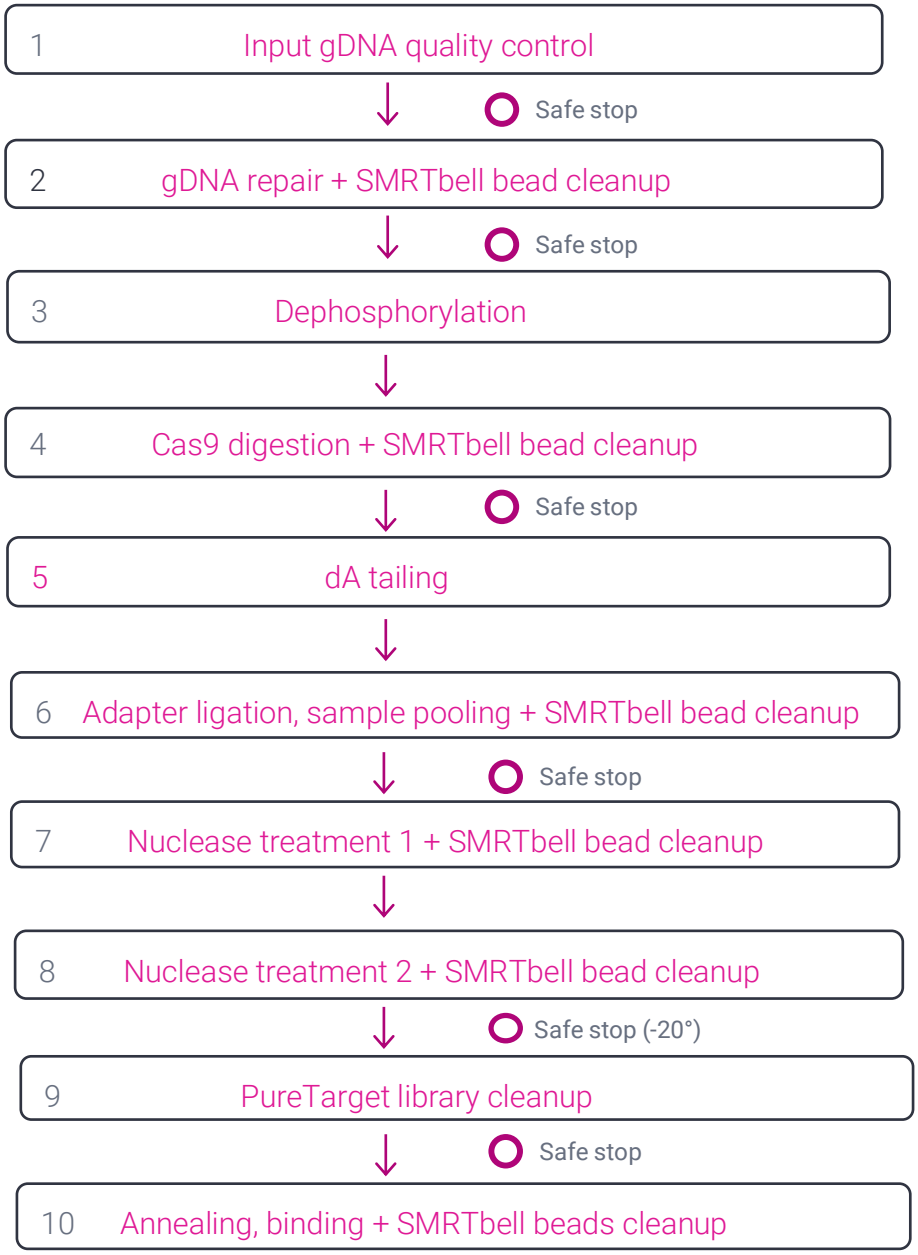
## Before you begin

This procedure describes the workflow for generating PureTarget repeat expansion or carrier libraries.

| Overview | |
|---|---|
| Samples | 96 |
| Library prep time | 16 hours with the Hamilton NGS STAR MOA system |
| **DNA input** | |
| Quantity | 1–1.5 µg in Elution buffer, TE buffer (pH 8, 0.1mM EDTA), or nuclease-free water |
| DNA size distribution | 50% ≥30 kb on Femto Pulse system; DIN >8 on TapeStation system |
| **Sample multiplexing** | |
| Revio® system with SPRQ™ chemistry | 96 samples |


PN 103-740-800 REV03 NOV2025

# Workflow overview





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# Materials and equipment

| | |
|---|---|
| **Recommended DNA sizing** | |
| Femto Pulse system | Agilent Technologies, Inc. M5330AA |
| Femto Pulse gDNA 165kb analysis kit | Agilent Technologies, Inc. FP-1002-0275 |
| TapeStation system | Agilent Technologies, Inc. G2992AA or G2991BA |
| **DNA quantitation** | |
| Qubit fluorometer | ThermoFisher Scientific Q33238 |
| Qubit 1X dsDNA HS assay kit | ThermoFisher Scientific Q33230 |
| **Target enrichment and library preparation** | |
| PureTarget™ repeat expansion panel 2.0 or<br>PureTarget™ carrier panel or<br>PureTarget™ repeat control panel | PacBio® 103-633-100<br>PacBio® 103-633-200<br>PacBio® 103-633-300 |
| PureTarget™ cleanup beads kit | PacBio® 103-633-000 |
| PureTarget™ cleanup buffer kit | PacBio® 103-682-800 |
| PureTarget™ kit 2.0 | PacBio® 103-632-900* |
| HiFi plex prep kit 96 | PacBio® 103-122-800* |
| SMRTbell® cleanup beads-10mL (qty 2) | PacBio® 102-158-300* |
| Elution buffer | PacBio® 101-633-500* |
| SMRTbell® adapter index plate 96A (for barcoding) or<br>SMRTbell® adapter index plate 96B (for barcoding) or<br>SMRTbell® adapter index plate 96C (for barcoding) or<br>SMRTbell® adapter index plate 96D (for barcoding) | PacBio® 102-009-200<br>PacBio® 102-547-800<br>PacBio® 102-547-900<br>PacBio® 102-548-000 |
| Revio® SPRQ™ polymerase kit (store at -20C upon arrival) | PacBio® 103-496-900 |
| **General lab supplies and equipment** | |
| 200 Proof ethanol, molecular biology or ACS grade | Any major lab supplier (MLS) |
| 8-channel pipettes | Any MLS |
| 96-well PCR plate | BioRad Hard-Shell 96-Well PCR Plates #HSP9601 |
| Abgene 96 Well 0.8 mL Polypropylene Deepwell Storage Plate | ThermoFisher Scientific, AB0859 |
| Microcentrifuge | Any MLS |
| Magnum FLX magnetic plate (springs removed) | Alpaqua, A000400 |
| Magnetic separation rack compatible with 1.5 mL tubes | Any MLS (e.g., Invitrogen DynaMag-2 Magnet 12321D) |
| Thermocycler compatible with 0.2 mL tube strips | Any MLS |
| Nuclease-free (NF) water | Any MLS, molecular biology grade |
| 1.5 mL DNA LoBind tubes | Eppendorf 022431021 |

*Sold together as part of the PureTarget™ kit 96 bundle


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# General best practices

## DNA input

For optimal performance, this protocol recommends high-quality, high molecular weight (HMW) human gDNA with at least 50% of the mass of DNA in molecules at ≥30 kb in length (genome quality number (GQN) of ≥5 at 30 kb) based on the Agilent Femto Pulse system, or a DIN value >8 on the Agilent TapeStation. For low-quality human gDNA, it is still feasible to proceed, but lower coverage is expected.

**Note**: Removal of RNA with RNAse is required for any DNA extraction method used. Failure to remove RNA may result in sequencing inhibition.

**The supported sample types are:**

- **Human blood genomic DNA**: extracted by Nanobind® RBC lysis PanDNA kit, Nanobind Whole blood manual CBB kit/PanDNA kit, Nanobind HT 200 µL protocol, Nanobind HT 1 mL protocol
- **Human B-lymphocyte cell lines**: extracted by Nanobind CBB kit/ PanDNA kit, Nanobind CBB HT; Coriell cell line gDNA ($GQN_{30kb}$ ≥5).

For information on the performance of other extraction methods, see Application note - Comprehensive genotyping with the PureTarget repeat expansion panel and HiFi sequencing.

**The protocol supports 1- 1.5 µg per sample. 1.3- 1.5 µg per sample is highly recommended for samples going through the gDNA repair step**, to ensure sufficient mass to achieve optimal target coverage. A maximum mass of 150 µg of gDNA input (across 96 samples) is recommended for a single Revio system + SPRQ chemistry SMRT® Cell.

## Multiplexing samples

### Revio system with SPRQ chemistry

Each PureTarget kit bundle supports one preparation of 96 samples using the automation protocol.

## Reagent and sample handling

- For a quick guide on master mix preparation and plating, see Appendix 1.
- Room temperature is defined as any temperature in the range of 18–23°C for this protocol.
- Do not vortex enzymes.
- Bring SMRTbell cleanup beads to room temperature. Always vortex immediately prior to use.
- Bring Qubit reagents to room temperature prior to use.
- Thaw frozen reagents at room temperature. Place on ice after thawing.
- Keep master mixes involving temperature-sensitive reagents on ice until use.
- Quick-spin all reagents in a microcentrifuge to collect liquid at the bottom prior to use.
- Samples can be stored at the specified temperature at the safe stopping points listed in the protocol.

## Library preparation reagents

Kits stored at -20°C.

| Step used | Tube | Reagent |
|---|---|---|
| **PureTarget kit 2.0** | | |
| Dephosphorylation | Blue | Phosphatase |
| Cas9 digestion | Green | Cas9 Nuclease |
| Dephosphorylation/Cas9 digestion | Red | Cas9 buffer |
| dA tailing/Nuclease treatment | Orange | dA tail buffer |
| dA tailing | Violet | *Taq* DNA Polymerase |
| dA tailing | Yellow | dATP |
| Nuclease treatment | White | Pure Target Nuclease |
| **PureTarget panel** | | |
| Cas9 digestion | White | Repeat expansion panel 2.0 *or* |
| | Purple | Carrier panel *or* |
| | Orange | Control panel |
| **HiFi plex prep kit 96** | | |
| gDNA repair | Purple | Repair buffer M96 |
| gDNA repair | Blue | End repair mix M96 |
| gDNA repair | Green | DNA repair mix M96 |
| Adapter ligation | Yellow | Ligation mix M96 |
| Adapter ligation | Red | Ligation enhancer M96 |
| Nuclease treatment | Light green | Nuclease mix M96 |
| **SMRTbell adapter index plate** | | |
| Adapter ligation | White plate | SMRTbell adapter index plate 96A, 96B, 96C, or 96D |

## PureTarget cleanup beads kit

Keep at room temperature.

| Component | Tube color |
|---|---|
| PureTarget cleanup wash buffer* | Bottle, white |
| PureTarget cleanup binding buffer | Bottle, white |
| PureTarget cleanup beads | Clear |

***Important**: Prior to the first use of the PureTarget cleanup beads kit, add 15 mL of 200 proof ethanol to PureTarget cleanup wash buffer and mix well. Store bottles upright to prevent leakage. Ensure bottle cap is tightly screwed on and store bottles upright to prevent leakage.

## PureTarget cleanup buffer kit

Keep at room temperature.

| Component | Tube color |
|---|---|
| PureTarget cleanup buffer 1 | Red |
| PureTarget cleanup buffer 2 | Blue |
| PureTarget cleanup buffer 3 | Green |

## Revio SPRQ polymerase kit

Kit stored at -20°C.

| Component | Tube color |
|---|---|
| Annealing buffer | Light blue |
| Standard sequencing primer | Light green |
| Polymerase buffer | Yellow |
| Loading buffer | Green |
| Dilution buffer | Blue |
| Sequencing polymerase | Purple |
| Sequencing control | Red |

**Note**: Bring the Loading buffer to room temperature prior to use. The Loading buffer is light sensitive and should be protected from light when not in use.
**Note**: The Revio SPRQ polymerase kit supplies reagent volumes sufficient for 12 SMRT Cells for PureTarget.

# Safety precautions

Refer to the Safety Data Sheet (SDS) for information on reagent hazards and protocols for safe handling, use, storage, and disposal.

# Thermocycler conditions

Program thermocycler(s) prior to beginning the protocol for the first time. If the lid temperature is not adjustable, set the lid temperature to "ON".

Note: Maintaining the lid temperature at 10°C higher than the incubation temperature is designed to minimize condensation. If the lid does not cool to the recommended values, performance will not be impacted. As long as the block temperature is accurate and the lid temperature is at least 10°C above the incubation temperature for heated steps, the enzymatic reactions will proceed as expected.

## 1.DNA repair program

Set the lid temperature to ≥65°C.

| Step | Time | Temperature |
|---|---|---|
| 1 | 60 min | 37°C |
| 2 | 30 min | 52°C |
| 3 | Hold | 4°C |

## 2. Dephosphorylation program

Set the lid temperature to ≥95°C.

| Step | Time | Temperature |
|---|---|---|
| 1 | 50 min | 37°C |
| 2 | 5 min | 85°C |
| 3 | Hold | 4°C |

## 3. Cas9 digestion program

Set the lid temperature to ≥65°C.

| Step | Time | Temperature |
|---|---|---|
| 1 | 60 min | 37°C |
| 2 | Hold | 4°C |

## 4. dA-tail program

Set the lid temperature to ≥95°C.

| Step | Time | Temperature |
|---|---|---|
| 1 | 1 min | 37°C |
| 2 | 5 min | 72°C |
| 3 | Hold | 4°C |

## 5. Adapter ligation program

Set the lid temperature to “OFF” or ≥30°C.

| Step | Time | Temperature |
|---|---|---|
| 1 | 30 min | 20°C or RT |
| 2 | Hold | 4°C |

## 6. Nuclease treatment-1/ -2 program

Set the lid temperature to ≥65°C.

| Step | Time | Temperature |
|---|---|---|
| 1 | (1) 30 min/ (2) 60 min | 37°C |
| 2 | Hold | 4°C |

# Workflow steps

## 1. Input DNA quality control and dilution

Before you begin, evaluate the quantity and size distribution of input DNA using Agilent Femto Pulse system or TapeStation system to determine whether it is suitable for the protocol.

| ✔ | Step | Instructions |
|---|---|---|
| | 1.1 | Bring the Qubit 1X dsDNA HS working solution and standards to **room temperature**. |
| | 1.2 | Pulse vortex and/or pipette-mix each sample 5 times to homogenize the DNA in solution.<br>**Note**: For viscous input DNA, it is important to homogenize the extracted DNA prior to start of the protocol. To homogenize the DNA, pulse-vortex and/or pipette-mix full sample volume with standard (not wide bore) tips. These steps will maintain HMW of your DNA but will improve accuracy of quantification and subsequent handling. |
| | 1.3 | Quick-spin each sample to collect liquid. |
| | 1.4 | Take a **1 µL** aliquot from each sample and dilute with **9 µL** of **Elution buffer or water.** |
| | 1.5 | Measure DNA concentration with a Qubit fluorometer using the **1X dsDNA HS kit.** |
| | 1.6 | Measure DNA size distribution with a Femto Pulse system using the **gDNA 165 kb analysis kit.**<br>Or<br>Measure DNA size distribution with a **TapeStation system** using the Genomic DNA ScreenTape Analysis |
| | 1.7 | Aliquot or bring **1- 1.5 µg DNA** to a final volume of 30 µL per sample **(33- 50 ng/µL)** with nuclease-free water or Elution buffer.<br>**This protocol supports 1- 1.5 µg per sample. However, 1.3- 1.5 µg per sample is highly recommended for samples requiring the gDNA repair step.** |

## 2. gDNA repair

This step repairs input genomic DNA, which increases target coverage when using lower quality gDNA. For Nanobind extracted blood (whole blood or RBC lysis method) gDNA, this step can be skipped.

| ✔ | Step | Instructions |
|---|---|---|
| | 2.1 | Add the following components to a microcentrifuge tube. |

**gDNA repair master mix**

| ✔ | Reagent | Component | Volume per sample | Volume per 96-plex (overage included) |
|---|---|---|---|---|
| | | Nuclease-free water | 0.94 µL | 117 µL |
| | Purple | Repair buffer M96 | 3.5 µL | 454 µL |
| | Green | DNA repair mix M96 | 0.5 µL | 70 µL |
| | Blue | End repair mix M96 | 0.06 µL | 8 µL |
| | | **Total volume** | **5 µL** | **649 µL** |

| | |
|---|---|
| 2.2 | Pulse-vortex mix and quick-spin the gDNA repair master mix. |
| 2.3 | Add **5 µL** of the **gDNA repair master mix** to each gDNA sample from step 1.8 for a total reaction volume of **35 µL**. |
| 2.4 | Pipette-mix and quick-spin. |
| 2.5 | Run the DNA repair thermocycler program. Set the lid temperature to ≥65°C. |

| Step | Time | Temperature |
|---|---|---|
| 1 | 60 min | 37°C |
| 2 | 30 min | 52°C |
| 3 | Hold | 4°C |

### 1.2X SMRTbell bead cleanup

| | |
|---|---|
| 2.6 | Add **42 µL** of resuspended, room-temperature SMRTbell cleanup beads to each sample. |
| 2.7 | Pipette-mix the beads until evenly distributed. **Bead clumping may occur and is not a concern. Avoid over-pipetting as it may cause DNA/bead mixture to stick to the pipette tip.** |
| 2.8 | Incubate at **room temperature** for **10 minutes** to allow DNA to bind beads. |
| 2.9 | Place on a magnetic separation rack and allow beads to separate fully from the solution. |
| 2.10 | Slowly remove the cleared supernatant without disturbing the beads. Discard the supernatant. |
| 2.11 | Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into each well. After **30 seconds**, remove the 80% ethanol and discard. |
| 2.12 | Repeat the previous step. |
| 2.13 | Remove residual 80% ethanol and discard. |

| | |
|---|---|
| 2.14 | Remove the plate from the magnetic rack. **Immediately** add **26 µL** of Elution buffer to each well and fully resuspend the beads. |
| 2.15 | Incubate at **room temperature** for **5 minutes** to elute DNA. |
| 2.16 | Place the plate on a magnetic separation rack and allow beads to separate fully from the solution. |
| 2.17 | Slowly transfer **26 µL** cleared eluate without disturbing the beads to a new plate. Discard the old plate with beads. |
| 2.18 | (Optional) QC step: Take a 1 µL each sample and measure DNA concentration with a Qubit fluorometer using the 1X dsDNA HS kit.<br>Expect a step recovery of 70-90% per sample. |
| 2.19 | Proceed to the next step of the protocol. |

SAFE STOPPING POINT – Store at 4°C overnight or -20°C long term

## 3. Dephosphorylation

This step dephosphorylates the genomic DNA 5' termini, which prevents adapter ligation to non-targeted genomic DNA.

| ✔ | Step | Instructions |
|---|---|---|
| | 3.1 | Add the following components to a new microcentrifuge tube. |

**Dephosphorylation master mix**

| ✔ | Tube color | Component | Volume per sample | Volume per 96-plex (overage included) |
|---|---|---|---|---|
| | | Nuclease-free water | 0.9 µL | 110 µL |
| | Red | Cas9 buffer | 3 µL | 389 µL |
| | Blue | Phosphatase | 1.1 µL | 150 µL |
| | | **Total volume** | **5 µL** | **674 µL** |

| | |
|---|---|
| 3.2 | Pulse vortex-mix the **Dephosphorylation master mix** and quick-spin in a microcentrifuge to collect liquid. |
| 3.3 | Add **5 µL** of the **Dephosphorylation master mix** to each sample from step 2.17 for a total reaction volume of **30 µL**. |
| 3.4 | Pipette-mix each sample. |
| 3.5 | Run the **Dephosphorylation** thermocycler program. Set the lid temperature to ≥95°C. |

| Step | Time | Temperature |
|---|---|---|
| 1 | 50 min | 37°C |
| 2 | 5 min | 85°C |
| 3 | Hold | 4°C |

| | |
|---|---|
| 3.6 | Proceed to the next step of the protocol. |

# 4. Cas9 digestion and SMRTbell cleanup

This step enables digestion of double-stranded DNA at targeted regions mediated by Cas9-gRNA ribonucleoprotein complex formation.

| ✔ | Step | Instructions |
|---|---|---|
| | 4.1 | Add the following components to a new microcentrifuge tube. |

**Cas9 digestion master mix**

| ✔ | Reagent | Component | Volume per sample | Volume per 96-plex (overage included) |
|---|---|---|---|---|
| | | Nuclease-free water | 3.75 µL | 489 µL |
| | Red | Cas9 buffer | 0.5 µL | 65 µL |
| | Green | Cas9 Nuclease | 0.15 µL | 19 µL |
| | Variable | PureTarget panel* | 0.6 µL | 76 µL |
| | | **Total volume** | **5 µL** | **649 µL** |

*Repeat expansion 2.0, Carrier, or Control panel

| | | |
|---|---|---|
| | 4.2 | Pulse vortex-mix **Cas9 digestion master mix** and quick-spin in a microcentrifuge to collect liquid. |
| | 4.3 | Add **5 µL** of the Cas9 digestion master mix to each sample for a total reaction volume of **35 µL**. |
| | 4.4 | Pipette-mix each sample |
| | 4.5 | Run the **Cas9 digestion** thermocycler program. Set the lid temperature to ≥65°C. |

| Step | Time | Temperature |
|---|---|---|
| 1 | 60 min | 37°C |
| 2 | Hold | 4°C |

## 1.2X SMRTbell bead cleanup

| | | |
|---|---|---|
| | 4.6 | Add **42 µL** of resuspended, room-temperature SMRTbell cleanup beads to each sample. |
| | 4.7 | Pipette-mix the beads slowly until evenly distributed. **Bead clumping may occur and is not a concern. Avoid over-pipetting as it may cause DNA/bead mixture to stick to the pipette tip.** |
| | 4.8 | Incubate at **room temperature** for **10 minutes** to allow DNA to bind beads. |
| | 4.9 | Place plate on a magnetic separation rack until beads separate fully from the solution. |
| | 4.10 | Slowly remove the cleared supernatant without disturbing the beads. Discard the supernatant. |
| | 4.11 | Slowly dispense **200 µL** of **freshly prepared 80% ethanol** into each tube. After **30 seconds**, remove the 80% ethanol and discard. |
| | 4.12 | Remove residual 80% ethanol and discard. |

| | |
|---|---|
| 4.13 | Remove plate from the magnetic rack. **Immediately** add **23 µL** of Elution buffer to each well and resuspend the beads. |
| 4.14 | Incubate at **room temperature** for **5 minutes** to elute DNA. |
| 4.15 | Place plate on a magnetic separation rack until beads separate fully from the solution. |
| 4.16 | Slowly transfer **23 µL** cleared eluate without disturbing the beads to a **new plate**. Discard old plate with beads. |
| 4.17 | (Optional) QC step: Take a 1 µL aliquot from each sample and dilute with 9 µL of Elution buffer or water. Measure DNA concentration with a Qubit fluorometer using the 1X dsDNA HS kit.<br><br>Expect a step recovery of 50-80% per sample. |

SAFE STOPPING POINT – Store at 4°C overnight or -20°C long term

## 5. dA-tail

This step enables A-tailing of DNA 3' ends after Cas9-gRNA digestion at targeted regions.

| ✔ | Step | Instructions |
|---|---|---|
| | 5.1 | Add the following components to a new microcentrifuge tube. |

**dA-tailing master mix**

| ✔ | Reagent | Component | Volume per sample | Volume per 96-plex (overage included) |
|---|---|---|---|---|
| | | Nuclease-free water | 1.125 µL | 152 µL |
| | Orange | dA tail buffer | 2.6 µL | 350 µL |
| | Yellow | dATP (100 mM) | 0.025 µL | 4 µL |
| | Violet | *Taq* DNA polymerase | 0.25 µL | 34 µL |
| | | **Total volume** | **4 µL** | **540 µL** |

| | |
|---|---|
| 5.2 | Pulse vortex-mix the **dA-tailing master mix** and quick-spin in a microcentrifuge to collect liquid. |
| 5.3 | Add **4 µL** of the **dA-tailing master mix** to **22 µL** of each sample for a total reaction volume of **26 µL**. |
| 5.4 | Pipette mix each sample. |
| 5.5 | Run the **dA-tail** thermocycler program. Set the lid temperature to ≥95°C. |

| Step | Time | Temperature |
|---|---|---|
| 1 | 1 min | 37°C |
| 2 | 5 min | 72°C |
| 3 | Hold | 4°C |

| | |
|---|---|
| 5.6 | Proceed to the next step of the protocol. |

# 6. Adapter ligation and SMRTbell cleanup

This step ligates the indexed SMRTbell adapter to the ends of each targeted DNA fragment.

| ✔ | Step | Instructions |
|---|---|---|
| | 6.1 | Add **4 µL SMRTbell adapter index** to each sample from Section 5 and pipette mix. |
| | 6.2 | Add the following components to a new microcentrifuge tube.<br>**Note**: Aspirate and dispense viscous ligation mix slowly to reduce liquid sticking to the exterior and interior wall of the pipette tip. |

**Ligation master mix**

| ✔ | Reagent | Component | Volume per sample | Volume Per 96-plex (overage included) |
|---|---|---|---|---|
| | | Nuclease-free water | 0.5 | 62 |
| | Yellow | Ligation mix M96 | 9.0 µL | 1124 µL (pipette 562 µL twice)* |
| | Red | Ligation enhancer M96 | 0.5 µL | 68 µL |
| | | **Total volume** | **10 µL** | **1260 µL** |

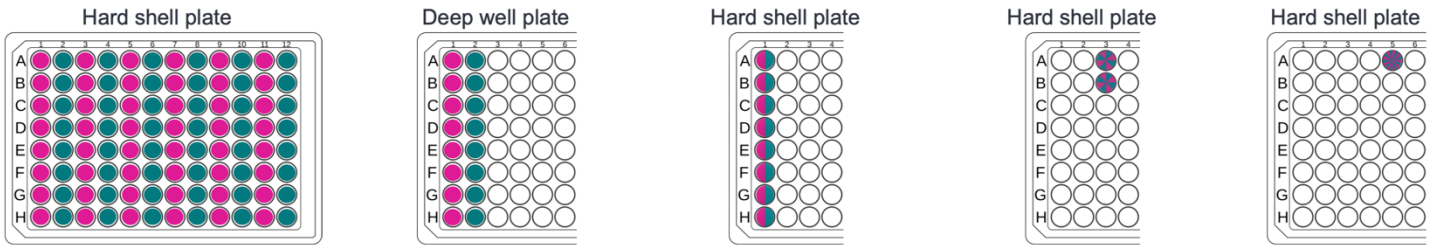
*Pipetting Ligation mix in a smaller volume twice minimizes liquid retention in the tip.

| | | |
|---|---|---|
| 6.3 | Pulse vortex-mix the **Ligation master mix** and quick-spin in a microcentrifuge to collect liquid. |
| 6.4 | Add **10 µL** of **Ligation master mix** to each sample from previous step for a total volume of **40 µL.** |
| 6.5 | Pipette-mix each sample |
| 6.6 | Run the Adapter ligation thermocycler program. Set the lid temperature to “OFF” or ≥30°C. |

| Step | Time | Temperature |
|---|---|---|
| 1 | 30 min | 20°C |
| 2 | Hold | 4°C |

## Sample pooling, cleanup with 1X SMRTbell cleanup beads

In the following steps, the 96 ligation reactions are pooled after the addition of SMRTbell beads into 16 pools for the post-ligation clean-up. Further pooling happens at each subsequent step.




| | |
|---|---|
| 6.7 | Add **40 µL** (1X) of resuspended, room-temperature SMRTbell cleanup beads to each sample. |

| | |
|---|---|
| 6.8 | Pipette-mix until evenly distributed. |
| 6.9 | Incubate at **room temperature** for **2 minutes** to allow DNA to bind beads. |
| 6.10 | Consolidate the ligated sample/SMRTbell cleanup bead mixture from step 6.9 into **16 wells** of a new deep well plate for a final sample volume of **480 µL per well.** |
| 6.11 | Incubate at **room temperature** for **10 minutes** to allow DNA to bind beads. |
| 6.12 | Place the plate on a magnetic separation rack for 5 minutes until beads separate fully from the solution. |
| 6.13 | Slowly remove the cleared supernatant without disturbing the beads. Discard the supernatant. |
| 6.14 | Slowly dispense **500 µL** of **freshly prepared 80% ethanol** into each tube. After **30 seconds**, remove the 80% ethanol and discard. |
| 6.15 | Remove residual 80% ethanol and discard. |
| 6.16 | Remove from the magnetic rack. **Immediately** add **32µL** of **Elution buffer** to each sample and resuspend the beads. |
| 6.17 | Incubate at **room temperature** for **5 minutes** to elute DNA. |
| 6.18 | Place in a magnetic separation rack until beads separate fully from the solution. |
| 6.30 | Slowly pool **31 µL** of the cleared eluate from the 2 columns into **8 new wells** on a new hard-shell plate (A1-H1), for a **total volume of 62 µL per well**. |
| 6.31 | Optional QC step: Take a **1 µL** aliquot from each pooled sample and **dilute with 9 µL of Elution buffer or water.** Measure DNA concentration with a Qubit fluorometer using the **1X dsDNA HS kit.** Expect a step recovery of 70-90% per sample. |

SAFE STOPPING POINT – Store at 4°C overnight or -20°C long term

## 7. Nuclease treatments 1 & 2

This step removes DNA fragments that have not formed SMRTbell libraries.

| ✔ | Step | Instructions |
|---|---|---|
| | 7.1 | Add the following components to a new microcentrifuge tube. |

**Nuclease master mix**

| ✔ Reagent | Component | Volume per sample | Volume per 96-plex (10 reactions, overage included) |
|---|---|---|---|
| Orange | dA tail buffer | 7 µL | 87 µL |
| Light Green | Nuclease mix M96 | 4.5 µL | 56 µL |
| White | PureTarget Nuclease* | 1.5 µL | 19 µL |
| | **Total volume** | **13 µL** | **162 µL** |

*Located in the PureTarget kit 2.0

**Important: this master mix will be used for BOTH Nuclease treatment 1 AND Nuclease treatment 2.**

7.2 Pulse-vortex mix the **Nuclease master mix** and quick-spin in a microcentrifuge to collect liquid.

## Nuclease treatment 1

7.3 Add **13 µL** of **Nuclease master mix** to 62 µL of each sample for a total volume of **75 µL**.
**Important: Save the remaining Nuclease master mix and place on ice at all times.**

7.4 Pipette-mix each sample.

7.5 Run the **Nuclease treatment 1** thermocycler program. Set the lid temperature to ≥65°C.

| Step | Time | Temperature |
|---|---|---|
| 1 | 30 min | 37°C |
| 2 | Hold | 4°C |

## 1.2X SMRTbell cleanup

7.6 Add **90 µL** of SMRTbell cleanup beads to each nuclease treated sample (8 total) for a total volume of 165 µL per well. Pipette-mix until the beads are evenly distributed. Pipette-mix full volume.

7.7 Incubate at **room temperature** for **10 minutes** to allow DNA to bind beads.

7.8 Place the tube in a magnetic separation rack until beads separate fully from the solution.

7.9 Slowly remove the cleared supernatant without disturbing the beads. Discard the supernatant.

7.10 Slowly dispense **200 µL** of **freshly prepared 80% ethanol** into each tube. After **30 seconds**, remove the 80% ethanol and discard.

7.11 Remove residual 80% ethanol and discard.

7.12 **Consolidate 8 samples down to 4.** Remove the plate from the magnetic rack. Immediately add **32 µL** of Elution buffer to the **2nd set of 4 wells (E1-H1)** and resuspend the beads by pipette mixing. Aspirate the entire volume (set at 35 µL) in each of the 4 wells and transfer to the **1st set of 4 wells (A1-D1)** and resuspend the beads by pipetting mixing.

7.13 Incubate at **room temperature** for **5 minutes** to elute DNA.

7.14 Place on a magnetic separation rack until beads separate fully from the solution.

7.15 **Consolidate 4 samples down to 2.** Slowly pool **31 µL** of cleared eluate from 4 wells (A1-D1) into 2 wells (A3, B3) on the same plate for **a final volume of 62 µL per well**.

7.16 (Optional) QC step: Take a **1 µL** aliquot from the pooled sample and measure DNA concentration with a Qubit fluorometer using the 1X dsDNA HS kit.
**Note: Expect a step recovery of 0.1-2%.**

## Nuclease treatment 2

7.17 Add **13 µL** of **Nuclease master mix** to **62 µL of the sample in wells (A3, B3)** that has gone through nuclease treatment 1 for a total volume of **75 µL.**

7.18 Pipette-mix the sample.

7.19 Run the **Nuclease treatment 2** thermocycler program. Set the lid temperature to ≥65°C.

| Step | Time | Temperature |
|---|---|---|
| 1 | 60 min | 37°C |
| 2 | Hold | 4°C |

## 1.2X SMRTbell bead cleanup

| | |
|---|---|
| 7.20 | Add **90 µL** of SMRTbell cleanup beads to each nuclease treated sample. |
| 7.21 | Pipette-mix until the beads are evenly distributed. |
| 7.22 | Incubate at **room temperature** for **10 minutes** to allow DNA to bind beads. |
| 7.23 | Place the plate on a magnetic separation rack until beads separate fully from the solution. |
| 7.24 | Slowly remove the cleared supernatant without disturbing the beads. Discard the supernatant. |
| 7.25 | Slowly dispense **200 µL** of **freshly prepared 80% ethanol** into each tube. After **30 seconds**, remove the 80% ethanol and discard. |
| 7.26 | Repeat the previous step. |
| 7.27 | Remove residual 80% ethanol and discard. |
| 7.28 | Remove from the magnetic rack. **Immediately** add **29 µL** of **Elution buffer** to the well and pipette mix until fully resuspended. |
| 7.29 | Incubate at **room temperature** for **5 minutes** to elute DNA. |
| 7.30 | Place the tube in a magnetic separation rack until beads separate fully from the solution. |
| 7.31 | **Consolidate 2 samples down to 1**. Pool **28 µL** eluate from each well without disturbing the beads to a new well (**A5**) for a final volume of **a final volume of 56 µL in a 1.5 ml Eppendorf**. |
| 7.32 | QC step: Take a **1 µL** aliquot from each sample and measure DNA concentration with a Qubit fluorometer using the **1X dsDNA HS kit.**<br>**Note**: Expect an overall recovery of 0.01-0.1% relative to the starting gDNA total mass. For example, starting with 1.5 µg input per sample, the final mass of a pooled 96-plex library recovered at this step is expected to be in the range of 15 ng–150 ng. A higher than the expected library yield may indicate inefficient nuclease digestion, or sample incompatibility with the protocol. |
| 7.33 | Proceed to the next step of the protocol. |

## SAFE STOPPING POINT – Store at -20°C

# 8. PureTarget cleanup of the SMRTbell library

This step will prepare the library ready for sequencing.

Perform one cleanup per final pool of 96 samples.

All reagents in PureTarget cleanup kit should be stored at room-temperature; do not chill or freeze any reagent.

Vortex buffers in PureTarget cleanup kit prior to use.

Prior to the 1st use of the PureTarget cleanup beads kit, add 15mL of 200 Proof ethanol to PureTarget cleanup wash buffer and mix well.

<table>
<tr><th>✔</th><th>Step</th><th>Instructions</th></tr>
<tr><td></td><td>8.1</td><td>Add 28 µL PureTarget cleanup buffer 1 (🔴) to 55 µL SMRTbell library from step 7.30 and pipette-mix.</td></tr>
<tr><td></td><td>8.2</td><td>Add the following components to a new microcentrifuge tube.
<table>
<tr><th colspan="4">PureTarget cleanup buffer 2+3 mix</th></tr>
<tr><th>✔ Reagent</th><th>Component</th><th>Volume per sample</th><th>Volume (overage included)</th></tr>
<tr><td>Blue</td><td>PureTarget cleanup buffer 2</td><td>14 µL</td><td>19 µL</td></tr>
<tr><td>Green</td><td>PureTarget cleanup buffer 3</td><td>14 µL</td><td>19 µL</td></tr>
<tr><td></td><td>Total volume</td><td>28 µL</td><td>38 µL</td></tr>
</table>
Pulse vortex to mix and quick-spin.<br><br>Important: PureTarget cleanup buffer 2 and buffer 3 must be pre-mixed prior to adding to sample. If added individually to sample, yield may be low.</td></tr>
<tr><td></td><td>8.3</td><td>Add 28 µL of pre-mixed PureTarget cleanup buffer 2+3 mix to the mixture from step 8.1 and pipette mix. The solution will become opaque and cloudy after homogenous mixing. Incubate at room temperature for 1 minute.</td></tr>
<tr><td></td><td>8.4</td><td>Centrifuge at 13,000 rcf for 2 minutes at room temperature.</td></tr>
<tr><td></td><td>8.5</td><td>Taking care to avoid the pellet, transfer 95 µL of the clear supernatant to a new 1.5 mL LoBind tube. Discard the tube with pellet.</td></tr>
</table>

| | |
|---|---|
| 8.6 | Add the following components to a new microcentrifuge tube.<br>**PureTarget cleanup bead binding master mix**<br>✔ **Reagent** / **Component** / **Volume per sample** / **Volume** (overage included)<br>Clear — PureTarget cleanup beads — 5 µL — 7 µL<br>White — PureTarget cleanup binding buffer — 100 µL — 140 µL<br>**Total volume** — **105 µL** — **147 µL**<br>Pulse vortex to mix and quick-spin. |
| 8.7 | Add 105 µL of fully resuspended PureTarget cleanup bead binding master mix to **95 µL** of the sample (step 8.5). |
| 8.8 | Pipette-mix 10 times until evenly distributed. Incubate at room temperature for 10 mins, with periodic pipette-mixing to keep beads in suspension. |
| 8.9 | Place in a magnetic separation rack until beads separate fully from the solution. |
| 8.10 | Slowly remove the cleared supernatant without disturbing the beads. Discard the supernatant. |
| 8.11 | Slowly dispense **150 µL** of **PureTarget cleanup wash buffer (with ethanol added)** into the tube. Remove the tube from the magnetic separation rack and gently resuspend the bead.<br>**Important: ensure that 15 mL of ethanol has been added to PureTarget Cleanup Wash Buffer prior to first use.** |
| 8.12 | Place the tube in a magnetic separation rack until beads separate fully from the solution. |
| 8.13 | Slowly remove the cleared supernatant without disturbing the beads. Discard the supernatant. |
| 8.14 | Slowly dispense **200 µL** of **PureTarget cleanup wash buffer (with ethanol added)** into the well without disturbing the bead pellet. After **30 seconds**, remove the **PureTarget cleanup wash buffer** and discard. |
| 8.15 | Slowly dispense **200 µL** of **fresh 80% ethanol** into the well without disturbing the bead pellet. After **30 seconds**, remove the 80% ethanol and discard. |
| 8.16 | It is critical to remove residual 80% ethanol for efficient elution:<br>• Remove the tube from the magnetic separation rack.<br>• Quick-spin the tube in a microcentrifuge.<br>• Place the tube back in a magnetic separation rack until beads separate fully from the solution.<br>• Remove residual 80% ethanol and discard.<br>• Air dry the bead pellet for 2 min. |
| 8.17 | Remove the tube from the magnetic rack. Add **26 µL** of **Elution buffer** and resuspend the beads. |
| 8.18 | Incubate at **room temperature** for **15 minutes** to elute DNA, with gentle vortexing every 5 mins to fully resuspend the beads. |
| 8.19 | Place the tube in a magnetic separation rack until beads separate fully from the solution. |

| | |
|---|---|
| 8.20 | Slowly transfer **26 µL** cleared supernatant without disturbing the beads to a new **1.5 mL DNA LoBind tube.** |
| | QC step: Take a **1 µL** aliquot from the sample and measure DNA concentration with a Qubit fluorometer using the **1X dsDNA HS kit.** |
| 8.21 | **Expect an overall recovery of 0.005-0.05% relative to starting gDNA total mass, and a step recovery of 40-60%.** For example, starting with 1.5 µg input per sample, the final mass of a pooled 96-plex library recovered at this step is expected to be in the range of 7.2 ng–72 ng. |

SAFE STOPPING POINT – Store at 4°C overnight or -20°C long term

## 9. Annealing, binding, and cleanup (ABC)

This step is for preparing the SMRTbell library (25 µL) for sequencing on Revio +SPRQ.

| ✔ | Step | Instructions |
|---|---|---|
| | 9.1 | Prepare the appropriate volume of master mix with 10% overage using the per reaction volumes listed below. |

**Annealing mix**

| ✔ | Tube | Component | Volume | Volume (overage included) |
|---|---|---|---|---|
| | Light blue | Annealing buffer | 12.5 µL | 15 µL |
| | Light green | Standard sequencing primer | 12.5 µL | 15 µL |
| | | **Total volume** | **25 µL** | **30 µL** |

| | |
|---|---|
| 9.2 | Pipette-mix the **Annealing mix** and quick spin to collect liquid. |
| 9.3 | Add **25 µL** of the **Annealing mix** to each library for a total volume of **50 µL.** |
| 9.4 | Pipette-mix each sample and quick spin to collect liquid. |
| 9.5 | Incubate at room temperature for **15 minutes**. |
| 9.6 | During primer incubation, prepare the polymerase dilution (see below) and store on ice. |

9.7 To prepare the polymerase, add the following components to a new microcentrifuge tube on ice. Adjust component volumes for the number of samples being prepared, plus 10% overage.

| Polymerase Dilution | | | | |
|---|---|---|---|---|
| ✔ | Tube | Component | Volume | Volume (overage included) |
| | Yellow | Polymerase buffer | 44 µL | 53 µL |
| | Purple | Sequencing polymerase | 6 µL | 7 µL |
| | | Total volume | 50 µL | 60 µL |

9.8 Pipette mix the **polymerase dilution** and quick-spin to collect liquid.

9.9 Add **50 µL of polymerase dilution** to primer annealed sample for a total volume of **100 µL**.

9.10 Pipette-mix each sample and quick-spin to collect liquid.

9.11 Incubate at **room temperature for 15 minutes.**

9.12 Proceed immediately to the next step of the protocol to remove excess polymerase.

## 1X SMRTbell bead cleanup

9.13 Add **100 µL** of resuspended, room-temperature SMRTbell cleanup beads to each sample

9.14 Pipette-mix the beads until evenly distributed and quick-spin if necessary to collect all liquid from the sides of the tube.

9.15 Incubate at **room temperature** for **10 minutes** to allow DNA to bind beads

9.16 Place sample on an appropriate magnet and allow beads to separate fully from the solution

9.17 Slowly remove the cleared supernatant without disturbing the beads. Discard the supernatant. **DO NOT USE EtOH.** Proceed immediately to the elution. It is important not to let the beads dry out.

9.18 Remove sample from the magnet and **immediately** add **Loading buffer** to each tube and resuspend the beads by pipette mixing.

| | Revio SPRQ Polymerase Kit |
|---|---|
| Loading buffer | 23 µL |

9.19 Quick-spin the samples to collect any liquid from the sides of the tube.

9.20 Incubate at **room temperature** for **15 minutes** to elute DNA

9.21 Place sample on magnet and allow beads to separate fully from the solution.

9.22 Slowly transfer 23 µL of cleared eluate without disturbing the beads to a **new tube protected from light**. Discard the old tube with beads. The sample is now ready for sequencing (see Section 10).

**PROTOCOL COMPLETE**

**Important:** Polymerase-bound libraries can be stored at 4°C for up to 1 week, or at -20°C for up to 6 months prior to sequencing. Note that the Loading buffer is light sensitive.

# 10. Preparing sequencing internal control and sample for sequencing

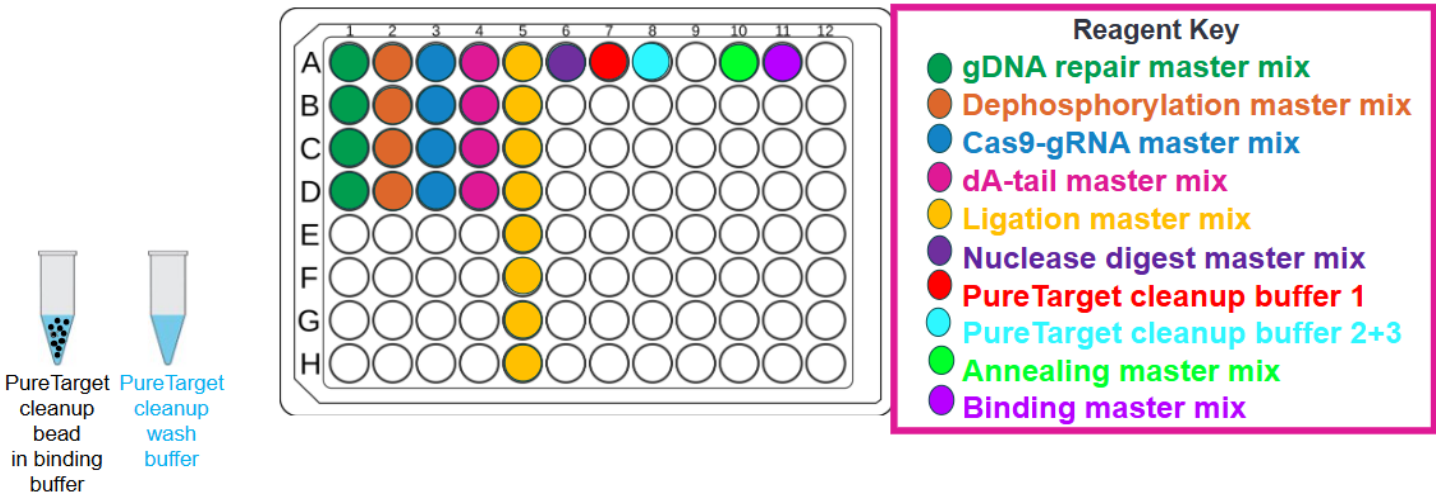
| Step | Instructions |
|---|---|
| 10.1 | Dilute the sequencing control using the volumes listed below.<br><br>**Dilution 1**<br>Component: Dilution buffer — Volume: 19 µL<br>Component: Sequencing control — Volume: 1 µL<br>Total volume: 20 µL<br><br>Pipette mix and quick-spin in a microcentrifuge to collect liquid. |
| 10.2 | Dilute Dilution 1 using the volumes listed below.<br>**Dilution 2**<br>Component: Dilution buffer — Volume: 19 µL<br>Component: Dilution 1 — Volume: 1 µL<br>Total volume: 20 µL<br><br>Pipette mix and quick-spin in a microcentrifuge to collect liquid. |
| 10.3 | Dilute Dilution 2 using the volumes listed below.<br>**Dilution 3**<br>Component: Dilution buffer — Volume: 19 µL<br>Component: Dilution 2 — Volume: 1 µL<br>Total volume: 20 µL<br><br>Pipette mix and quick-spin in a microcentrifuge to collect liquid. |
| 10.4 | Prepare sample for sequencing. Add the following components to the Revio SPRQ sequencing reagent plate.<br><br>**Sequencing reagent plate sample**<br>Component: Sample from step 10.22 — Volume: 23 µL<br>Component: Diluted internal sequencing control (Dilution 3) — Volume: 1 µL<br>Total volume: 24 µL |

| | |
|---|---|
| 10.5 | Load exactly 23 µL of sample prepared in 10.4 per sequencing well or store at 4°C for up to 24 hours before use. For long-term storage, store the sample at -20°C. |
| 10.6 | For Run Design, ensure the following specifications are selected:<br>Use Adaptive Loading: *Off*<br>Application: *PureTarget repeat expansion/carrier/custom*<br>Analysis Workflow:<br>• PureTarget repeat expansion: *PureTarget repeat expansion*<br>• PureTarget carrier: *Target Enrichment*<br>• PureTarget custom: *Target Enrichment*<br>Target and repeat definitions:<br>• PureTarget repeat expansion: *PureTarget repeat expansion panel 2.0*<br>• PureTarget carrier: *PureTarget carrier panel 1.0*<br>• PureTarget custom: user provided bed file. Please reference the SMRT Link user guide for bed file format specifications<br>If setting up Target Enrichment analysis for PureTarget custom or in SMRT Analysis after sequencing, the user must select the following specifications under "Advanced":<br>• Include Fail Reads: On<br>• Padding around regions (bp): 0 |

# Appendix

## Master mix preparation and plating quick guide

Reagent master mix plate layout –plate positions of master mixes for gDNA repair, dephosphorylation, Cas9-gRNA digestion, dA tailing, adapter ligation, nuclease digestion, PureTarget cleanup, and ABC.





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## Quick guide - reagent master mix preparation for automation:

- Quick pulse vortex master mixes 5 times with an end-to-end flip in between, and spin down
- When pipetting the master mix into the well, only dispense to the 1st stop
- Ligation master mix is viscous, pipette slowly to minimize liquid retention in the tip
- PureTarget cleanup bead is heavy and requires thorough resuspension by vortexing
- Master mix overage calculation includes minor adjustments to increase enzyme utilization

| Day1 | | | |
|---|---|---|---|
| DNA repair (5 µL/rxn) | 1X | 96X+overage | Plate 151 µL each well to A1-D1 |
| Nuclease free water | 0.94 | 117 | |
| Repair buffer | 3.5 | 454 | |
| End repair mix | 0.06 | 8 | |
| DNA repair mix | 0.5 | 70 | |

| Dephosphorylation (5 µL/rxn) | 1X | 96X+overage | Plate 151 µL each well to A2-D2 |
|---|---|---|---|
| Nuclease free water | 0.9 | 110 | |
| cas9 buffer | 3 | 389 | |
| Phosphatase | 1.1 | 150 | |

| Cas9-gRNA (5 µL/rxn) | 1X | 96X+overage | Plate 151 µL each well to A3-D3 |
|---|---|---|---|
| Nuclease free water | 3.75 | 489 | |
| cas9 buffer | 0.5 | 65 | |
| cas9 nuclease (20uM) | 0.15 | 19 | |
| gRNA mix (5uM) | 0.6 | 76 | |

| Day2 | | | |
|---|---|---|---|
| dA tailing (4 µL/rxn) | 1X | 96X+overage | Plate 126 µL each well to A4-D4 |
| Nuclease free water | 1.125 | 152 | |
| dA tail buffer | 2.6 | 350 | |
| dATP (100mM) | 0.025 | 4 | |
| Taq DNA pol | 0.25 | 34 | |

| Adapter ligation (10 µL/rxn) | 1X | 96X+overage | Plate 151 µL each well to A5-H5 |
|---|---|---|---|
| Nuclease free water | 0.5 | 62 | |
| Ligation mix* | 9.0 | 565 µL twice | |
| Ligation enhancer | 0.5 | 68 | |

| Nuclease digestion (13 µL/rxn) | 1X | 10X+overage | Plate 151 µL to A6 |
|---|---|---|---|
| dA tail buffer | 7 | 87 | |
| Nuclease mix | 4.5 | 56 | |
| PureTarget nuclease | 1.5 | 19 | |

| Master mix plate will be moved to be at room temperature after 2nd nuclease incubation | | | |
|---|---|---|---|
| PureTarget cleanup | 1X | 1X+overage | Plate 38 µL to A7 |
| PureTarget cleanup buffer 1 | 28 | 38 | |

| PureTarget cleanup buffer 2+3 | 1X | 1X+overage | Plate 38 µL to A8 |
|---|---|---|---|
| PureTarget cleanup buffer 2 | 14 | 19 | |
| PureTarget cleanup buffer 3 | 14 | 19 | |

| PureTarget cleanup bead binding master mix (105 µL/rxn) | 1X | 1X+overage | |
|---|---|---|---|
| PureTarget cleanup bead | 5 | 7 | Add to a 2 mL tube and load 147 µL |
| PureTarget cleanup binding buffer | 100 | 140 | |
| PureTarget cleanup wash buffer (EtOH added) | 400 | 600 | Add to a 2 mL tube and load 600 µL |

| Annealing master mix (25 µL/rxn) | 1X | 1X+overage | |
|---|---|---|---|
| Annealing buffer | 12.5 | 15 | Plate 30 µL to A10 |
| Standard sequencing primer | 12.5 | 15 | |

| Binding master mix (50 µL/rxn) | 1X | 1X+overage | |
|---|---|---|---|
| Polymerase dilution buffer | 44 | 53 | Plate 30 µL to A11 |
| Sequencing polymerase | 6 | 7 | |
| Loading buffer | 23 | 74 | Add to a 2 mL amber tube and load 74 µL |

| Revision history (description) | Version | Date |
|---|---|---|
| Initial release | 01 | September 2025 |
| Minor updates and updated DIN to >8 | 02 | October 2025 |
| Updated library complex storage condition | 03 | November 2025 |