Takara Bio USA, Inc.

SMART-Seq® v4 Reagent Kit for the SMARTer™ Apollo™ System User Manual

Cat. Nos. 640170, 640171 & 640078
(011018)

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I. Introduction

The SMARTer Apollo Automation Platform

The **SMARTer Apollo** library prep system (Cat. No. 640078) provides automated liquid handling capabilities that streamline repetitive, labor-intensive procedures for an array of applications, including NGS library preparation. By automating critical steps such as reaction setup and cleanup, as well as sample purification and enrichment, the SMARTer Apollo system reduces hands-on time and ensures greater precision relative to manual workflows, dramatically expanding the scale and scope of experiments that can be performed. Implementation of validated protocols and chemistries on the SMARTer Apollo system reliably yields consistent libraries and reproducible data for diverse NGS applications such as DNA-seq, RNA-seq, and ChIP-seq.

The SMARTer Apollo system can also run flexible batch sizes accommodating low- and high-throughput experiments, without wasting reagents or time. While batch sizes smaller than 48 samples can be processed on the SMARTer Apollo system, performing fewer than 48 reactions using the workflow described in this user manual may not provide time savings over manual sample processing. For information on the deck layout when using between 12 and 96 samples, please see Appendix A.

With the workflow described in this user manual, synthesis and purification of cDNA from any number of samples can be completed in approximately 9–10 hours, prior to validation (Figure 1).

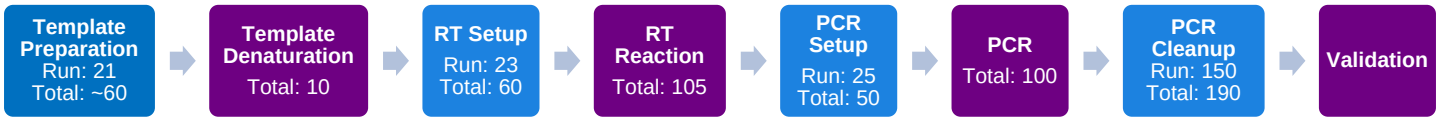


Figure 1. Workflow for cDNA preparation with the SMARTer Apollo system and the SMART-Seq v4 Reagent Kit for the SMARTer Apollo System. Blue and purple boxes indicate subprotocols performed on and off the SMARTer Apollo system, respectively. Run = the run time in minutes on the SMARTer Apollo system, if applicable. Total = total time in minutes spent, including thawing of reagents, reagent and equipment setup, heating and cooling of thermal blocks, incubation of reactions, and automated liquid-handling processes, if applicable. Run time is independent of sample number. Validation time varies depending on the method used.

cDNA Synthesis Using Template-Switching Technology

The **SMART-Seq v4 Reagent Kit for the SMARTer Apollo System** (Cat. No. 640170) is designed to generate high-quality, full-length cDNA directly from 1–1,000 cells or 10 pg–10 ng of total RNA. The protocol and corresponding SMARTer Apollo script described in this user manual have been specifically validated using a 10-pg input of total RNA at an input volume of 7 µl, followed by sequencing library construction from the resulting cDNA with the Nextera® XT Library Preparation Kit. While the 7-µl input volume is an absolute requirement for use of this protocol on the SMARTer Apollo system, this automated protocol performs comparably to the manual SMART-Seq v4 protocol across the same recommended input ranges of total RNA or cells. cDNA generated using the SMART-Seq v4 Reagent Kit for the SMARTer Apollo System with the protocol and script described here can also be processed with Ion Torrent and other Illumina® platform-specific library preparation kits. Final sequencing libraries produced with these kits are compatible with Ion Personal Genome Machine (PGM), Ion Proton, Illumina HiSeq®, Illumina MiSeq®, Illumina MiniSeq™, and Illumina NextSeq® platforms.

The SMART-Seq v4 Reagent Kit for the SMARTer Apollo System incorporates our proprietary SMART® (**S**witching **M**echanism **a**t 5’ End of **R**NA **T**emplate) technology. This technology relies on the template-switching activity of MMLV-derived reverse transcriptases to enrich for full-length cDNAs and to add defined PCR adapters directly to both ends of the first-strand cDNAs (Chenchik et al. 1998). This ensures that final cDNA libraries contain the 5’ end of the mRNA and maintain a true representation of the original mRNA transcripts; these factors are critical for transcriptome sequencing and gene expression analysis. SMART technology offers

unparalleled sensitivity and unbiased amplification of cDNA transcripts, and it allows direct cDNA synthesis from intact cells.

Adaptations to SMART technology for next-generation sequencing (NGS) were incorporated into the first-generation of our kit for ultra-low input mRNA-seq (the SMARTer® Ultra® Low RNA Kit for Illumina Sequencing) and published as the SMART-Seq method (Ramsköld et al. 2012). Improvements were included in subsequent generations of SMARTer Ultra Low kits, and the SMART-Seq method was updated to SMART-Seq2 (Picelli et al. 2013). The SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing improves upon the SMART-Seq2 method by incorporating the novel use of locked nucleic acid (LNA) technology into an optimized template-switching oligo, and other advancements developed by our scientists. The enhancements in this kit result in single-cell mRNA-seq libraries that clearly outperform previously published protocols (including SMART-Seq2) and existing kits. The SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing provides higher sensitivity and reproducibility—meaning more genes are identified from libraries produced with this kit—and significantly lower background than the SMART-Seq2 method. For more information on SMART technology, please visit takarabio.com. A schematic outline of SMART-Seq v4 cDNA synthesis technology is shown in Figure 2.

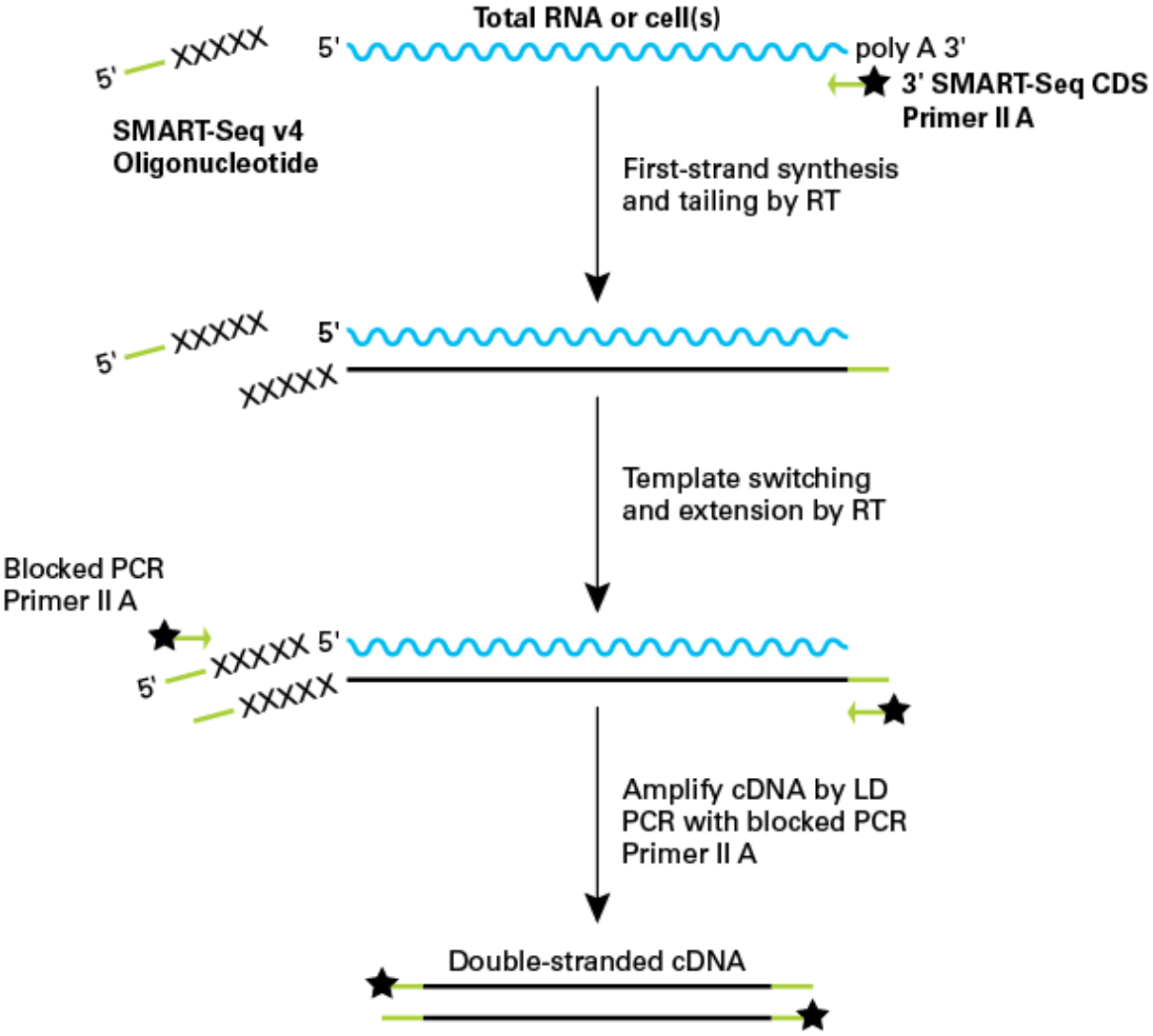


Figure 2. Flowchart of SMART cDNA synthesis. The SMART-Seq v4 Oligonucleotide, 3' SMART-Seq CDS Primer II A, and PCR Primer II A all contain a stretch of identical sequence. The black star indicates a chemical block on the 5' end of the oligonucleotide.

Complete Workflow for NGS Library Preparation with the SMARTer Apollo System

cDNA prepared using this SMART-Seq v4 reagent kit as described in this user manual can be processed into Illumina-ready sequencing libraries using a protocol and script we have developed for NGS library preparation on the SMARTer Apollo system with the Nextera XT DNA Library Preparation Kit (Illumina, Cat. No. FC-1311096). Together, these protocols comprise a complete workflow for automated, high-throughput NGS library preparation (Figure 3).

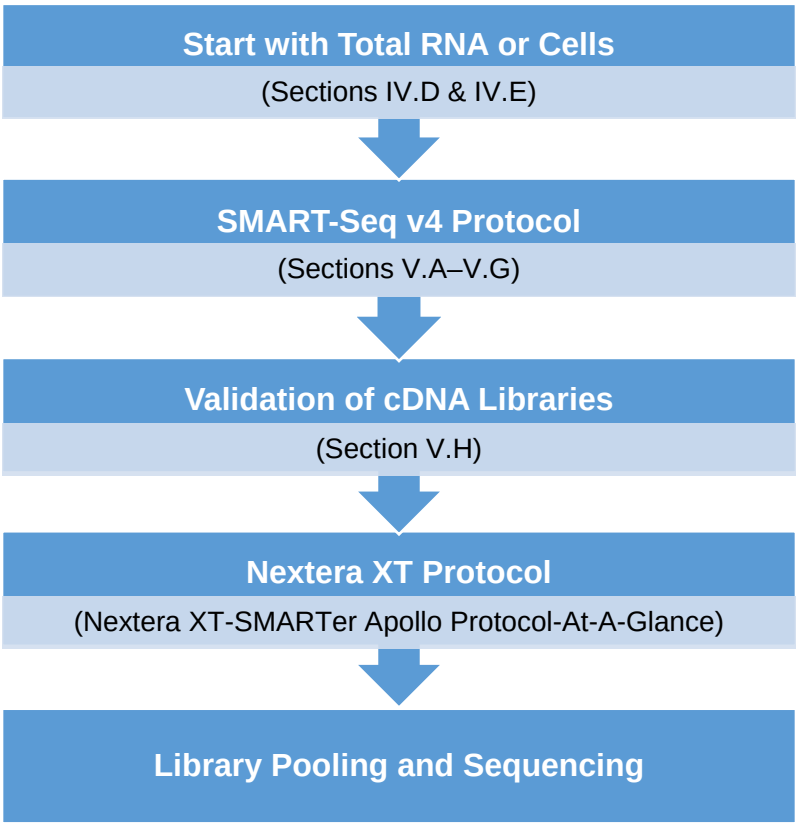


Figure 3. Overview of complete NGS library preparation workflow with the SMARTer Apollo system.

II. List of Components

SMART-Seq v4 Reagent Kit for the SMARTer Apollo System

These components have been specifically designed to work together and are optimized for this particular automation protocol. Please do not make any substitutions. The substitution of reagents in the kit and/or a modification of the protocol may lead to unexpected results.

SMART-Seq v4 Reagent Kit for the SMARTer Apollo System (Not sold separately. Storage conditions are listed below for Box 1 and Box 2.)	640170 (96 rxns)
Box 1 (Store at –70°C.)	
Control Total RNA (1 µg/µl)	5 µl
Box 2 (Store at –20°C. Once thawed, store Lysis 1 at 4°C and EB at room temperature. Continue storing all other reagents at –20°C.)	
Lysis 1	1.85 ml
Lysis 2	350 µl
RT 1	150 µl
RT 2	600 µl
RT 3	300 µl
PCR 1	135 µl
PCR 2	200 µl
PCR 3	4 x 1.25 ml
RI	240 µl
EB	2 x 6.8 ml
NFW	4 ml

SMARTer Apollo Consumables

The protocol and script described in this user manual have been validated using the following consumables. For equivalent Wafergen Bio-systems part numbers, please see Appendix A for a conversion table. **Please do not make any substitutions, or the attempted run may fail.**

Consumables	Source	Cat. No.	Quantity	Usage/96-rxn run	Usage/12-rxn run
SMARTer Apollo Filter Tips	Takara Bio	640084	Box of 960 tips	480 tips	60 tips
SMARTer Apollo 0.2 ml PCR 8-Tube Strips, Clear	Takara Bio	640082	Box of 125 strips	64 strips	9 strips
SMARTer Apollo Caps for 0.2 ml PCR 8-Tube Strips, Clear	Takara Bio	640086	Box of 125 strips	64 strips	9 strips
SMARTer Apollo Reservoirs	Takara Bio	640087	Box of 100 reservoirs	4 reservoirs	4 reservoirs
Hard-Shell Plate	Bio-Rad	HSP-9601	Box of 50 plates	3 plates	3 plates

SMARTer Apollo Subscripts

The following SMARTer Apollo subscripts are required for completion of the workflow described in this manual:

- `SetTemp10C_v1-1.scb`—chills temperature-controlled blocks to 10°C
- `SSv4_1_Lysis_96_v1-1.scb`—runs the liquid-handling procedure for Template Preparation (Section V.A)
- `SSv4_2_RT_96_v1-1.scb`—runs the liquid-handling procedure for RT Setup (Section V.C)
- `SSv4_3_PCR_96_v1-1.scb`—runs the liquid-handling procedure for PCR Setup (Section V.E)
- `SSv4_4_PCRCleanup_96_v1-1.scb`—runs the liquid-handling procedure for PCR Cleanup (Section V.G)

III. Additional Materials Required

The following reagents and materials are required but not supplied. They have been validated to work with this protocol. Please do not make any substitutions because you may not obtain the expected results:

- Single-channel pipette: 10 µl, 20 µl, 200 µl, and 1,000 µl
- Eight-channel pipette (recommended): 20 µl and 200 µl
- Filter pipette tips: 2 µl, 20 µl, 200 µl, and 1,000 µl
- Minicentrifuge for 1.5-ml tubes
- Minicentrifuge for 0.2-ml tubes or strips or low-speed benchtop centrifuge for 0.2-ml strips (recommended; e.g., Eppendorf 5804 R, Cat. No. 5805000017)

For cDNA synthesis, PCR Amplification, and Validation:

- One dedicated thermal cycler used only for first-strand cDNA synthesis (Section V.D)
- One dedicated thermal cycler used only for double-stranded cDNA amplification by PCR (Section V.F)
- High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626; Section V.H)

For SPRI (Solid Phase Reversible Immobilization) Bead Purifications (Section V.G):

- Agencourt AMPure XP PCR purification kit (60 ml, Beckman Coulter, Item No. A63881)

  NOTE: Agencourt AMPure XP beads need to come to room temperature before the container is opened. Therefore, we strongly recommend preparing 10-ml aliquots upon receipt, and then refrigerating the aliquots. Individual tubes can be removed for each experiment, allowing them to come to room temperature more quickly (≥30 minutes). This will also decrease the chance of bead contamination. Mix well to disperse the beads before adding them to your reactions. The beads are viscous, so pipette slowly.
- 100% ethanol (molecular biology grade)
- RNase decontamination solution (e.g., RNase Away, MilliporeSigma, Cat. No. 83931)
- Nuclease-free water (molecular biology grade)

IV. General Considerations

A. SMARTer Apollo System Best Practices

- Clean the work surfaces, including the retention plates, with 70% ethanol followed by RNase decontamination solution at least once a week.
- Discard any deformed plastics.
- Separate partial tube strips with scissors and remove resulting plastic overhangs.
- Spin down reagents before placing them on the deck to avoid air bubbles. **Bubbles at the bottoms of tubes must be removed to ensure accurate volume delivery.**
- Ensure plastics are properly seated on the deck surface with caps/lids removed. Be sure to push any tubes down completely and evenly prior to installing the metal retention plates.
- Ensure the instrument is on and ready for the run. If the instrument is off, turn it on. If it is already on, perform a power cycle by turning the instrument off, then on.

B. Requirements for Preventing Contamination

Before you set up the experiment, **make sure you have two physically separated work stations**:

- **A PCR Clean Work Station** for all pre-PCR experiments that require clean room conditions, e.g., first-strand cDNA synthesis (Sections V.A & V.C).

  NOTES:
  - The PCR Clean Work Station must be located in a clean room with positive air flow, as contamination may occur very easily. Once contamination occurs it can be difficult to remove.
  - Strictly obey clean room operation rules.

- **A second work station located in the general laboratory** where you will perform PCR (Section V.F) and measure cDNA concentration (Section V.H).

C. General Requirements

- **The success of your experiment depends on the quality of your input RNA. Prior to cDNA synthesis, please make sure that your RNA is intact and free of contaminants.**
- All lab supplies related to SMARTer cDNA synthesis need to be stored in a DNA-free, closed cabinet. Reagents for SMARTer cDNA synthesis need to be stored in a freezer/refrigerator that has not previously been used to store PCR amplicons.
- Briefly centrifuge all tubes containing reagent to collect contents at the bottom before opening each tube for the first time.
- Do not increase (or decrease) the amount or concentration of any reaction component. The amounts and concentrations have been carefully optimized for the amplification reagents and protocol.
- If you are using this protocol for the first time, we strongly recommend that you perform negative and positive control reactions to verify that kit components are working properly.
- The scripts in these protocols are designed to accommodate reactions in sets of 12. Therefore, regardless of sample number, **master mixes must be prepared with total volumes in multiples of 12.** In each subprotocol, we have provided master mix recipes for 96 samples and 12 samples. To calculate the total master mix volume needed for sample numbers other than 96 and 12, round up to the nearest multiple of 12. For example, if using between 13 and 24 reactions, multiply the 12-reaction master mix values by 2; if 25–36 reactions, multiply by 3; if 37–48, by 4; and so forth.

- When loading consumables such as filter tips, load the same number of tips as the number of samples. It is not necessary to load the consumables in multiples of 12; however, make sure to set up the consumables in the same positions as your samples. See Figure 5 as an example.
- To keep track of your samples, it is important to load the samples onto the deck "from the bottom up;" for example, if you have 16 samples, load each column from Row 1 to Row 12, then start again at Row 1 when loading the next column. Thus, Sample 1 would be in position D1 and Sample 13 would be in E1. (See Figure 5 for the layout of block columns and rows. For more information on deck setup, see Appendix A.)

D. Sample Recommendations

- **Total RNA Extraction**
  The sequence complexity and the average length of cDNA are noticeably dependent on the quality of starting RNA material. Due to the limited sample size, most traditional RNA isolation methods may not be applicable. There are several commercially available products that enable purification of total RNA preparations from extremely small samples. We offer the NucleoSpin RNA XS kit (Cat. No. 740902.10) for purification of RNA from ≥100 cells. When choosing a purification method (kit), ensure that it is appropriate for your sample amount. Input RNA should be free from poly A+ carrier RNA or glycogen that will interfere with oligo(dT)-primed cDNA synthesis.
- **Evaluation of RNA Quality**
  After RNA extraction, if your sample size is not limiting, we recommend evaluating total RNA quality using the Agilent RNA 6000 Pico Kit (Cat. No. 5067-1513). Refer to the manufacturer's instructions for information on how to use the Agilent RNA 6000 Pico Kit.
- **Cell Culture Media**
  When working with cultured cells, it is important to select a cell culture medium and a cell suspension solution that do not inhibit first-strand cDNA synthesis.

E. Sample Requirements

cDNA preparation on the SMARTer Apollo system using the SMART-Seq v4 Reagent Kit for the SMARTer Apollo System **requires an input volume of 7 µl** of cells or RNA.

IMPORTANT: Regardless of whether cells or total RNA are used as input material, a 7-µl input volume is an absolute requirement for successful implementation of this protocol on the SMARTer Apollo system.

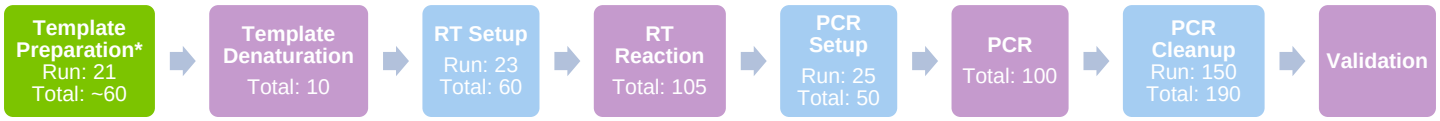
- **Total RNA**
  This protocol has been optimized for cDNA synthesis starting from 10 pg of total RNA. However, if your RNA sample is not limiting, we recommend that you start with more total RNA (up to 10 ng). Purified total RNA should be in nuclease-free water.
- **Cells**
  - We recommend starting from intact cells, but it is possible to use the protocol described in this user manual with previously frozen or freshly cultured cells. **This protocol cannot be used with cells that have undergone fixation.**
  - Cells should be washed and resuspended prior to lysis. The presence of media can interfere with first-strand synthesis. If necessary, test the effect of your media on cDNA synthesis by performing a reaction with control RNA and the estimated amount of media that you expect to accompany your cell(s).

V. Protocols

NOTES:

- Please read the entire protocol before starting. The SMART-Seq v4 Reagent Kit for the SMARTer Apollo System is optimized for cDNA synthesis from 1–1,000 intact cells or ultra-low input amounts of total RNA. Due to the sensitivity of the protocol, the input material (total RNA or cells) needs to be collected and purified under clean-room conditions to avoid contamination.
- This full protocol was validated using a 10-pg input of total RNA in a 7-µl volume and using the reagent volumes defined in each subprotocol. Any deviation in reagent volumes may affect the performance of the kit and script.

A. Protocol: Template Preparation



*For each protocol, the corresponding step in the workflow diagram is indicated in green. Steps performed manually are indicated in purple. Steps performed on the SMARTer Apollo system are indicated in blue. Run = the run time on the SMARTer Apollo system, if applicable. Total = total time spent including reagent setup and run time, if applicable. Times are indicated in minutes.

Lysis buffer and RNase inhibitor are added to cells or RNA along with a primer that will be annealed in the next protocol.

IMPORTANT: To avoid introducing contaminants into your sample, Step 3 of this protocol should be performed in a PCR clean work station, ideally in a clean room.

Materials Required

Table I. Reagents and Consumables Required for Template Preparation.

Reagents	Storage conditions	Source
Sample (7 µl of cells or RNA)	–70°C	User
Lysis 1	See note*	SMART-Seq v4 Reagent Kit for the SMARTer Apollo System
Lysis 2	–20°C	
RI	–20°C	
NFW	–20°C	

Consumables	Source	Cat. No.	Quantity	Usage/ 96-rxn run	Usage/12-rxn run
SMARTer Apollo Filter Tips	Takara Bio	640084	Box of 960	96 tips	12 tips
SMARTer Apollo 0.2 ml PCR 8-Tube Strips, Clear	Takara Bio	640082	Box of 125	13 strips	2 strips
SMARTer Apollo Caps for 0.2 ml PCR 8-Tube Strips, Clear	Takara Bio	640086	Box of 125	13 strips	2 strips

*Lysis 1 ships at –20°C. Once thawed, store at 4°C.

Protocol: Prepare Template from Controls and Your Samples

Before you begin, review the best practices in Section IV.A for proper use of the SMARTer Apollo instrument.

1. Before you begin, check that:
   - All work surfaces have been cleaned with 70% ethanol followed by RNase decontamination solution within the past week.
   - Any deformed plastics are discarded.
   - Power-cycle the instrument between each script.
2. Prechill SMARTer Apollo blocks:
   a. On the touchscreen, press **Maintenance** and then press **User Maintenance**.
   b. Select the following subscript: `SetTemp10C_v1-1.scb`. Do not exit the **User Maintenance** menu.
   c. Proceed to Step 3 (below) while the instrument is chilling. Chilling takes about 2 minutes.
3. Prepare reagents:
   a. Thaw the reagents needed for template preparation on ice. Gently vortex each reagent to mix and spin down briefly. Keep all reagents on ice.
   b. Prepare a stock solution of Template Preparation Master Mix. Mix the Template Preparation Master Mix briefly, then spin down. The stock solutions shown below are for 96 or 12 reactions. If using between 13 and 24 reactions, multiply the 12-reaction values by 2; if 25–36 reactions, multiply by 3; if 37–48, by 4; and so forth. This can be applied to the amounts throughout this protocol.

	Less than 17 cycles*		17 or more cycles*	
	96 reactions	**12 reactions**	**96 reactions**	**12 reactions**
Lysis 1**	136.8 µl	17.1 µl	136.8 µl	17.1 µl
RI	7.2 µl	0.9 µl	7.2 µl	0.9 µl
Lysis 2	288.0 µl	36.0 µl	144.0 µl	18.0 µl
NFW***	288.0 µl	36.0 µl	432.0 µl	54.0 µl
Total volume	720.0 µl	90.0 µl	720.0 µl	90 µl

*See Table III in Section V. E for PCR cycling guidelines.

**Lysis 1 contains a detergent. It is critical to avoid bubbles when mixing.

***Please use the NFW included with this SMART-Seq v4 reagent kit.

4. Load consumables onto the SMARTer Apollo work surface according to the layout in Figure 4 (96 samples) or Figure 5 (12 samples). See Appendix A for deck setup using other sample numbers. First, load the consumables that do not initially hold reagents. Next, on the benchtop, aliquot reagents and samples into the indicated consumables according to the table below, and then load them onto the system just before the run.

Component	Consumable	Volume
Sample (RNA or cells)	SMARTer Apollo 0.2 ml PCR 8-Tube Strips, Clear	7 µl per tube
Template Preparation Master Mix (TP MM)	SMARTer Apollo 0.2 ml PCR 8-Tube Strips, Clear	85 µl per tube (12 reactions per tube)

NOTES:
- The SMARTer Apollo system is calibrated for SMARTer Apollo PCR tubes only. Using other tubes may cause the run to fail.
- Ensure that there are no air bubbles. Keep all reagents on ice.
- Do not place reagents on the SMARTer Apollo deck until you are ready to start the run.

5. Install the metal retention plates on Blocks 3 and 4.
6. Empty the waste box.
   NOTE: An accumulation of tips in the waste box may cause the run to fail.
7. Before you begin the run, check that:
   - No plastic overhangs from partial strips are present.
   - Reagents were spun down to remove bubbles. **Bubbles at the bottoms of tubes must be removed to ensure accurate volume delivery.**
   - Plastic consumables are properly seated on the deck surface with caps/lids removed.
8. Set up the run.
   a. From the chill-down run in Step 2, press **OK** to return to the **User Maintenance** menu.
   b. Select the following subscript: `SSv4_1_Lysis_96_v1-1.scb`.
9. Start the template preparation run.
   a. Close the instrument door.
   b. Press **Run**. **The run time is 21 minutes.**
10. While the SMARTer Apollo system is running, preheat and pause the thermal cycler used for the next protocol (Template Denaturation, Section V.B, below) at 72°C.
11. When the run is complete, remove the products from Block 3 and discard the consumables in Block 4.
12. Cap the tubes containing product and spin down briefly.
13. Turn off the instrument.
14. Proceed immediately to the Template Denaturation protocol (Section V.B, below).
    NOTE: This is **NOT** a safe stopping point to store the samples. Proceed immediately to Template Denaturation.

Template Preparation (96 samples)

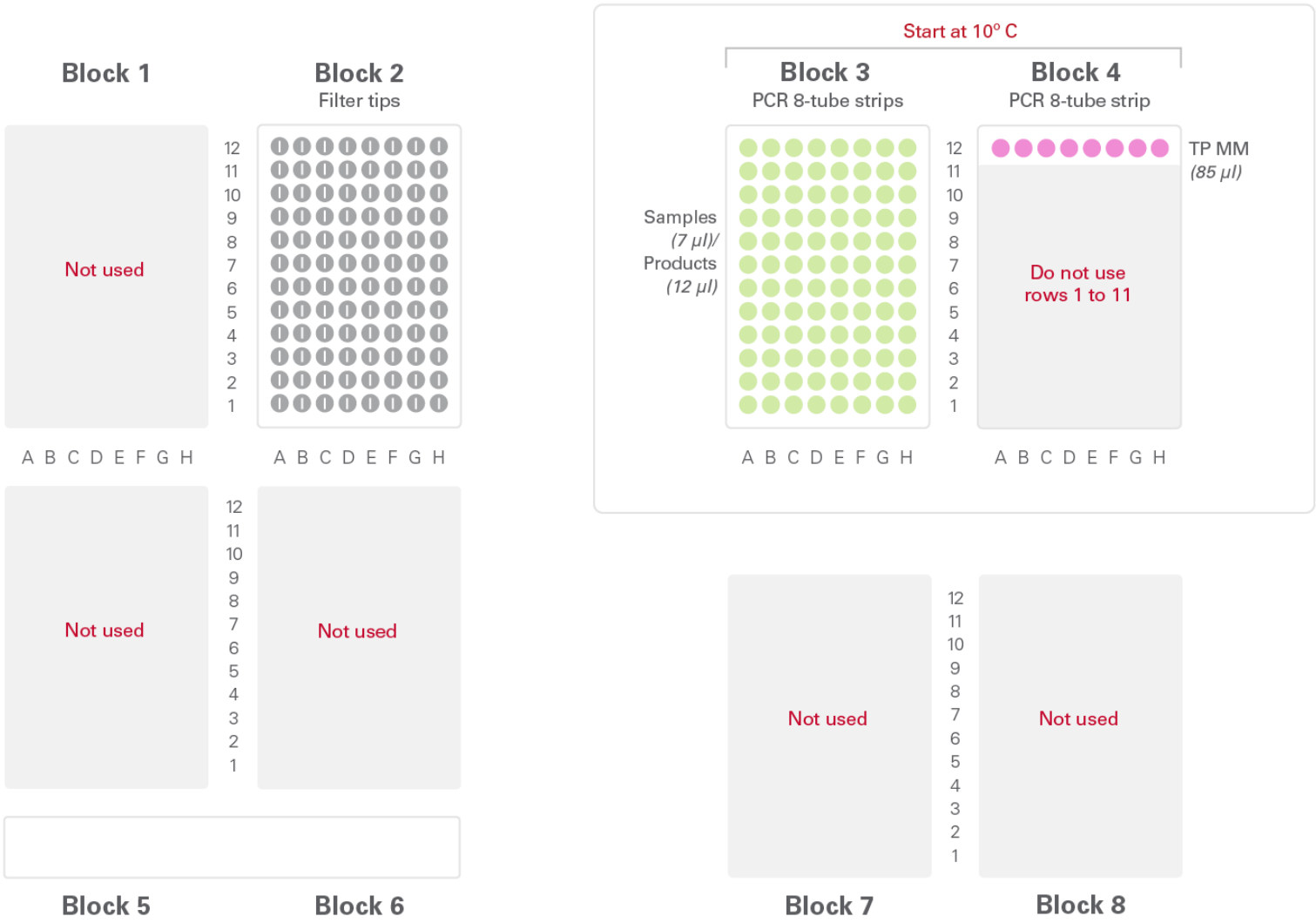


Figure 4. Deck layout for template preparation from 96 samples. For this protocol, use script `SSv4_1_Lysis_96_v1-1.scb`. Run time is 21 minutes.

Template Preparation (12 samples)

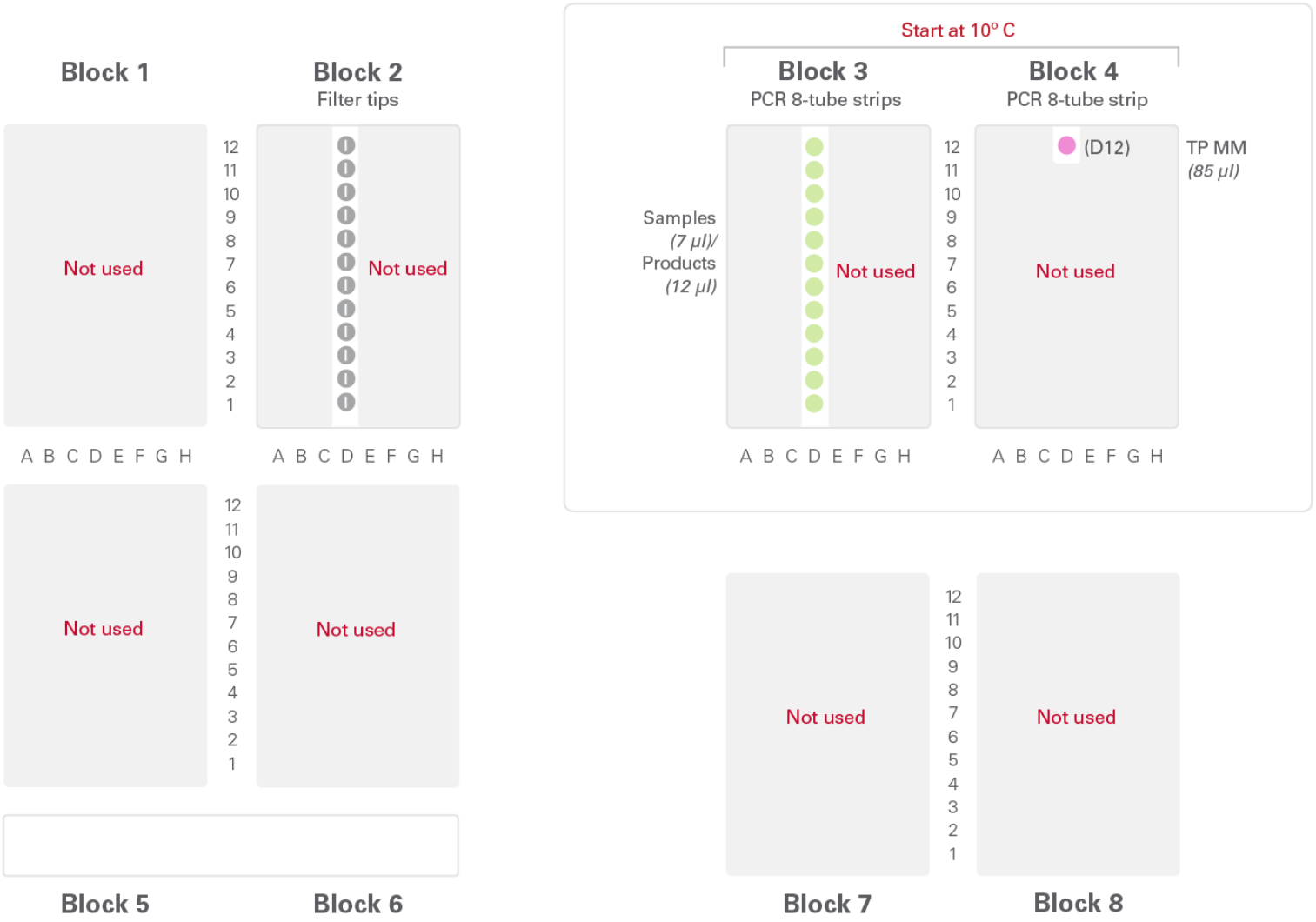
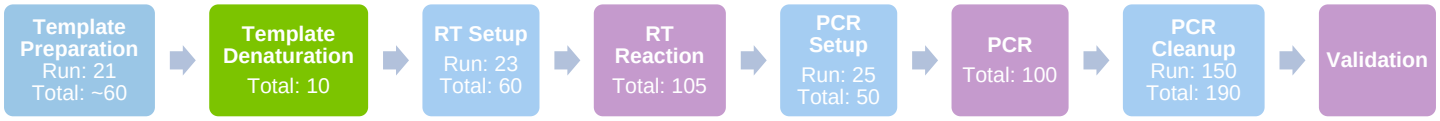


Figure 5. Deck layout for template preparation from 12 samples. For this protocol, use script `SSv4_1_Lysis_96_v1-1.scb`. Run time is 21 minutes.

- Sample/Product
- Filter tip
- Template Preparation Master Mix (TP MM)

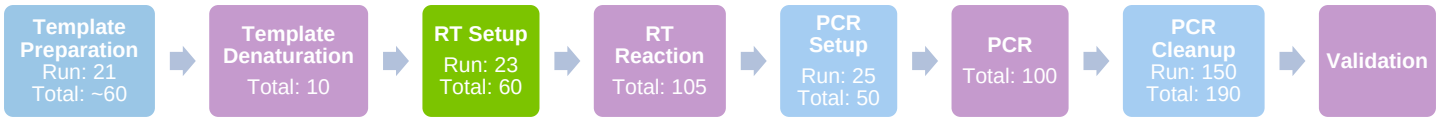
B. Protocol: Template Denaturation



The template is denatured and primer is annealed to the template.

1. Before beginning this protocol, thaw all reagents needed for the next protocol (Section V.C, RT Setup) on ice or at room temperature, as indicated in Step V.C.3.
2. Incubate the tubes from Step V.A.10 at 72°C in a preheated, hot-lid thermal cycler for 3 minutes.
   NOTE: Begin preparing Reverse Transcriptase Master Mix for the next protocol (Step V.C.3) during this incubation step.
3. Immediately after the 3-min incubation at 72°C, place the samples on ice for 2 minutes.
4. Proceed immediately to the Reverse Transcription Reaction (RT) Setup protocol (Section V.C, below).
   NOTE: This is **NOT** a safe stopping point to store the template. Proceed immediately to the RT Setup protocol.

C. Protocol: RT Setup



Reverse transcriptase and template-switching oligo are combined in a master mix and then added to template prepared in the previous section.

IMPORTANT: To avoid introducing contaminants into your sample, Step 3 of this protocol should be performed at a PCR clean work station, ideally in a clean room.

Materials Required

Table II. Reagents and Consumables Required for RT Setup.

Reagents	Storage conditions	Source
Prepared template	–	Section V.B
RT 1	–20°C	SMART-Seq v4 Reagent Kit for the SMARTer Apollo System
RT 2	–20°C	
RT 3	–20°C	
RI	–20°C	
NFW	–20°C	

Consumables	Source	Cat. No.	Quantity	Usage/96-rxn run	Usage/12-rxn run
SMARTer Apollo Filter Tips	Takara Bio	640084	Box of 960	96 tips	12 tips
SMARTer Apollo 0.2 ml PCR 8-Tube Strips, Clear	Takara Bio	640082	Box of 125	13 strips	2 strips
SMARTer Apollo Caps for 0.2 ml PCR 8-Tube Strips, Clear	Takara Bio	640086	Box of 125	13 strips	2 strips

Protocol: Set Up RT Reaction

Before you begin, review the Best Practices in Section IV.A.

1. Before you begin, check that:
   - All work surfaces have been cleaned with 70% ethanol followed by RNase decontamination solution within the past week.
   - Any deformed plastics are discarded.
   - The instrument has been power-cycled since the prior script.
2. Prechill the SMARTer Apollo blocks. The blocks should be chilled as follows:
   a. On the touchscreen, press **Maintenance** and then press **User Maintenance**.
   b. Select the following subscript: `SetTemp10C_v1-1.scb`. Do not exit the **User Maintenance** menu.
   c. Proceed to Step 3 (below) while the instrument is chilling. Chilling takes about 2 minutes.

   NOTE: The Reverse Transcriptase Master Mix (Step V.C.3, next) should be prepared during the incubation step in the previous section (Step V.B.2). RT 3 will be added just before use. Setting up the RT reaction as soon as possible is critical for first-strand cDNA synthesis and should not be delayed after completing Section V.B (Template Denaturation).
3. Prepare reagents.
   a. Thaw RT 2 at room temperature. Thaw all the remaining reagents needed for first-strand cDNA synthesis (except RT 3, which should be kept at –20°C until use) on ice. Gently vortex each reagent to mix and spin down briefly. Store all but the RT 2 on ice.

   NOTE: RT 2 may form precipitates. Thaw this buffer at room temperature and vortex before using to ensure all components are completely in solution.

   b. Prepare a stock solution of Reverse Transcriptase Master Mix to generate first-strand cDNA. Add RT 3 to the Master Mix just prior to use, making sure to gently mix the RT 3 tube without vortexing before adding it. The stock solutions shown below are for 96 or 12 reactions. If using between 13 and 24 reactions, multiply the 12-reaction values by 2; if 25–36 reactions, multiply by 3; if 37–48, by 4; and so forth.

	96 reactions	12 reactions
RT 2	516.0 µl	64.0 µl
RT 1	129.0 µl	16.0 µl
RI	64.5 µl	8.0 µl
NFW*	64.5 µl	8.0 µl
RT 3	258.0 µl	32.0 µl
Total volume	1,032.0 µl	128.0 µl

   *Please use the NFW included with this SMART-Seq v4 reagent kit.

   NOTE: Mix the Master Mix well by gently vortexing and then spin the tube(s) briefly in a minicentrifuge to collect the contents at the bottom of the tube.
4. Load consumables onto the SMARTer Apollo work surface according to the layout in Figure 6 (96 samples) or Figure 7 (12 samples). See Appendix A for deck setup using other sample numbers. First, load the consumables that do not initially hold reagents. Next, on the benchtop, aliquot reagents and samples into the indicated consumables according to the table below, and then load them onto the system just before the run.

Component	Consumable	Volume
Sample (from Section V.B)	SMARTer Apollo 0.2 ml PCR 8-Tube Strips, Clear	12 µl per tube
Reverse Transcriptase Master Mix (RT MM)	SMARTer Apollo 0.2 ml PCR 8-Tube Strips, Clear	120 µl per tube (12 reactions per tube)

NOTES:
- The SMARTer Apollo system is calibrated for SMARTer Apollo PCR tubes only. Using other tubes may cause the run to fail.
- Ensure that there are no air bubbles. Keep all reagents on ice.
- Do not place reagents on the SMARTer Apollo deck until you are ready to start the run.

5. Install the metal retention plates on Blocks 3 and 4.
6. Empty the waste box.
   NOTE: An accumulation of tips in the waste box may cause the run to fail.
7. Before you begin the run, check that:
   - No overhangs from partial strips are present.
   - Reagents were spun down to remove bubbles. **Bubbles at the bottoms of tubes must be removed to ensure accurate volume delivery.**
   - Plastic consumables are properly seated on the deck surface with caps/lids removed.
8. Set up the run.
   a. From the chill-down run in Step 2, or from the template preparation run completed in Section V.A, press **OK** to return to the **User Maintenance** menu.
   b. Select the following subscript: `SSv4_2_RT_96_v1-1.scb`.
9. Start the RT setup run.
   a. Close the instrument door.
   b. Press **Run**. **The run time is 23 minutes.**
10. While the SMARTer Apollo system is running, preheat and pause the thermal cycler used for the next protocol (RT Reaction, Section V.D, below) at 42°C.
11. When the run is complete, remove the products from Block 3 and discard the consumables in Block 4.
12. Cap the tubes containing product and spin down briefly.
13. From the touchscreen, press **OK** to return to the **User Maintenance** menu. Do not exit the **User Maintenance** menu or turn off the instrument if you are continuing to the PCR Setup protocol after the RT Reaction protocol.
14. Proceed to the RT Reaction protocol (Section V.D, below).
    NOTE: This is **NOT** a safe stopping point to store the reaction. Proceed immediately to RT Reaction.

RT Setup (96 samples)

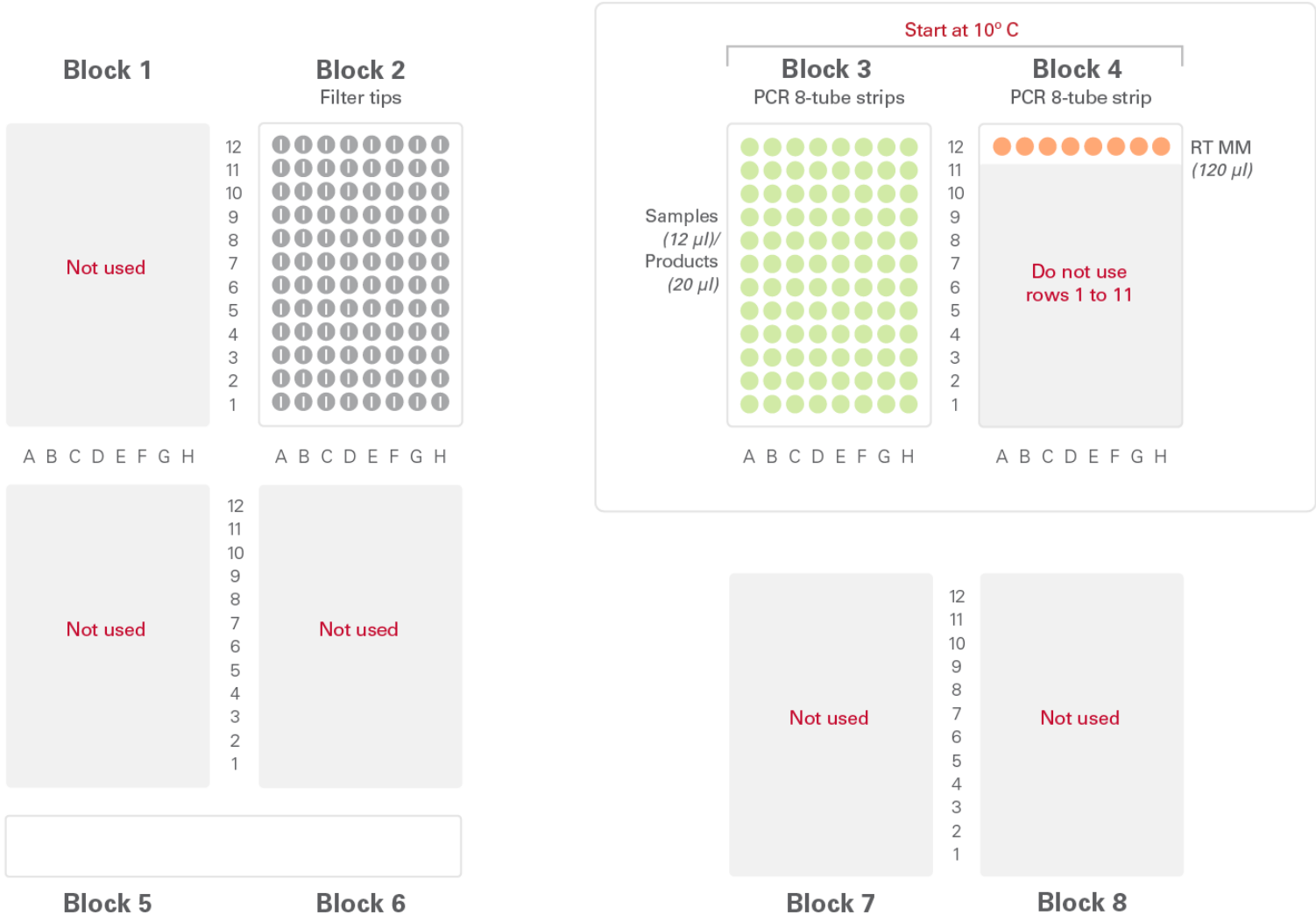


Figure 6. Deck layout for RT setup from 96 samples. For this protocol, use script `SSv4_2_RT_96_v1-1.scb`. Run time is 23 minutes.

RT Setup (12 samples)

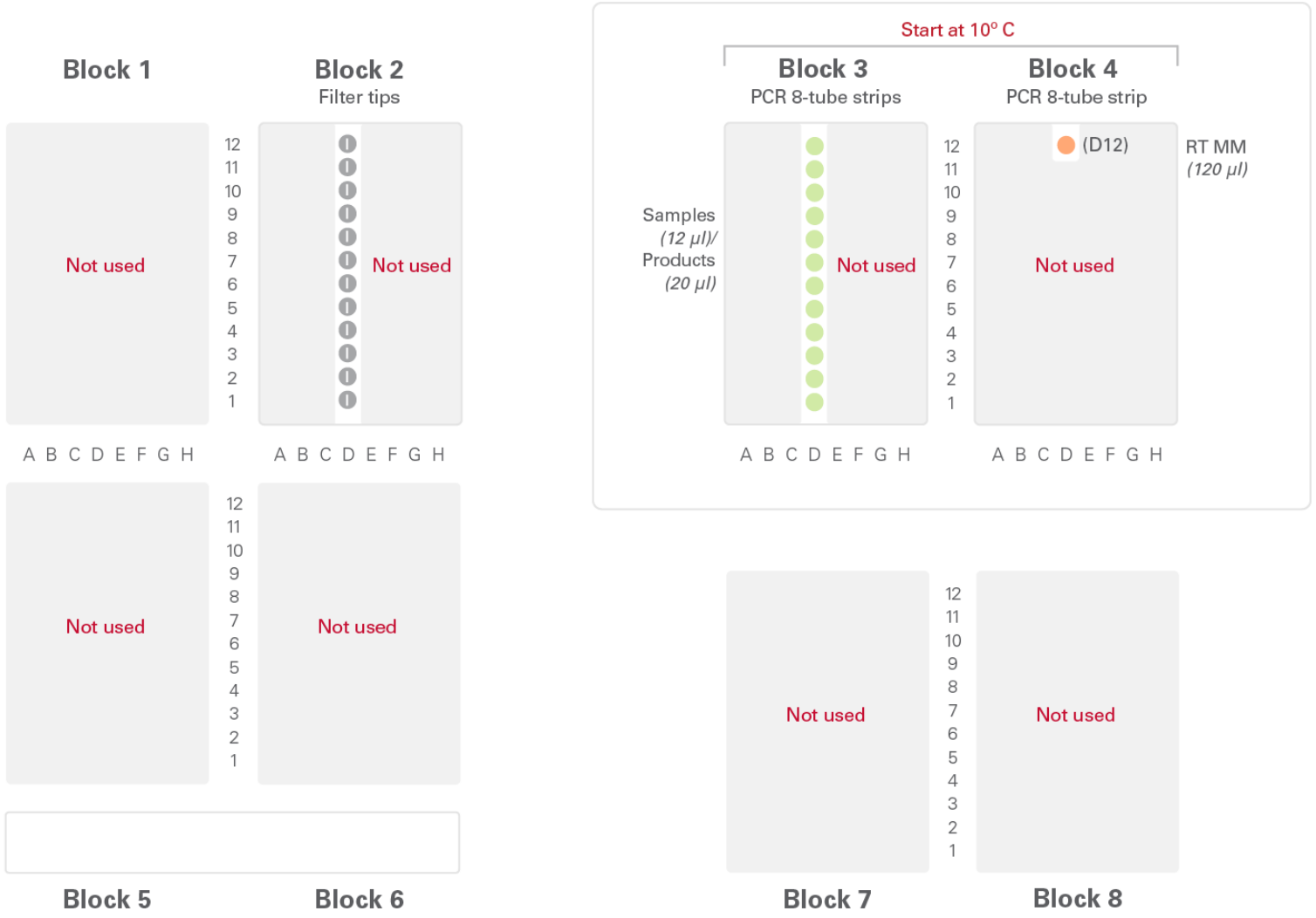
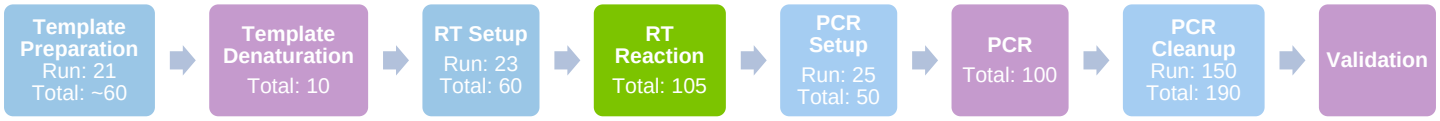


Figure 7. Deck layout for RT setup from 12 samples. For this protocol, use script `SSv4_2_RT_96_v1-1.scb`. Run time is 23 minutes.

D. Protocol: RT Reaction



First-strand cDNA synthesis (from total RNA or cells) is primed by the 3' SMART-Seq CDS Primer II A and uses the SMART-Seq v4 Oligonucleotide (included in the provided kit reagents) for template switching at the 5' end of the transcript.

1. Place the tubes in a thermal cycler with a heated lid, preheated to 42°C. Run the following program:

42°C	90 min
70°C	10 min
4°C	forever

SAFE STOPPING POINT: If you do not plan to proceed immediately to PCR Setup (Section V.E, below), samples can be stored at 4°C overnight.

E. Protocol: PCR Setup

Primer, PCR polymerase, and buffer are combined in a master mix and then added to the first-strand cDNA prepared in the previous section.

IMPORTANT: Table III provides guidelines for PCR optimization, depending on the amount of total RNA or cells used for first-strand cDNA synthesis. These guidelines were determined using the Control Total RNA. Typical cycle numbers are provided as a rough guide for working with small amounts of RNA. Optimal parameters may vary for different templates, different cell types, and different thermal cyclers. To determine the optimal number of cycles for your sample and conditions, we strongly recommend that you perform a range of cycles.

Table III. Cycling Guidelines Based on Amount of Starting Material.

Input amount of total RNA	Input amount of cells	Typical number of PCR cycles
10 ng	1,000 cells	7–8
1 ng	100 cells	10–11
100 pg	10 cells	14–15
10 pg	1 cell	17–18

Materials Required

Table IV. Reagents and Consumables Required for PCR Setup.

Reagents	Storage conditions	Source
First-strand cDNA	4°C	Section V.D
PCR 1	–20°C	SMART-Seq v4 Reagent Kit for the SMARTer Apollo System
PCR 2	–20°C	
PCR 3	–20°C	
NFW	–20°C	

Consumables	Source	Cat. No.	Quantity	Usage/96-rxn run	Usage/12-rxn run
SMARTer Apollo Filter Tips	Takara Bio	640084	Box of 960	96 tips	12 tips
SMARTer Apollo 0.2 ml PCR 8-Tube Strips, Clear	Takara Bio	640082	Box of 125	14 strips	2 strips
SMARTer Apollo Caps for 0.2 ml PCR 8-Tube Strips, Clear	Takara Bio	640086	Box of 125	14 strips	2 strips

Protocol: Set Up PCR

Before you begin, review the Best Practices in Section IV.A.

1. Before you begin, check that:
   - All work surfaces have been cleaned with 70% ethanol within the past week.
   - Any deformed plastics are discarded.
   - The instrument has been power-cycled since the prior script.
2. Prechill the SMARTer Apollo blocks. The blocks should be chilled as follows:
   a. On the touchscreen, press **Maintenance** and then press **User Maintenance**.
   b. Select the following subscript: `SetTemp10C_v1-1.scb`. Do not exit the **User Maintenance** menu.
   c. Proceed to Step 3 (below) while the instrument is chilling. Chilling takes about 2 minutes.
3. Prepare reagents:
   a. Thaw all the reagents needed for PCR (except PCR 2, which should be kept at –20°C until use) on ice. Gently vortex each reagent to mix and spin down briefly. Keep all reagents on ice.
   b. Prepare a stock solution of PCR Master Mix. The stock solutions shown below are for 96 or 12 reactions. If using between 13 and 24 reactions, multiply the 12-reaction values by 2; if 25–36 reactions, multiply by 3; if 37–48, by 4; and so forth.

	96 reactions	12 reactions
PCR 3	2,760.0 µl	350.0 µl
PCR 1	110.4 µl	14.0 µl
PCR 2	110.4 µl	14.0 µl
NFW*	331.2 µl	42.0 µl
Total volume	3,312.0 µl	420.0 µl

*Please use the NFW included with this SMART-Seq v4 reagent kit.

NOTE: Remove PCR 2 from the freezer, gently mix the tube without vortexing, and add to the Master Mix just before use. Mix the Master Mix well by vortexing gently and spin the tube briefly to collect the contents at the bottom of the tube.

4. Load consumables onto the SMARTer Apollo work surface according to the layout in Figure 8 (96 samples) or Figure 9 (12 samples). See Appendix A for deck setup using other sample numbers. First, load the consumables that do not initially hold reagents. Next, on the benchtop, aliquot reagents and samples into the indicated consumables according to the table below, and then load them onto the system just before the run.

Component	Consumable	Volume
Sample (from Section V.D)	SMARTer Apollo 0.2 ml PCR 8-Tube Strips, Clear	20 µl per tube
PCR Master Mix (PCR MM)	SMARTer Apollo 0.2 ml PCR 8-Tube Strips, Clear	200 µl per tube (6 reactions per tube)

NOTES:
- The SMARTer Apollo system is calibrated for SMARTer Apollo PCR tubes only. Using other tubes may cause the run to fail.
- Ensure that there are no air bubbles. Keep all reagents on ice.
- Do not place reagents on the SMARTer Apollo deck until you are ready to start the run.

5. Install the metal retention plates on Blocks 3 and 4.
6. Empty the waste box.
   NOTE: An accumulation of tips in the waste box may cause the run to fail.
7. Before you begin the run, check that:
   - No overhangs from partial strips are present.
   - Reagents were spun down to remove bubbles.
   - Plastic consumables are properly seated on the deck surface with caps/lids removed.
8. Set up the run.
   a. From the chill-down run in Step 2, or from the RT setup run completed in Section V.C, press **OK** to return to the **User Maintenance** menu.
   b. Select the following subscript: `SSv4_3_PCR_96_v1-1.scb`.
9. Start the PCR setup run.
   a. Close the instrument door.
   b. Press **Run**. **The run time is 25 minutes.**
10. While the SMARTer Apollo system is running, preheat and pause the thermal cycler used for the next protocol (PCR, Section V.F, below) at 95°C.
11. When the run is complete, remove the products from Block 3 and discard the consumables in Block 4.
12. Cap the tubes containing product and spin down briefly.
13. From the touchscreen, press **OK** to return to the **User Maintenance** menu. Do not exit the **User Maintenance** menu or turn off the instrument if you are continuing to the PCR Cleanup protocol after PCR.
14. Proceed to PCR (Section V.F, below) on a benchtop thermal cycler.
    NOTE: This is **NOT** a safe stopping point to store the DNA. Proceed immediately to PCR.

PCR Setup (96 samples)

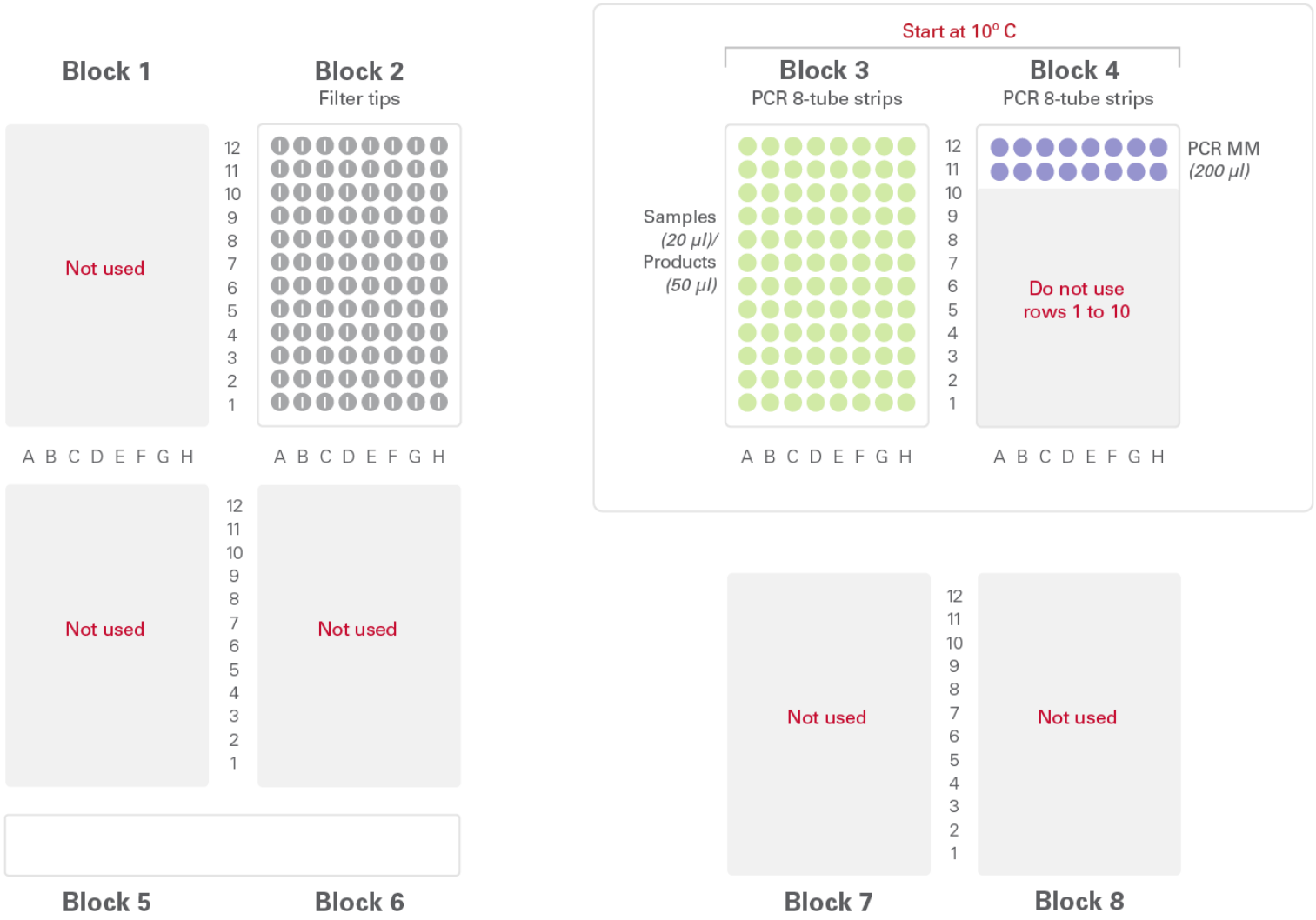


Figure 8. Deck layout for PCR setup from 96 samples. For this protocol, use script `SSv4_3_PCR_96_v1-1.scb`. Run time is 25 minutes.

PCR Setup (12 samples)

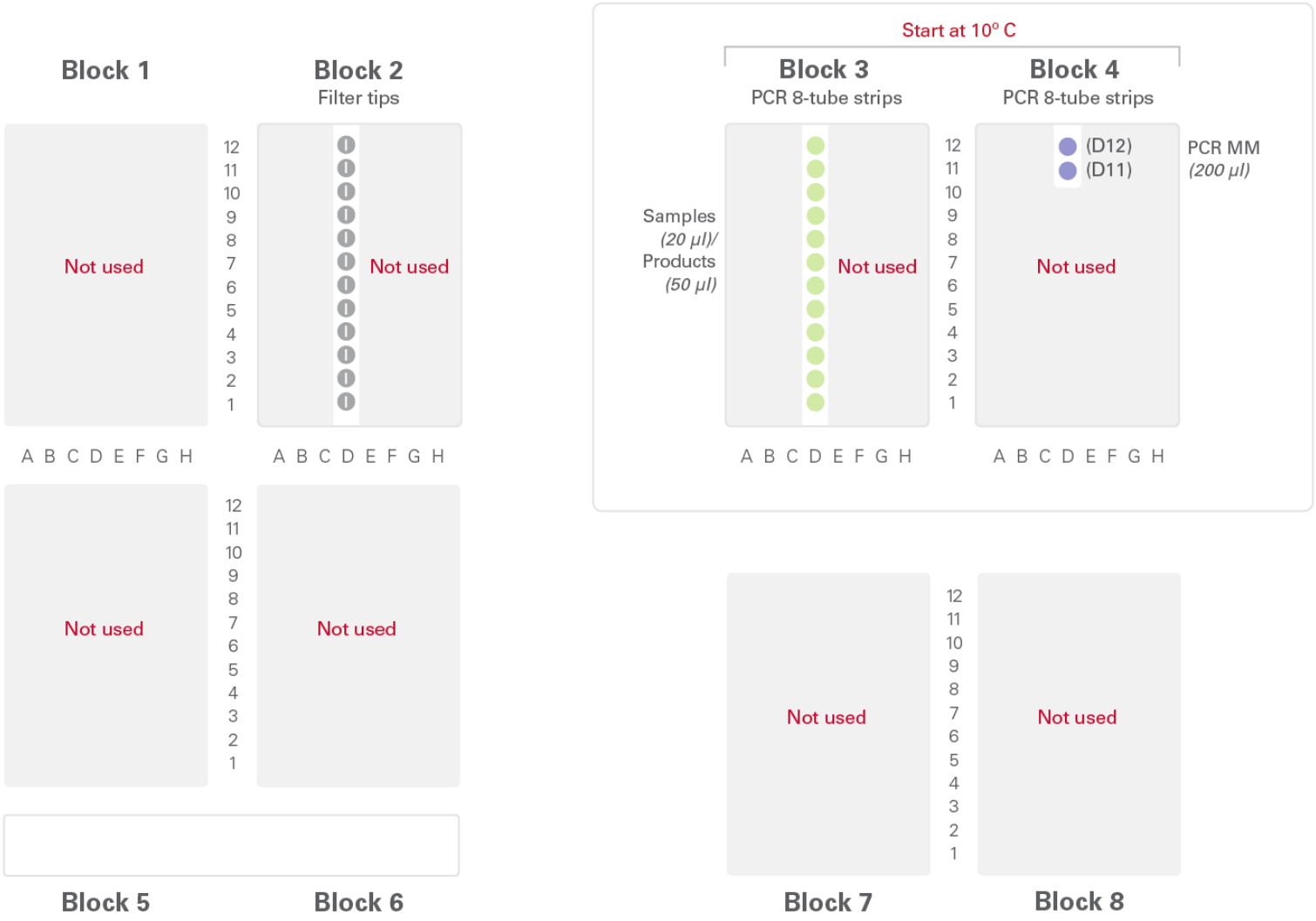
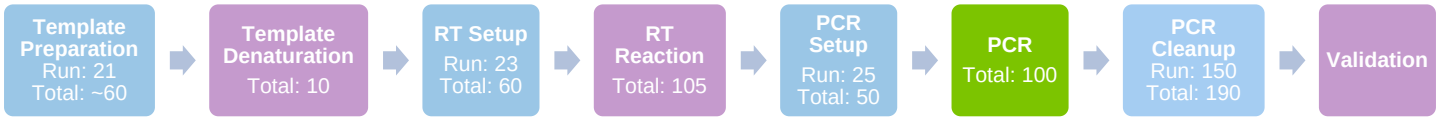


Figure 9. Deck layout for PCR setup from 12 samples. For this protocol, use script `SSv4_3_PCR_96_v1-1.scb`. Run time is 25 minutes.

F. Protocol: PCR



PCR Primer II A amplifies cDNA from the sequences introduced by the 3' SMART-Seq CDS Primer II A and the SMART-Seq v4 Oligonucleotide (included in the provided kit reagents).

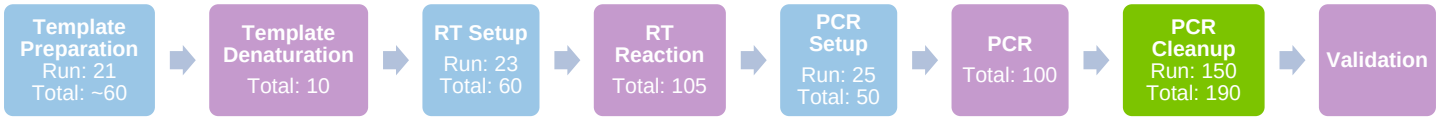
1. Place the tubes from Step V.E.12 in a preheated thermal cycler and run the following program:

95°C	1 min
X cycles:[a]	
98°C	10 sec
65°C	30 sec
68°C	3 min
72°C	10 min
4°C	forever

[a]Consult Table III for PCR cycle number guidelines.

SAFE STOPPING POINT: Proceed to PCR Cleanup (Section V.G) or store samples at 4°C overnight.

G. Protocol: PCR Cleanup



PCR-amplified cDNA is purified by immobilization on AMPure XP beads. The beads are then washed with 80% ethanol and cDNA is eluted with EB.

NOTES:

- Before each use, bring bead aliquots to room temperature for at least 30 min and mix well to disperse.

Materials Required

Table V. Reagents and Consumables Required for PCR Cleanup.

Reagents	Storage conditions	Source
PCR product	4°C	Section V.F
EB	Room temperature	SMART-Seq v4 Reagent Kit for the SMARTer Apollo System
AMPure XP Beads	2°C to 8°C	User
100% ethanol	Room temperature	User
Nuclease-free water	Room temperature	User

Consumables	Source	Cat. No.	Quantity	Usage/96-rxn run	Usage/12-rxn run
SMARTer Apollo Filter Tips	Takara Bio	640084	Box of 960	192 tips	24 tips
SMARTer Apollo 0.2 ml PCR 8-Tube Strips, Clear	Takara Bio	640082	Box of 125	24 strips	3 strips
SMARTer Apollo Caps for 0.2 ml PCR 8-Tube Strips	Takara Bio	640086	Box of 125	24 strips	3 strips
SMARTer Apollo Reservoirs	Takara Bio	640087	Box of 100	2 reservoirs	2 reservoirs
Hard-Shell Plate	Bio-Rad	HSP-9601	Box of 50	3 plates	3 plates

Protocol: Clean Up PCR Products

Before you begin, review the Best Practices in Section IV.A.

1. Before you begin, check that:
   - All work surfaces have been cleaned with 70% ethanol within the past week.
   - Any deformed plastics are discarded.
   - The instrument has been power-cycled since the prior script.
2. Prechill the Apollo blocks. The blocks should be chilled as follows:
   a. On the touchscreen, press **Maintenance** and then press **User Maintenance**.
   b. Select the following subscript: `SetTemp10C_v1-1.scb`. Do not exit the **User Maintenance** menu.
   c. Proceed to Step 3 (below) while the instrument is chilling. Chilling takes about 2 minutes.
3. Vortex the tubes containing the room-temperature AMPure XP beads for 30 seconds to make sure that the beads are evenly dispersed prior to use.
4. Load consumables onto the SMARTer Apollo work surface (see Figure 10 or 11). First, load the consumables that do not initially hold reagents. Next, on the benchtop, aliquot reagents and samples into the indicated consumables according to the table below. Load them onto the system just before the run.

Component	Consumable	Volume
Elution Buffer	Hard-Shell Plate, Rows 11–12	120 µl per well (6 reactions well)
AMPure XP beads	Hard-Shell Plate, Rows 1–4	165 µl per well (3 reactions per well)
100% ethanol	SMARTer Apollo Reservoir	15 ml
Nuclease-free water*	SMARTer Apollo Reservoir	12 ml

*Nuclease-free water (molecular biology grade) required for this step is not included with this SMART-Seq v4 reagent kit and must be provided separately by the user.

NOTES:
- The SMARTer Apollo system is specifically calibrated for the consumables indicated in the table above. Using alternative consumables may cause the run to fail.
- Ensure that there are no air bubbles.
- Do not place reagents on the SMARTer Apollo deck until you are ready to start the run.

5. Install the metal retention plates on Blocks 3 and 4.
6. Empty the waste box.

NOTE: An accumulation of tips in the waste box may cause the run to fail.

7. Before you begin the run, check that:
   - No overhangs from partial strips are present.
   - Reagents were spun down to remove bubbles. **Bubbles at the bottoms of tubes must be removed to ensure accurate volume delivery.**
   - Plastic consumables are properly seated on the deck surface with caps/lids removed.
8. Set up the run.
   a. From the chill-down run in Step 2, or from the PCR setup run completed in Section V.E, press **OK** to return to the **User Maintenance** menu.
   b. Select the following subscript: `SSv4_4_PCRCleanup_96_v1-1.scb`.
9. Start the PCR cleanup run.
   a. Close the instrument door.
   b. Press **Run**. **The run time is 2 hours, 30 minutes.**
10. When the run is complete, leave the products on Block 4 and discard the consumables in Blocks 2, 3, 6, 7, and 8. Visually inspect the final products and spin down, as necessary.
11. Cap the tubes containing product and spin down briefly.
12. Validate the purified cDNA using your preferred method or as described below (Section V.H).

PCR Cleanup (96 samples)

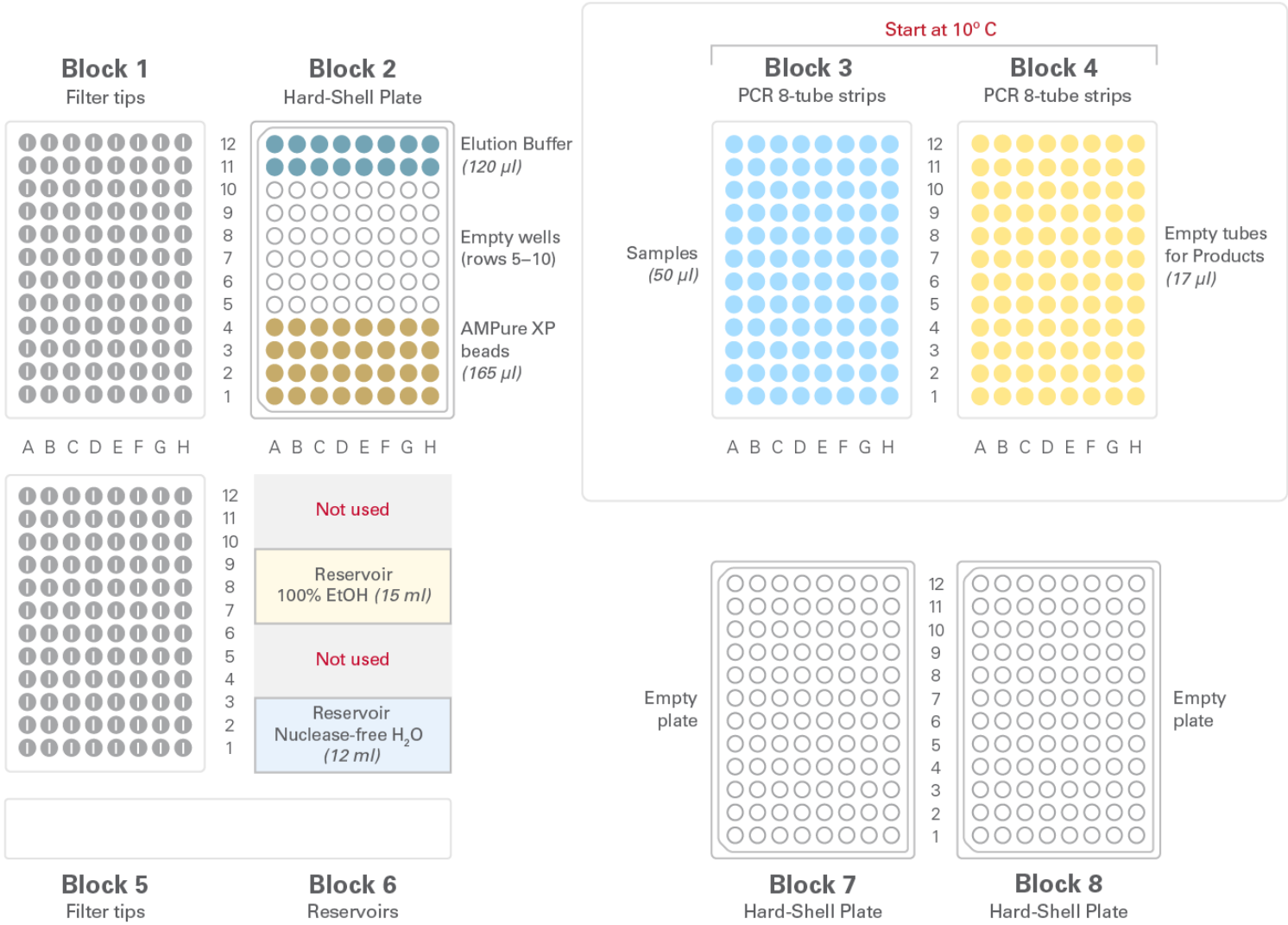


Figure 10. Deck layout for PCR cleanup from 96 samples. For this protocol, use script `SSv4_4_PCRCleanup_96_v1-1.scb`. Run time is 2.5 hr.

PCR Cleanup (12 samples)

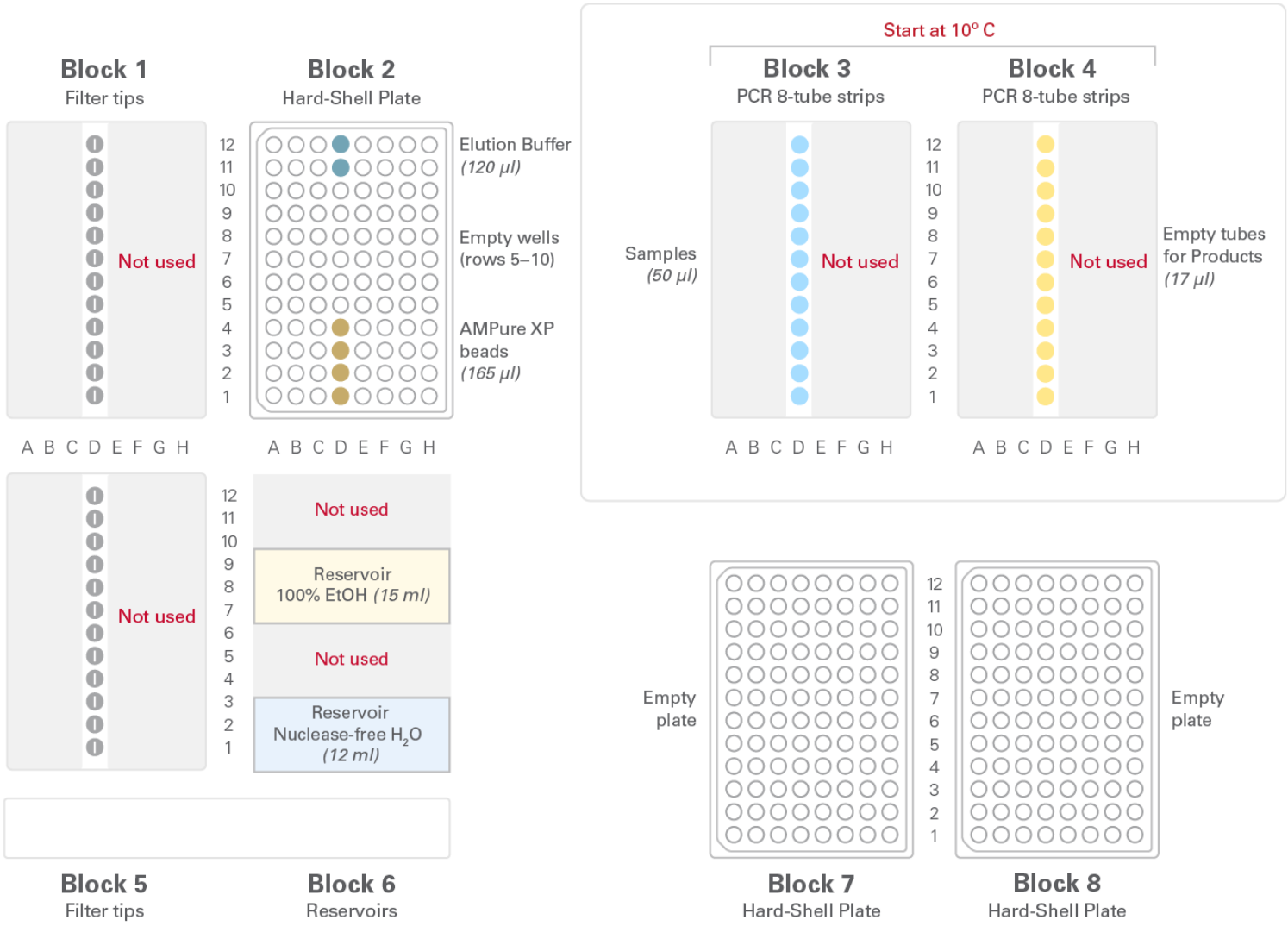


Figure 11. Deck layout for PCR cleanup from 12 samples. For this protocol, use script `SSv4_4_PCRCleanup_96_v1-1.scb`. Run time is 2.5 hr.

H. Protocol: Validation Using the Agilent 2100 Bioanalyzer

Yields of amplified cDNA may vary with different input types and amounts, affecting downstream sequencing library preparation. Sequencing library yield—and for some library preparation methods such as Illumina's Nextera XT kit, the library size distribution—may vary with input cDNA concentration. To minimize this variation and to achieve high sequencing library quality, the concentration of each cDNA library must be carefully determined. There are a few options for quantification of amplified cDNA, including the PicoGreen assay (Thermo Fisher Scientific), Qubit fluorometer (Thermo Fisher Scientific), or the Fragment Analyzer (Advanced Analytical). Here, we provide a protocol using the Agilent 2100 Bioanalyzer.

1. Aliquot 1 µl of the amplified cDNA for validation using the Agilent 2100 Bioanalyzer and Agilent's High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626). See the Agilent High Sensitivity DNA Kit User Manual for instructions.
2. Compare the results for your samples and controls (see Figure 12, below) to verify whether the sample is suitable for further processing. Successful cDNA synthesis and amplification should yield no product in the negative control (Figure 12, Panel B) and a distinct peak spanning 400 bp to 10,000 bp, peaked at ~2,500 bp, for the positive control RNA sample (Figure 12, Panel A), yielding approximately 3.4–17 ng of cDNA (depending on the input type and amount).

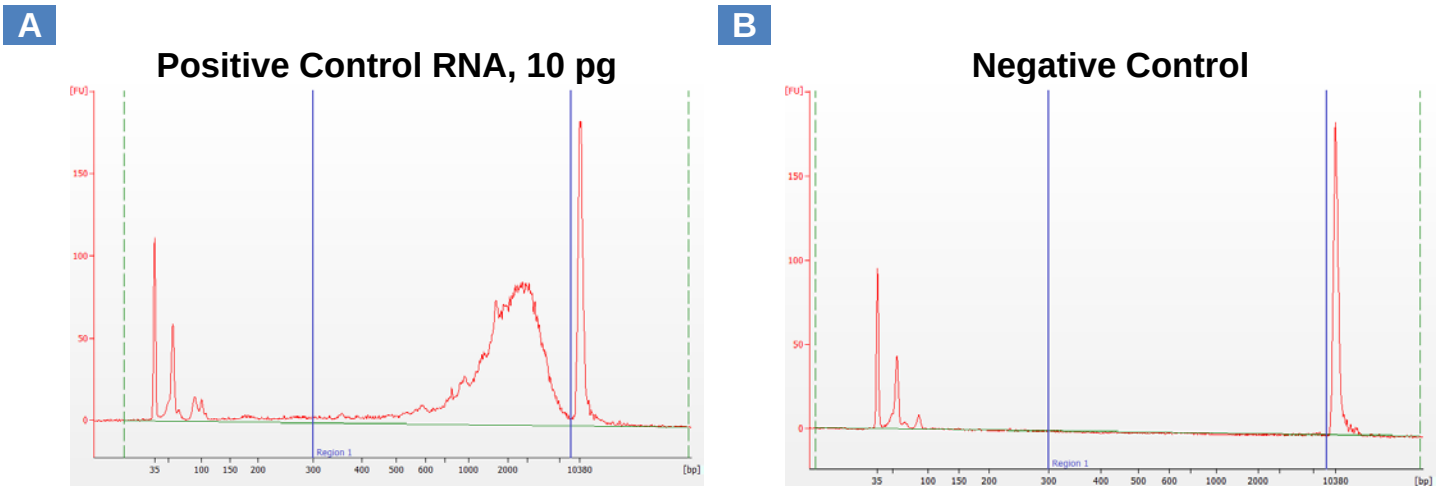


Figure 12. Example electropherogram results from Agilent 2100 Bioanalyzer. All samples were subjected to SMART cDNA synthesis and amplification as described in the protocol. Panel A shows a clean product following cDNA synthesis and amplification (17 PCR cycles). Panel B shows no product in the negative control following cDNA synthesis and amplification (17 PCR cycles).

VI. References

Chenchik, A., Zhu, Y., Diatchenko, L., Li., R., Hill, J. & Siebert, P. Generation and use of high-quality cDNA from small amounts of total RNA by SMART PCR. In *RT-PCR Methods for Gene Cloning and Analysis*. Eds. Siebert, P. & Larrick, J. (BioTechniques Books, MA), pp. 305–319 (1998).

Picelli, S., Björklund, Å. K., Faridani, O. R., Sagasser, S., Winberg, G., Sandberg, R. Smart-seq2 for sensitive full-length transcriptome profiling in single cells. *Nat. Methods* **10,** 1096–1098 (2013).

Ramsköld, D., Luo, S., Wang, Y.-C., Li, R., Deng, Q., Faridani, O.R., Daniels, G.A., Khrebtukova, I., Loring, J.F., Laurent, L.C., Schroth, G.P. & Sandberg, R. Full-length mRNA-seq from single-cell levels of RNA and individual circulating tumor cells. *Nature Biotechnology* **30,** 777–782 (2012).

Appendix A: Deck Setup for Additional Reaction Sizes

The SMARTer Apollo system can run flexible batch sizes, in multiples of 12, up to 96 samples. The 96-reaction master mix and deck layout can accommodate between 85 and 96 samples. Sample amounts that are not exact multiples of 12 can be run on the system; however, master mix volumes must be prepared in multiples of 12, and a specific deck setup must be used for each multiple of 12, determined by rounding up the number of samples to the nearest multiple of 12. For example, if you have 37 samples, you will need to prepare enough master mix for 48 reactions, and use the 48-reaction plate layout described in table below. Please note: while batch sizes smaller than 48 samples can be processed on the SMARTer Apollo system, performing fewer than 48 reactions using the workflow described in this user manual may not provide time savings over manual sample processing.

Table VI. Deck Layout Options for Various Sample Numbers

Setting up less than 96 samples	
# of samples	Columns to load
1–12	D or E*
13–24	D, E
25–36	C, D, E or D, E, F
37–48	C, D, E, F
49–60	B, C, D, E, F or C, D, E, F, G
61–72	B, C, D, E, F, G
73–84	A, B, C, D, E, F, G or B, C, D, E, F, G, H

*The user can choose between a deck setup centered around column D or E. The scripts are designed to accommodate either layout.

Appendix B: WaferGen-Takara Bio Part Number Conversion

If you have a WaferGen catalog number that you have previously used for ordering, please use the corresponding Takara Bio catalog number to place an order.

Table VII. WaferGen-Takara Part Numbers

WaferGen Cat. No.	Takara Bio Cat. No.	Product
300019	**640082**	SMARTer Apollo 0.2 ml PCR 8-Tube Strips, Clear
300029	**640086**	SMARTer Apollo Caps for 0.2 ml PCR 8-Tube Strips, Clear
300027	**640084**	SMARTer Apollo Filter Tips
300031	**640087**	SMARTer Apollo Reservoirs
100003	**640078**	SMARTer Apollo

This table only includes the products used in this series of protocols. Additional SMARTer Apollo products formerly available from WaferGen are available on our website, takarabio.com.

Contact Us	
Customer Service/Ordering	**Technical Support**
tel: 800.662.2566 (toll-free)	tel: 800.662.2566 (toll-free)
fax: 800.424.1350 (toll-free)	fax: 800.424.1350 (toll-free)
web: takarabio.com	web: takarabio.com
e-mail: ordersUS@takarabio.com	e-mail: techUS@takarabio.com

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This document has been reviewed and approved by the Quality Department.