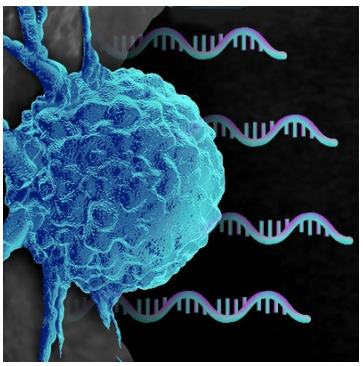
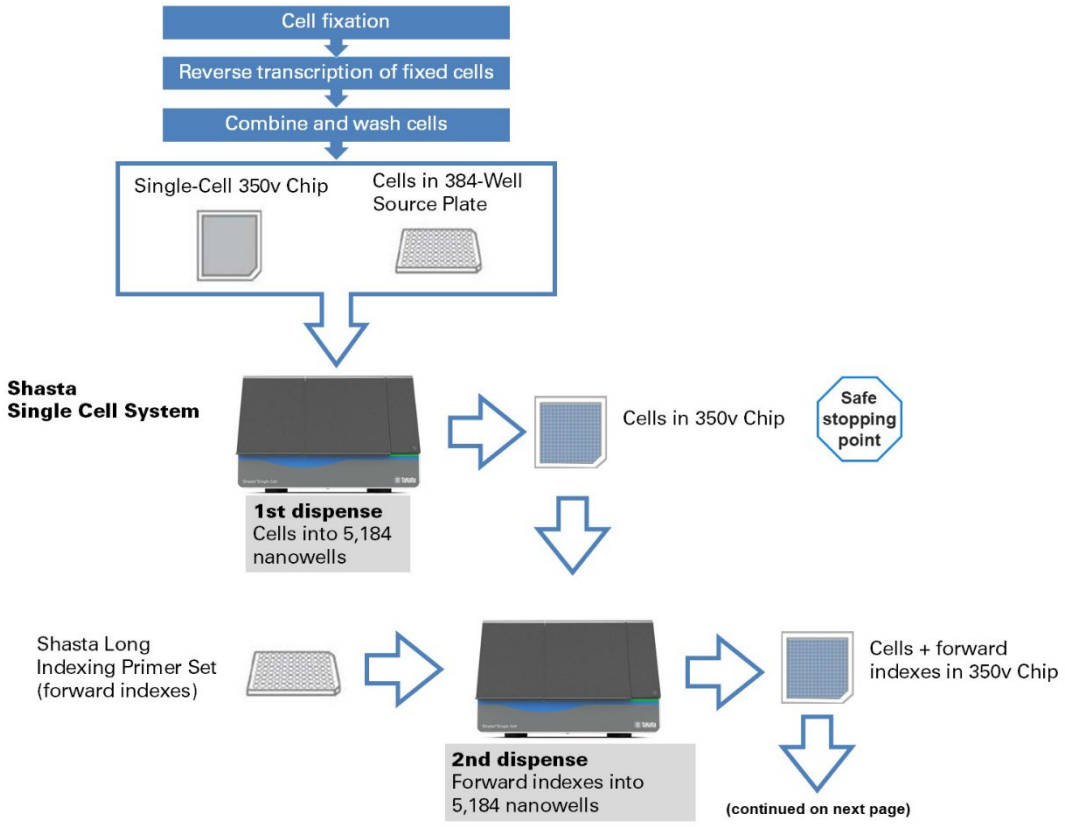


Shasta™ Total RNA-Seq Experiment Planner for the Shasta Single Cell System

I. Introduction

This Experiment Planner offers guidance on the experimental design of the key steps in the Shasta Total RNA-Seq workflow, including cell concentration adjustment, reverse transcription (RT) plate layout, cell dilution for dispensing, and PCR 2 cycles. Examples of modifications based on these criteria are included in this document.

The planner is intended for use as a supplement to the Shasta Total RNA-Seq Kit User Manual for the Shasta Single Cell System and cannot be used on its own to perform the workflow. For more information on the procedure, please use the manual.



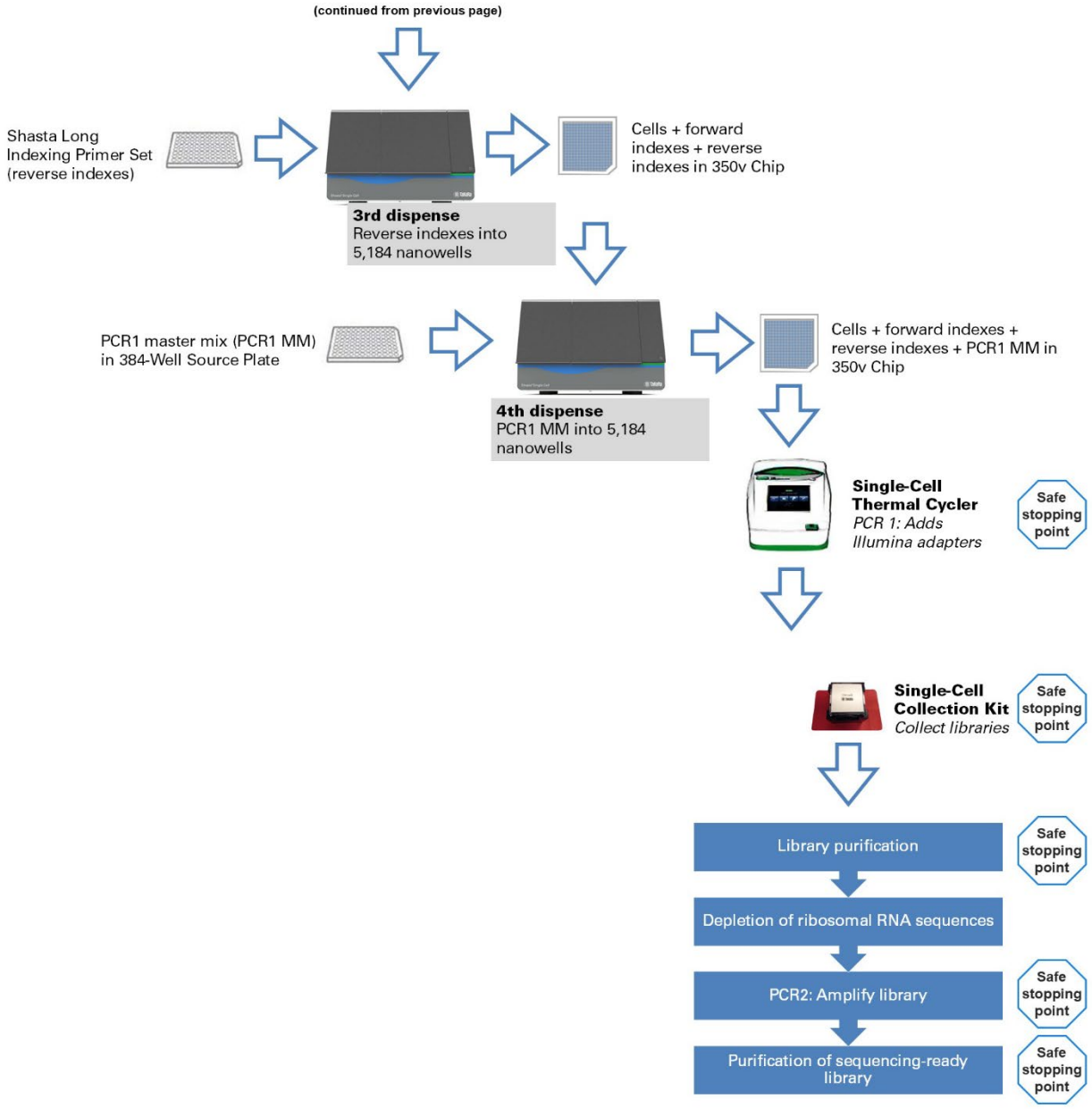


Figure 1. Complete Shasta Total RNA-Seq Kit workflow. For details on the steps outlined here, please refer to the user manual.

II. Experiment Planner

A. Starting Sample Requirements

- There will be cell loss during cell fixation, washing, RT, and combining cells from the RT plate (Figure 2). We recommend having ≥2 x 10^6 cells if starting with 1 single-cell suspension sample and ≥1 x 10^6 cells/sample when starting with ≥2 samples. Samples with low cell numbers are not recommended but may be acceptable with conditions (See Section II.D).
- To achieve a maximum output of 100,000 cells per experiment, the entire RT plate requires 1 x 10^6 cells/plate for the RT reaction (about 10,000 cells per RT well). If less than 1 x 10^6 cells/plate is used, you may end up with an output of less than 100,00 cells per experiment.

Section II.B provides additional information about the sample number and the RT plate layout.

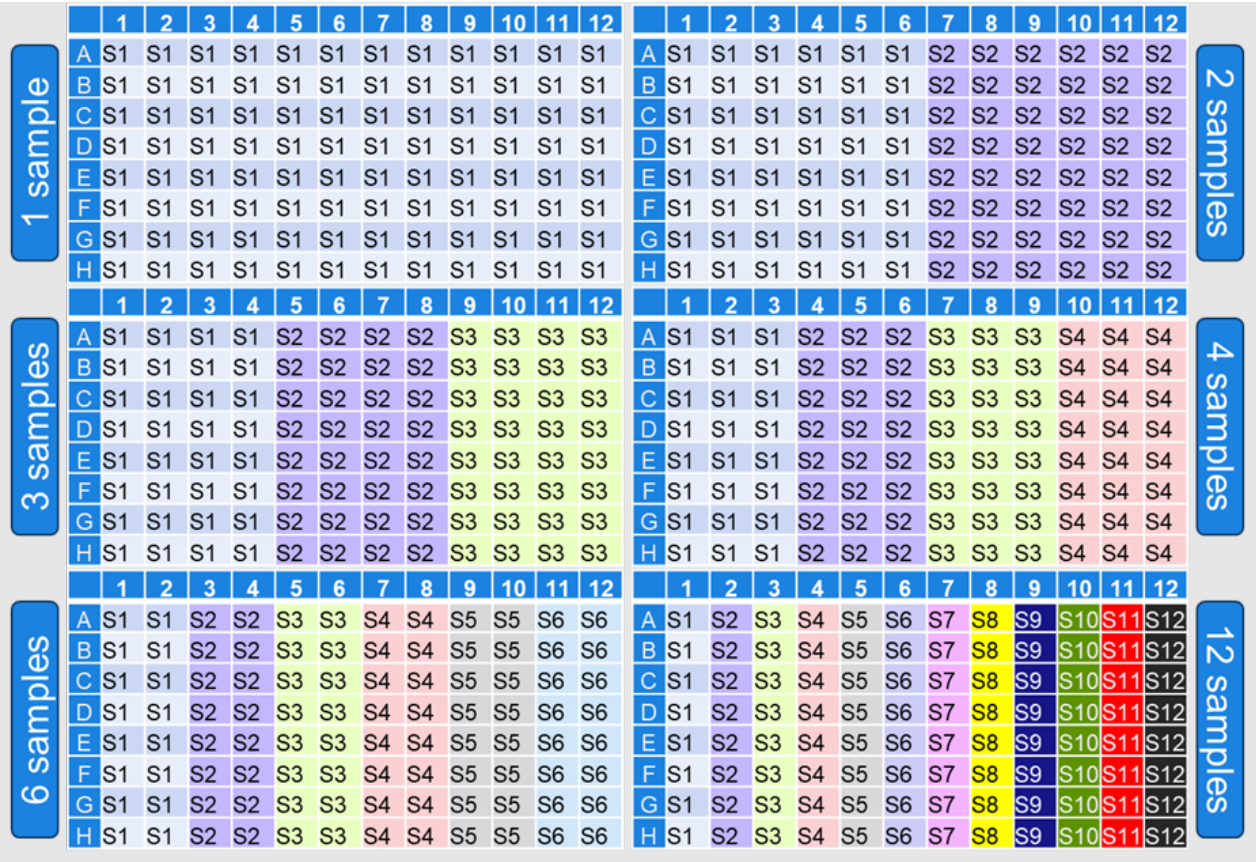


Figure 2. Examples of RT plate layouts with different sample numbers.

B. Sample Differentiation During RT with TSO Barcodes

The Shasta Total RNA-Seq kit differentiates samples using the sample barcodes in the TSO-index plate. One experiment uses one TSO plate containing 96 sample barcodes.

The current Shasta Total RNA-Seq kit provides reagents supporting up to 12 samples per experiment. Since the maximum output of the kit is about 100,000 cells per experiment across all samples, fewer cells per sample will be output for analysis if more samples are used.

Example TSO plate layouts based on sample numbers are shown in Figure 2 (above). You can adjust the sample layout and well numbers per sample based on your own research needs.

NOTE: The entire 96-well plate for the RT reaction should contain around 1 x 10^6 cells/plate so that sufficient cells will be recovered for the downstream cell dispensing to achieve a maximum output of 100,000 cells/experiment.

C. Fragmentation Mix

1. Prepare the fragmentation mix with 25% overage after determining the plate layout and the number of wells needed for each sample. The formula below outlines the components and volumes needed for one well in the 96-well plate. Scale-up as necessary.

5.0 µl	5X Fragmentation Buffer
5.0 µl	Fixed cells per sample
0.6 µl	RNase Inhibitor (40 U/µl)
10.6 µl*	**Total volume per well**

*This includes +25% for overage.

Example 1

If six samples are to be tested and each sample will be loaded with an equal number into 16 wells for the RT reaction, a minimum of 80 µl of fixed cells for each sample is required. The fragmentation mix formula is:

5.0 x 16 =	80.0 µl	5X Fragmentation Buffer
5.0 x 16 =	80.0 µl	Fixed cells per sample
0.6 x 16 =	9.6 µl	RNase Inhibitor (40 U/µl)
10.6 x 16 =	**169.6 µl**	**Total volume per sample**

2. Use a 200 µl wide-bore pipette tip to pipette the fragmentation mix up and down 4–5 times to mix. Load 8.5 µl of the fragmentation mix into the bottom of each well of a fresh 96-well plate to create the fragmentation cell plate. DO NOT vortex.

D. Adjusting Sample Distribution for Samples with Low Cell Numbers

Lower cell input (for example, 1 x 10^5 fixed cells) is not recommended but may be acceptable with the following conditions:

In the case where certain samples have a low number of cells, other samples will need to be added to the remaining wells of the RT plate. The goal is to have around 1 x 10^6 cells in the RT plate. The low-cell input sample will be a smaller proportion of the final data (see the example below).

Example:
Three samples are to be tested. After fixation of the samples, the fixed cell numbers for each sample are as follows:

1. Sample 1 (S1): 10,000 cells
2. Sample 2 (S2): 500,000 cells
3. Sample 3 (S3): 500,000 cells

The distribution would follow the logic outlined below:

- S1 (10,000 cells) has only enough cells for one well. S2 and S3 will be loaded to the rest of the plate so that the plate contains a total of 1 x 10^6 cells (see Figure 3).
- Therefore, the three samples would have the following proportion in the final output total of 100,000 cells:
  - S1 = 10,000 / 1 x 10^6 X 100,000 cells = 1,000 cells
  - S2 = 490,000 / 1 x 10^6 X 100,000 cells = 49,000 cells
  - S3 = 500,000 / 1 x 10^6 X 100,000 cells = 50,000 cells

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S2	S2	S2	S2	S2	S3	S3	S3	S3	S3	S3
B	S2	S2	S2	S2	S2	S2	S3	S3	S3	S3	S3	S3
C	S2	S2	S2	S2	S2	S2	S3	S3	S3	S3	S3	S3
D	S2	S2	S2	S2	S2	S2	S3	S3	S3	S3	S3	S3
E	S2	S2	S2	S2	S2	S2	S3	S3	S3	S3	S3	S3
F	S2	S2	S2	S2	S2	S2	S3	S3	S3	S3	S3	S3
G	S2	S2	S2	S2	S2	S2	S3	S3	S3	S3	S3	S3
H	S2	S2	S2	S2	S2	S2	S3	S3	S3	S3	S3	S3

S1 = 10k cells | S2 = 490k cells | S3 = 500k cells

Figure 3. An example of RT plate layout with non-uniform distribution with one low cell number sample.

E. Before Cell Dispense

After the RT reaction and before cell dispensing, count the combined cells and check the cell morphology under a microscope. Use Table 1 as a guide to dilute cells before cell dispensing.

Table 1. Cell dilution guidance for cell dispensing.

Cell concentration for dispensing (cells/ml)*	Final volume	Cell count per nanowell	Expected cell number output in final data per chip
20,000–50,000	1 ml	1–2	5,000–10,000
50,000–80,000	1 ml	2–4	10,000–20,000
80,000–200,000	1 ml	4–10	20,000–50,000
200,000–400,000	1 ml	10–20	50,000–100,000

*If the cell concentration is lower than 20,000 cells/ml, the library may not have enough yield. If the cell concentration is greater than 400,000 cells/ml, it may result in higher doublets and should be diluted to 400,000 cells/ml or less.

Example: To achieve the maximum throughput, the cell concentration needs to be 400,000 cells/ml in a 1 ml volume for cell dispensing.

F. PCR 2 Cycle Number Recommendations

IMPORTANT: We do not recommend adjusting the number of cycles in the first PCR (PCR 1) step.

For the number of cycles in the second PCR (PCR 2) step, please use Table 2 as a guide.

Table 2. PCR 2 cycle number guidelines.

Cell type	Cell concentration for dispensing	Expected cell number output in final data per chip	PCR 2 cycles
High mRNA content cells	20,000–50,000	5,000–10,000	18–20
	50,000–80,000	10,000–20,000	17–18
	80,000–200,000	20,000–50,000	15–17
	200,000–400,000	50,000–100,000	15
Low mRNA content cells	20,000–50,000	5,000–10,000	22–25
	50,000–80,000	10,000–20,000	20–22
	80,000–200,000	20,000–50,000	18–20
	200,000–400,000	50,000–100,000	17–18

It is possible that the suggested number of cycles will not generate enough material for downstream sequencing. In these cases, we recommend adding 100 µl of magnetic beads (1X) to the library and eluting in a final volume of 10–15 µl to enrich the library.

If this still does not give sufficient yield, we recommend further increasing the number of PCR 2 cycles.

Products

Cat. #	Product	Size
640288	Shasta Total RNA-Seq Kit - 2 Chip	2 Chips

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