Positive and negative controls in scRNA-seq

Whether you've done single-cell RNA-seq one or one thousand times before, control reactions are invaluable for troubleshooting your experiments. All of Takara Bio's NGS kits include positive control samples to make this as easy as possible. The best positive control has an RNA input mass similar to your experimental samples (e.g., 10 pg of RNA as a starting point for single cells). Similarly, the best negative control is one treated the same as your actual samples (e.g., mock FACS sample buffer). Due to the difficulties of working with very small input masses, some new users may see low cDNA yield in their positive control samples. Until you are comfortable with the protocol, you may want to test two positive control inputs (e.g., 10 pg and 100 pg). New users can sometimes also see a high background in their negative controls, which is a critical problem. This article can help explain how these controls should perform and help you troubleshoot when they do not. As different kits produce different results, please go to the appropriate section for SMART-Seq HT, SMART-Seq v4, or SMART-Seq Stranded.

SMART-Seq v4 and SMART-Seq HT: expected results

Successful cDNA synthesis and amplification in the positive control RNA sample should yield ≥ 200 pg/µl of cDNA. The cDNA should show a distinct peak that spans 400 to 10,000 bp and has a maximum between 2,000 and 2,500 bp (Figure 1, Panel A) when analyzed with an Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit (Agilent, Cat. # 5067-4626). For the negative control, cDNA synthesis and amplification should yield very little to no product using the Agilent High Sensitivity DNA Kit (Figure 1, Panel C). If using a fluorescent nucleic acid binding dye such as PicoGreen (Thermo Fisher Scientific, Cat. # P11496), the yield in your negative control should be ≤100 pg/µl.

These values and shapes may differ if you overload your chip or use an alternative capillary electrophoresis instrument. The Fragment Analyzer generally gives results similar to the Bioanalyzer, though the same samples may look different when analyzed with the Bioanalyzer versus the Agilent 2200 TapeStation (Figure 2). Unfortunately, we have found that the PerkinElmer LabChip system is not sensitive enough for analysis of the cDNA produced by the SMART-Seq v4 or SMART-Seq HT kits.

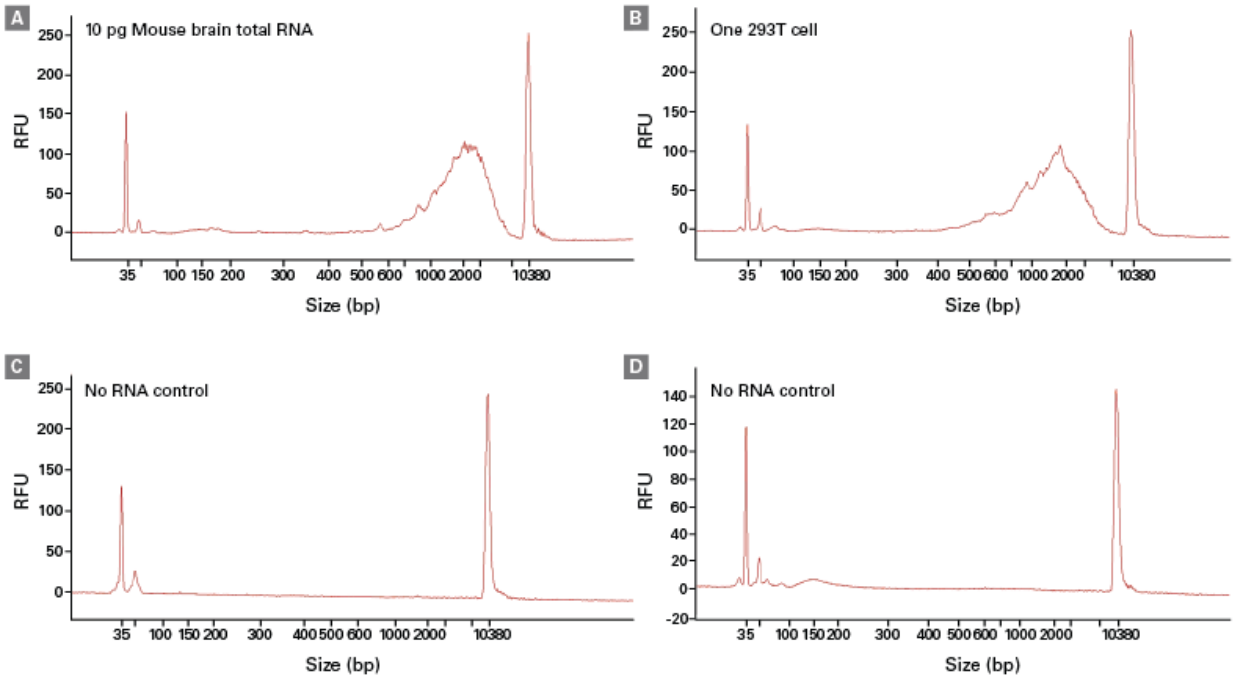

Figure 1. Example electropherogram traces from Agilent 2100 Bioanalyzer. These traces are representative of results obtained from SMART-Seq HT and SMART-Seq v4 kits. These specific samples were processed with the SMART-Seq HT kit as described in the user manual, including one-step RT-PCR and amplified with 17 cycles of PCR. After bead purification, 1 µl of the sample was analyzed using the Agilent High Sensitivity DNA Kit. **Panel A.** Representative results from 10 pg of positive control (Mouse Brain Total RNA control, included in both SMART-Seq HT and SMART-Seq v4 kits). **Panel B.** Example results from one 293T cell (isolated using FACS). **Panels C and D.** Representative results generated from negative controls, showing either a totally flat profile (C) or a small number of unspecific products between 100 and 300 bp (D). These products do not affect the quality of the sequencing data.

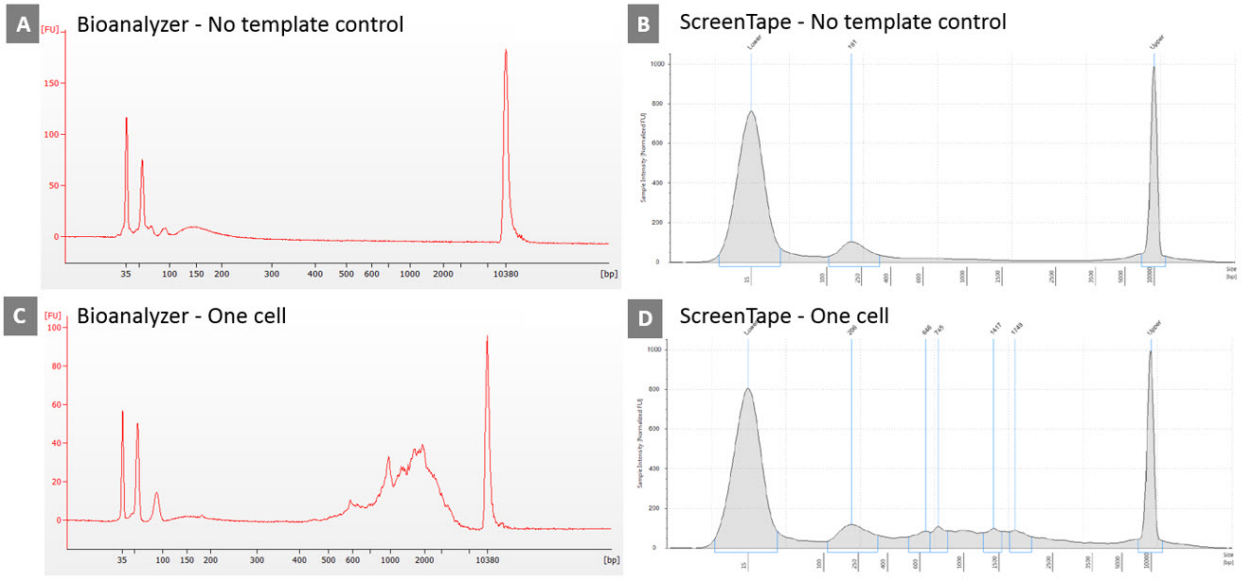

Figure 2. Comparison of electropherogram traces from Agilent 2100 Bioanalyzer and Agilent TapeStation systems. These traces are representative of results from SMART-HT and SMART-Seq v4 kits. These specific samples were processed with the SMART-Seq HT kit as described in the user manual, including one-step RT-PCR and amplified with 18 cycles of PCR. After bead purification, 1 µl of the same sample was analyzed using the Agilent High Sensitivity DNA Kit (**Panels A and B**) or the High Sensitivity D5000 ScreenTape (**Panels C and D**).

Troubleshooting unexpected results from SMART-Seq v4 or SMART-Seq HT

If your results are not similar to those described and shown above, the following information can help you narrow down what went wrong.

1. My positive control sample has a much lower yield and/or a smear of products much smaller than shown in Figure 1, Panel A.

Possible causes:

- A small shift in the cDNA size such as that shown in Figure 1B is acceptable, but large shifts in the size of cDNA products-or a yield of <200 pg/µl indicates that your RNA is likely degraded.

Recommendations:

- Ensure that the PCR cycling parameters you used are appropriate to the input mass of control RNA that you used (see Table I) and adjust if needed.
- Test a higher positive control input (e.g., 100 pg in addition to 10 pg) until you're comfortable with the protocol.
- Ensure you are making fresh dilutions of the control RNA immediately before each experiment in RNase-free water containing RNase inhibitor(s).
- Minimize sample loss by using RNase- and DNase-free, low RNA- and DNA-binding plasticware (pipette tips, plates/tube strips, etc.)
- Carefully follow the instructions in the protocol for bead cleanup steps, which can be a significant cause of sample loss. Ensure beads are fully separated before removing supernatant. Using a strong magnetic rack or device will improve and speed this separation. Following the recommended protocol for post-ethanol drying and hydration times can also improve yield.
- Minimize sample handling time to reduce sample contamination and degradation.
- Always wear a lab coat, sleeve covers, and gloves throughout the protocol. Change gloves often, especially between each step in the protocol, to minimize contamination.

Table I. Recommended PCR cycles for control RNA

Input of Control RNA	Recommended PCR cycles
10 ng	7–8*
1 ng	10–11
100 pg	14–15
10 pg	17–18

*The SMART-Seq HT kit is not recommended for inputs greater than 1 ng.

2. There is a signal in my negative control.

Possible causes:

- A small amount of signal, as shown in Figure 1, Panel D, is acceptable. However, if you have a significant signal in your negative control (≥100 pg/µl), you may have significant environmental or amplicon contamination.

Recommendations:

- Maintain separate pre- and post-PCR workspaces to minimize contamination. Ideally, the pre-PCR workstation should be in a clean room with positive airflow, which will greatly decrease the risk of amplicon or environmental contamination.
- Minimize sample handling time to reduce sample contamination and degradation.
- Always wear a lab coat, sleeve covers, and gloves throughout the protocol. Change gloves often, especially between each step in the protocol, to minimize contamination.

A successful positive control should produce >3 ng/µl of final sequencing library. Its size distribution should span 20–2,000bp with a maximum at ~300–450 bp (Figure 3, Panels A and C) when diluted to 1.5 ng/µl and analyzed with an Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit. The size distribution may differ if you overload your chip or use an alternative capillary electrophoresis instrument (e.g., Agilent TapeStation, PerkinElmer LabChip, or Fragment Analyzer).

The negative control samples should show little or no background over the corresponding range (Figure 3, Panel B). Negative controls typically display a higher amount of PCR products between 1,000 and 2,000 bp than between 200 and 1,000 bp (Figure 3, Panel D).

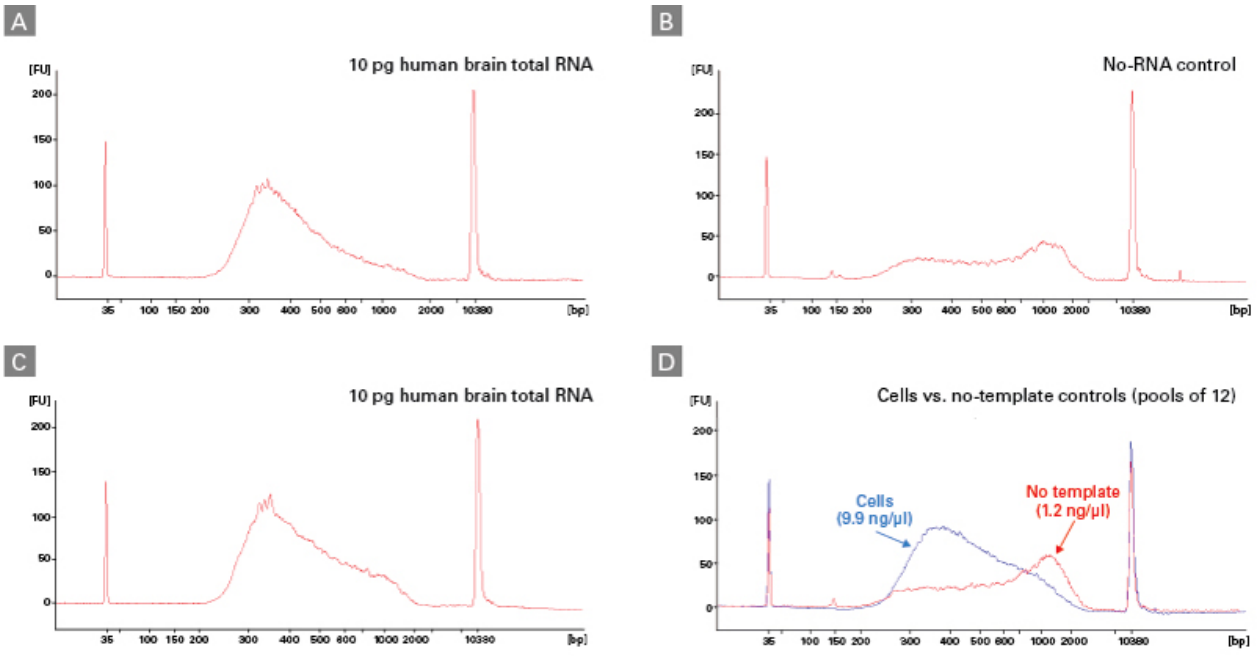

Figure 3. Example electropherogram results from the Agilent 2100 Bioanalyzer. Libraries were generated using 10 pg of Control Total RNA (human brain)

(**Panels A and C**) or a no-RNA control (**Panel B**) using the ultra-low workflow. **Panel D** shows an example of libraries generated directly from either single cells or a no-template control using the ultra-low workflow. Note that the no-template control exhibits a small amount of background <200 bp, and typically shows some amount of PCR product between 200 and 1,000 bp, in addition to a larger amount of product >1,000 bp. This background is typically unavoidable but is acceptable as long as libraries from RNA samples contain ≥3 times more material than no-template controls.

Troubleshooting unexpected results from SMART-Seq Stranded

If your results are not similar to those described and shown above, the following information can help you narrow down what went wrong.

1. My positive control sample has signal in the 150–200 bp size range.

Possible causes:

- A small amount of products of this size (such as those seen in the positive control in Figure 3, Panel D) may arise from the final amplification reaction and will not interfere with sequencing.

Recommendations:

- You may consider adding an additional AMPure bead cleanup step with a 1:1 bead:sample ratio.

2. My positive control sample has a smear of products smaller than shown in Figure 3, Panel A

Possible causes:

- Your RNA may be degraded, or you may have over-sheared your sample.

Recommendations:

- Test a higher positive control input (e.g., 100 pg in addition to 10 pg) until you're comfortable with the protocol.
- Ensure you are making fresh dilutions of the control RNA immediately before each experiment in RNase-free water containing RNase inhibitor(s). Minimize sample loss by using RNase- and DNase-free, low RNA- and DNA-binding plastics (pipette tips, plates/tube strips, etc.).
- Carefully follow the instructions in the protocol for bead cleanup steps, which can be a significant cause of sample loss. Ensure beads are fully separated before removing supernatant. Using a strong magnetic rack or device will improve and speed this separation. Following the recommended protocol for post-ethanol drying and hydration times can also improve yield.
- Minimize sample handling time to reduce sample contamination and degradation.
- Always wear a lab coat, sleeve covers, and gloves throughout the protocol. Change gloves often, especially between each step in the protocol, to minimize contamination.

3. There is a signal in my negative control.

Possible causes:

- Background signal may be visible in your negative controls (Figure 3D) due to the large number of PCR cycles used in this protocol, particularly when using 22–23 total cycles. In this case, a negative control that yields 0.5–1 ng/µl is not unusual. Background signal is acceptable as long as positive control samples are 3x the value of the negative control. If the signal in your negative control is similar to your positive control, see below.

Recommendations:

- Maintain three workstations to minimize amplicon contamination: a cDNA synthesis station (pre-PCR), a PCR station, and a PCR purification and library quantitation station. Ideally, the cDNA synthesis station should be in a clean room with positive airflow to minimize the risk of amplicon or environmental contamination.
- Minimize sample handling time to reduce sample contamination and degradation.
- Always wear a lab coat, sleeve covers, and gloves throughout the protocol. Change gloves often, especially between each step in the protocol, to minimize contamination.

4. The signal in my positive control is similar to my negative control.

Possible causes:

- Because this kit relies on random priming and includes a large number of PCR cycles, it is extraordinarily sensitive to environmental contaminants. When the signals from the positive and negative controls are similar, this indicates a failed library preparation.

Recommendations:

- Maintain three workstations to minimize amplicon contamination: a cDNA synthesis station (pre-PCR), a PCR station, and a PCR purification and library quantitation station. Ideally, the cDNA synthesis station should be in a clean room with positive airflow to minimize the risk of amplicon or environmental contamination.
- Ensure you are making fresh dilutions of the control RNA immediately before each experiment in RNase-free water containing RNase

inhibitor(s).

- Minimize sample loss by using RNase- and DNase-free, low RNA- and DNA-binding plastics (pipette tips, plates/tube strips, etc.)
- Carefully follow the instructions in the protocol for bead cleanup steps, which can be a significant cause of sample loss. Ensure beads are fully separated before removing supernatant. Using a strong magnetic rack or device will improve and speed this separation. Following the recommended protocol for post-ethanol drying and hydration times can also improve yield.
- Minimize sample handling time to reduce sample contamination and degradation.
- Always wear a lab coat, sleeve covers, and gloves throughout the protocol. Change gloves often, especially between each step in the protocol, to minimize contamination.

Additional support

Takara Bio is an expert in the field of single-cell RNA-seq, and we have pioneered and systematically advanced this technology for years. We offer a range of single-cell RNA-seq kits, including oligo-dT and random priming solutions. If you have specific questions about our kits or are running into experimental issues, our technical support team is standing by and ready to help.

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