Takara Bio USA, Inc.

SMARTer® PicoPLEX® Single Cell WGA Kit User Manual

Cat. No. R300671, R300672, R300673
(091818)

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I. Introduction

The SMARTer PicoPLEX Single Cell WGA Kit has been developed specifically for reproducible amplification of DNA from single cells, as well as for reproducible amplification of picogram quantities of DNA. Cell lysis and a custom DNA pre-amplification is followed by very low background amplification to yield micrograms of product in under three hours.

A. Overview

The SMARTer PicoPLEX Single Cell WGA Kit includes all necessary reagents for its designed purpose of extracting DNA and amplifying entire genomes in a single tube from a single cell in less than 3 hrs.

Suitable applications for the PicoPLEX WGA Kit include:

- Copy Number Variation (CNV) analysis using NGS, oligonucleotide aCGH, BAC aCGH, and qPCR
- Target enrichment or amplicon enrichment for mutation detection
- SNP Genotyping

B. Principle

SMARTer PicoPLEX Single Cell WGA is based on Takara Bio's patented PicoPLEX technology for single-cell genomic DNA (gDNA) amplification, which uses multiple cycles of quasi-random priming for reproducible sample preparation (Figure 1, below). The quasi-random nature of the primers ensure that the primers consistently bind to many sites on the genome and therefore lead to reproducible amplification of the genome.

The SMARTer PicoPLEX Single Cell WGA Kit follows a single-tube, 3-hour workflow. In the first step, a single cell or up to 10 cells are efficiently lysed to release gDNA (note that naked DNA may also be used in this step). In the second step, proprietary quasi-random primers bind to selective sites on the gDNA which is then pre-amplified in a linear manner. In the final step, the DNA is further amplified exponentially.

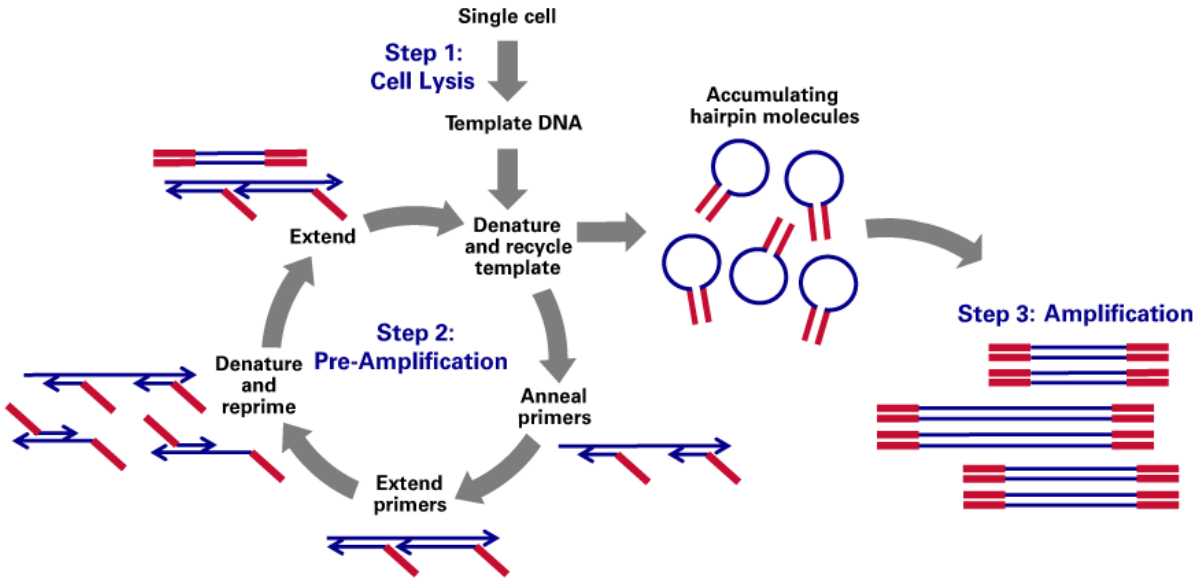

Figure 1. SMARTer PicoPLEX Single Cell WGA technology. A three-step, single-tube reaction that starts with a single cell. Cellular gDNA extracted in Step 1 is used as template for multiple cycles of quasi-random priming and linear amplification (Step 2) followed by exponential amplification (Step 3).

C. SMARTer PicoPLEX Single Cell WGA Workflow

The SMARTer PicoPLEX Single Cell WGA Kit workflow (Figure 2, below) is highly streamlined and consists of the following three steps:

- **Cell Lysis Step** for efficient lysis and release of gDNA
- **Pre-Amplification Step** for reproducible and consistent priming and multiple cycles of linear amplification of the released DNA
- **Amplification Step** for exponential amplification

The three-step SMARTer PicoPLEX Single Cell WGA workflow takes place in the same tube or plate and is completed in less than 3 hours.

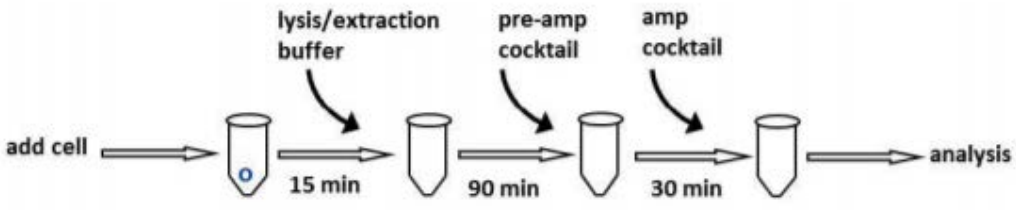

Figure 2. SMARTer PicoPLEX Single Cell WGA workflow overview. Steps involved in PicoPLEX Single Cell WGA sample preparation starting from a single cell

II. List of Components

The SMARTer PicoPLEX Single Cell WGA Kit consists of the following components.

NOTE: These components have been specifically designed to work together and are optimized for this particular 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.

Please make sure to spin down tubes to collect all the liquid at the bottom before first use.

Table I. SMARTer PicoPLEX Single Cell WGA Kit Contents

Cap Color	Component Name	R300671 (24 rxns)	R300672 (96 rxns)	R300673 (480 rxns) (5X R300672)
Green	Cell Extraction Buffer	120 µl	485 µl	5 x 485 µl
Violet	Extraction Enzyme Dilution Buffer	120 µl	485 µl	5 x 485 µl
Yellow	Cell Extraction Enzyme	5 µl	20 µl	5 x 20 µl
Red	Pre-Amp W Buffer	120 µl	485 µl	5 x 485 µl
White	Pre-Amp W Enzyme	5 µl	20 µl	5 x 20 µl
Orange	Amplification Buffer	630 µl	2 x 1,300 µl	10 x 1,300 µl
Blue	Amplification Enzyme	25 µl	80 µl	5 x 80 µl
Clear	Nuclease-Free Water	1000 µl	4 x 1,000 µl	20 x 1,000 µl
	Quick Protocol			

IMPORTANT: The SMARTer PicoPLEX Single Cell WGA Kit is shipped on dry ice and should be stored at –20ºC upon arrival.

III. Getting Started

A. Required Materials

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

- Hot-lid PCR thermal cycler (real-time instrument optional)

  NOTE: See Thermal Cycler Considerations in Section III. F.

- Centrifuge
- 96-well nuclease-free thin-wall PCR plates or PCR tubes

  NOTE: Select appropriate tubes or plates that are compatible with the thermal cyclers and/or real-time thermal cyclers used. Use appropriate caps or sealing films and seal thoroughly to eliminate evaporation during cycling conditions. Evaporation could reduce robustness and reproducibility of the reactions.

- Nuclease-free nonsticky 1.5-ml tubes (USA Scientific, Cat. No. 1415-2600)
- PCR plate seals
- Single-channel pipette: 10 µl, 20 µl, and 200 µl
- Multi-channel pipettes: 20 µl and 200 µl
- Low-binding filter pipette tips: 10 µl, 20 µl, 200 µl

- Phosphate-buffered saline (1X PBS free of Mg^{2+}, Ca^{2+}, and BSA, for washing cells if starting from single cells)
- 80% ethanol: freshly made for each experiment
- Nuclease-free water

B. Optional Materials

The following reagents are not required but recommended for monitoring amplification in real time.

- EvaGreen fluorescent dye (Biotium, Cat. No. 31000-T)
- Fluorescein Calibration Dye (Bio-Rad Laboratories, Cat. No. 170-8780)

C. Starting Material

Cells

Single mammalian cells (1–10 cells) from a broad range of sources can be used.

Genomic DNA

In place of whole cells, small amounts (15 pg to 60 pg) of purified genomic DNA can be used as starting material for sample preparation. Purified eukaryotic, prokaryotic, fungal, or viral DNA can also be used as starting material.

- 1–10 human cells (e.g., blastomeres, polar bodies, trophoblastic cells, amniocytes, CTCs, cultured cells, flow-sorted cells)
- 1,000–10,000 bacterial cells
- Isolated DNA (15–60 pg of human DNA)
- Sorted chromosomes
- Intact or fragmented, single- or double-stranded DNA
- Maximum sample volume of 5 µl

D. Key Considerations for Cell Preparation

Cell collection

Single cells collected by dilution, micro-manipulation and flow-sorted (stained by surface antibodies or unstained) are suitable to use with this kit. Cell fixation can be used but should be avoided for optimal results.

Clonally expanded cells

Use of clonally expanded cells with genetic homogeneity will help achieve optimal results as many cultured cell lines have unstable genomes not evident when averaging analysis even over a few cells.

Number of cells

Up to 10 cells can be used per reaction; however, the major advantage of the SMARTer PicoPLEX Single Cell WGA Kit is that it provides robust and reproducible amplification with a single cell.

Washing cultured cells

Minimize non-cellular DNA contaminations by washing cells with sterile, nuclease-free 1X PBS buffer (free of Mg^{2+}, Ca^{2+}, and BSA) freshly prepared from a 10X PBS stock. The carryover PBS volume must not exceed 2.5 µl in the cell lysis step of the reaction.

Cells obtained by approaches described above can be stored for future use at –80ºC by flash freezing or processed directly following the SMARTer PicoPLEX Single Cell WGA Protocol.

E. Safety Guidelines

Follow standard laboratory safety procedures and wear a suitable lab coat, protective goggles, and disposable gloves to ensure personal safety as well as to limit potential cross contaminations during the sample preparation and subsequent amplification reactions. For more information please refer to the appropriate Material Safety Data Sheets (MSDS) available online at takarabio.com.

F. Thermal Cycler Considerations

Thermal cycling and heated lid

Use a thermal cycler equipped with a heated lid that can handle 75-µl reaction volumes. Set the temperature of the heated lid to 100–105ºC to avoid sample evaporation during incubation and cycling.

Thermal cycler ramp rates

We recommend a ramp rate of 3°C/s–5°C/s; higher ramp rates are not recommended and could impact the quality of the amplified product

Monitoring amplification during the Amplification Reaction

Amplification can be monitored using a real-time thermal cycler with the addition of fluorescent dyes to the reaction (not provided with the kit, see Optional Materials in Section III.B) (Figure 3). If a regular thermal cycler is used instead, there is no need to add the dyes; substitute an appropriate amount of nuclease-free water to adjust the volumes in the Amplification Master Mix. In the absence of real-time monitoring, amplification can be analyzed by gel or by analysis of an aliquot of the product using the Agilent Bioanalyzer (see Quantification, Section IV.D).

Depending on the real-time instrument used, select an appropriate calibration dye and mix with EvaGreen detection dye mix (see Amplification Step, Section IV. C). For some real-time instruments calibration dye may not be needed; please refer to the real-time thermal cycler instrument's user manual.

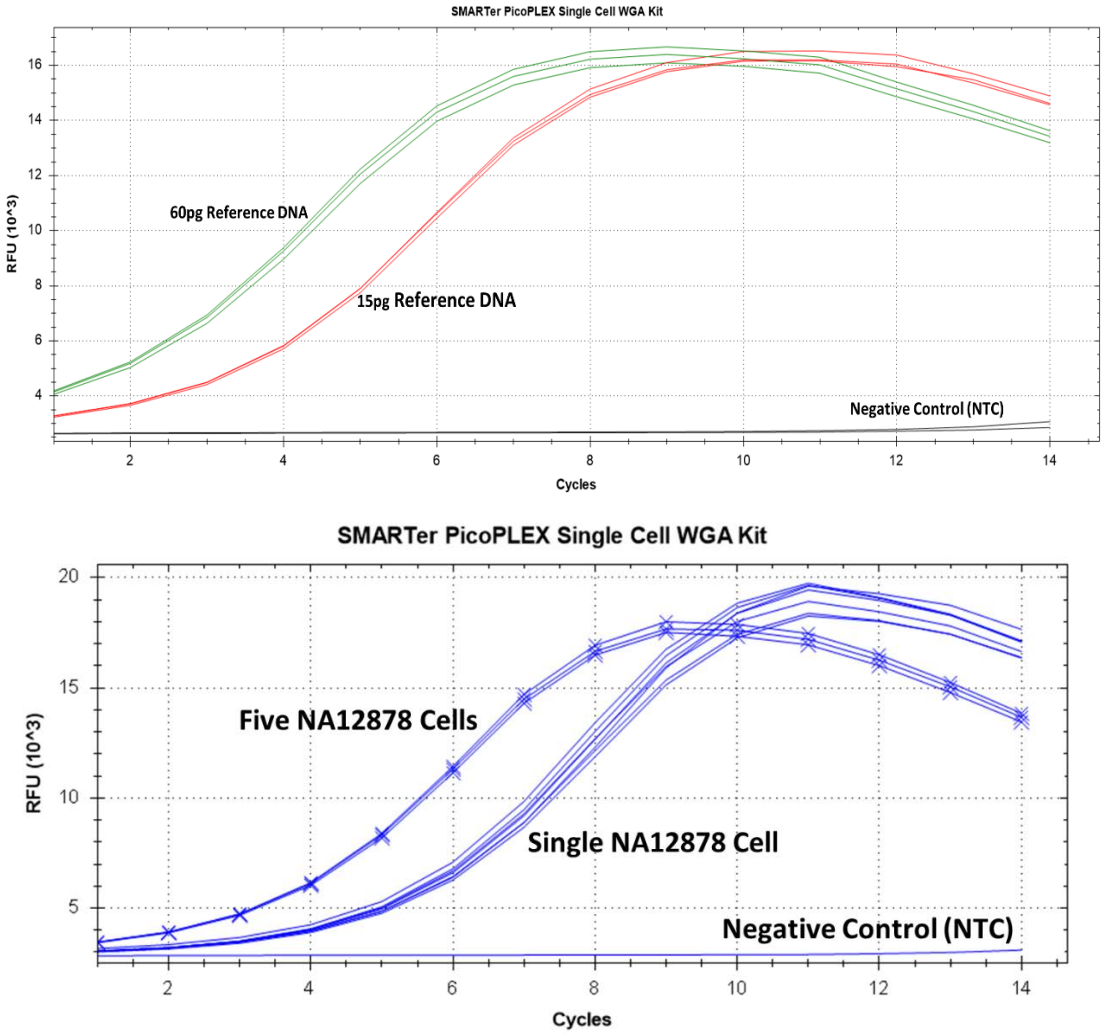

Figure 3. Real-time analysis of amplification products using SMARTer PicoPLEX Single Cell WGA. A typical real-time amplification analysis of products prepared with the SMARTer PicoPLEX Single Cell WGA Kit using (top) three reference DNA samples (NA12878) at 15 pg (red) or 60 pg (green) or (bottom) five or one GM12878 cells (blue), relative to their No Template Controls (NTC, black or blue). Results were visualized using a CFX96 Touch Real-Time PCR Detection System with EvaGreen as the dye.

G. Positive and Negative Controls

Include appropriate positive and negative controls in the experimental design to help verify that the test reactions proceeded as expected. A good choice for the positive (reference) control is single donor gDNA. Note that a diploid human cell contains ~6 pg of genomic DNA. At single-molecule levels, stochastic sampling will result in aliquots receiving zero, one, two, or more copies of the intended target region as dictated by the Poisson distribution. Therefore, it is suggested to use a minimum of 15 pg as a surrogate for single cell equivalence.

Always prepare fresh dilutions of gDNA to use as the positive control, and include a negative control (No Template Control, NTC) without cells or gDNA, containing only 2.5 µl of PBS or TE buffer (10 mM Tris pH 8.0, 0.1 mM EDTA).

The reference DNA positive control and experimental samples (cells) should work equally well. If the experimental samples contain any carryover contaminant(s) in the buffer, the downstream reactions may be impacted; inclusion of controls would help explain such problems. Please refer to Appendix A for details on preparing working dilutions of the reference gDNA from stock solutions.

H. Preparation of Master Mixes

A master mix with appropriate buffers and enzymes must be prepared at each workflow step based on the number of reactions to be performed Prepare ~5% excess of each master mix to allow for pipetting losses. Transfer the enzymes to ice just prior to use and centrifuge briefly to ensure all the contents are at the bottom of the tube. Thaw the buffers, vortex briefly, and centrifuge prior to use. Keep all the components and master mixes on ice. Once the master mix is prepared, mix the contents several times gently with a pipettor while avoiding introduction of excessive air bubbles and briefly centrifuge prior to dispensing into the PCR plate or tube(s).

IV. Protocols

A. Protocol: Cell-Lysis Step

1. Template Preparation Step

Template Preparation Reagents

Reagent	Cap Color
Cell Extraction Buffer	Green
Extraction Enzyme Dilution Buffer	Violet
Cell Extraction Enzyme	Yellow

NOTE: Assemble all reactions in thin-wall 96-well PCR plates or tubes that are compatible with the thermal cycler and/or the real-time cycler used.

1. Prepare samples as described below:
   - **Test samples:** Equilibrate cells (1 to 10) or DNA (15–60 pg) to a final volume of 5 µl by adding an appropriate amount of Cell Extraction Buffer
     NOTE: If a single cell is isolated in PBS, do not exceed 2.5 µl of PBS; adjust the final volume to 5 µl with Cell Extraction Buffer.
   - **Positive control reaction using reference DNA:** Assemble reactions using freshly diluted reference gDNA at an input amount of 15 pg (refer to Appendix A for preparing dilutions of reference DNA) by adding 2 µl of a 7.5 pg/µl dilution. Add 3 µl of Cell Extraction Buffer to each tube to bring the final volume of each reaction to 5 µl.
   - **Negative control reactions/no-template controls (NTCs):** Assemble NTC with 2.5 µl of PBS or TE buffer (10 mM Tris pH 8.0, 0.1 mM EDTA) and adjust the final volume to 5 µl with Cell Extraction Buffer.
2. Prepare **Cell Extraction Master Mix** as described below for the chosen number of reactions plus 5% extra. Mix thoroughly with a pipette. Keep on ice until used.

   4.8 µl Extraction Enzyme Dilution Buffer
   0.2 µl Cell Extraction Enzyme
   5 µl Total Volume

3. Assemble the **Lysis Reactions Mixture** as shown in the table below. To each 5 µl of equilibrated sample from Step 1 above, add 5 µl of **Cell Extraction Master Mix**. The final reaction volume at this stage will be 10 µl.

NOTE: Do not touch the cell or DNA sample with the pipet tip.

5 µl	Sample
5 µl	Cell Extraction Master Mix
10 µl	Total Volume

4. Seal the PCR plate using an appropriate sealing film or close the tube(s) tightly.
5. Centrifuge briefly to ensure the entire volume of the reaction is collected at the bottom of each well.
6. Place the plate or tube(s) in a thermal cycler with heated lid set to 100°C–105°C. Perform the **Lysis Reaction** using the conditions in the table below:

Lysis Reaction	
Temperature	**Time**
75°C	10 min
95°C	4 min
22°C	Indefinitely

7. After the cycler reaches 22ºC, remove the plate or tube(s) and centrifuge briefly.
8. Proceed to the **Pre-Amplifcation Step**.

NOTE: Following the Cell-Lysis Step, continue to the Pre-Amplification Step in the same plate or tube(s).
It is recommended to complete the experiment without any interruptions. If you must pause, the Lysis Reaction Mixture can be stored at 4°C for up to 3 hours.

B. Protocol: Pre-Amplification Step

Pre-Amplification Reagents

Reagent	Cap Color
Pre-Amp W Buffer	Red
Pre-Amp W Enzyme	White

1. Prepare **Pre-Amplification Master Mix** on ice as indicated below for the chosen number of reactions plus 5% extra. Mix gently several times and keep on ice until used.

4.8 µl	Pre – Amp W Buffer
0.2 µl	Pre – Amp W Enzyme
5 µl	Total Volume

2. Remove the seal on the plate or open the tube(s) containing the Lysis Reaction Mixture.
3. Add 5 µl of **Pre-Amplification Master Mix** to each **Lysis Reaction Mixture** to assemble the Pre-Amplifications as shown in the table below. Final reaction volume at this stage is 15 µl.

10 µl from Lysis Reaction
5 µl Pre – Amplification Master Mix
15 µl Total Volume

4. Seal the plate or tube(s) tightly.
5. Centrifuge briefly to collect the contents to the bottom of each well.
6. Return the plate or tube(s) to the thermal cycler with the heated lid set to 101°C–105°C. Perform the **Pre-Amplification Reaction** using the following cycling conditions:

Pre-Amplification Reaction		
Temperature	**Time**	**Number of cycles**
95°C	2 min	1 cycle
95°C	15 sec	12 cycles
15°C	50 sec	
25°C	40 sec	
35°C	30 sec	
65°C	40 sec	
75°C	40 sec	
4°C	Indefinitely	1 cycle

NOTE: Following the Pre-Amplification Reaction, continue the Amplification Step (Section IV.C, below) in the same plate or tube(s) maintained at 4°C.

It is recommended to complete the experiment without any interruptions. If you must pause, the Pre-Amplified Mixture can be stored under the following conditions:

- overnight at 4°C
- Up to 2 weeks at –20°C

C. Protocol: Amplification Step

Amplification Reagents

Reagent	Cap Color
Amplification Buffer	Orange
Amplification Enzyme	Blue
Nuclease-Free Water	Clear
Fluorescent Dyes (optional)	

NOTE: The yield of amplified product can vary depending upon sample condition, sample type, input amount, the thermal cycler used and the PCR product cleanup procedure. For 15 pg starting inputs of intact, purified gDNA, the number of amplification cycles recommended in this protocol will typically yield 8–12 µg of amplified genetic material (e.g. recovery after Ampure cleanup is ~8–12 µg, and recovery after other methods typically yields 10–12 µg). Average size of the amplified product ranges between 100 bp to 1 kb.

Amplification Protocol

1. Prepare the **Amplification Master Mix** as described below for the chosen number of reactions. Mix gently several times, and keep on ice until used.

25.0 µl	Amplification Buffer
0.8 µl	Amplification Enzyme
34.2 µl	Nuclease – Free Water (plus fluorescent dyes *)
60 µl	Total Volume

NOTES:

- **If monitoring in real-time:** Fluorescence dyes* (for detection and optical calibration) are added when monitoring amplification in real time during cycling. Please refer to the real-time PCR instrument's user manual for calibration dye recommendations. The volume of detection and calibration dyes plus nuclease-free water should not exceed 34.2 µl.
  Example: Mix 90 µl of 20X EvaGreen dye (Biotium, Cat. No. 31000-T, EvaGreen Dye, 20X in water) with 10 µl of 1:500 dilution of Fluorescein (Bio-Rad Laboratories, Cat. No. 170-8780, Fluorescein Calibration Dye). Add 2.5 µl of this mix and 31.7 µl of nuclease-free water per reaction to prepare the Amplification Master Mix.
- **If not monitoring in real-time:** If a regular thermal cycler is used, there is no need to add the dyes; use 34.2 µl of nuclease-free water per reaction in the Amplification Master Mix.

2. Remove the seal on the PCR plate or open the tube(s).
3. Add 60 µl of the **Amplification Master Mix** to each well.

15 µl	Pre – Amplification Reaction Product
60 µl	Amplification Master Mix
75 µl	Total Volume

4. Seal the PCR plate or tubes tightly and centrifuge briefly to collect the contents to the bottom of each well.

NOTE: Use optical sealing tape if a real-time thermal cycler is used.

5. Return the plate or tube(s) to the real-time thermal cycler with the heated lid on and perform the **Amplification Reaction** using the cycling conditions indicated below:

Amplification Reaction		
Temperature	**Time**	**Number of cycles**
95°C	2 min	1 cycle
95°C	15 sec	14 cycles**
65°C	1 min	
75°C*	1 min	
4°C	Indefinitely	1 cycle

*Acquire fluorescence data at this step, if monitoring amplification in real-time.

** The number of cycles in the Amplification Reaction may be modified by the Amplification Guide in Table II.

Table II. Cycles in the Amplification Reaction Guide

Amplification Guide	
# of Cycles	**Expected Yield (µg)**
6	0.5-2
8	1-3
10	4-6
12	8-10
14	9-12

NOTE: Actual yield may vary depending on input quantity and purification method. Values shown here correspond to an input of 15 pg of intact, purified DNA.

6. At the end of amplification, remove the PCR plate or tube(s) from the thermal cycler and centrifuge briefly to collect the contents to the bottom of each well.

NOTE: At this stage, samples can be processed for purification immediately or stored frozen at –20°C for later processing.

D. Protocol: Product Quantification

There are several approaches available for quantification, including UV absorption, fluorescence detection, and sizing and quantification using the Agilent Bioanalyzer. It is important to understand the benefits and limitations of each approach. UV absorption/fluorescence detection-based methods (e.g., Nanodrop, Qubit 2.0 Fluorometer, or Quant-iT PicoGreen dsDNA Assay Kit; Thermo Fisher Scientific) simply quantify total nucleic acid concentration. The Agilent Bioanalyzer system provides sizing and quantitation information about the genetic material analyzed.

To quantify SMARTer PicoPLEX Single Cell WGA material by using the BioAnalzyer (Figure 6, below), remove an aliquot of each sample product and dilute 1:20. Load a 1-µl aliquot of this diluted sample onto a Bioanalyzer high sensitivity DNA chip (Agilent Technologies, Inc; Cat. No. 5067-4626).

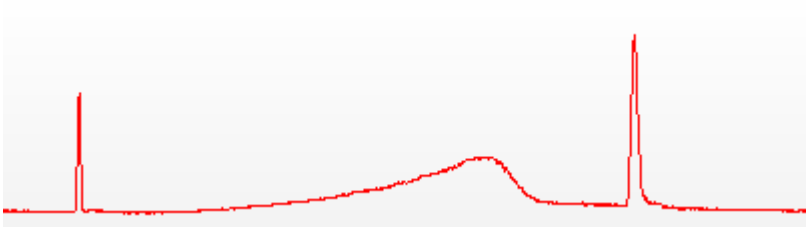

Figure 4. Bioanalyzer analysis of products prepared using SMARTer PicoPLEX Single Cell WGA. Samples were prepared using 15 pg of reference DNA with the SMARTer PicoPLEX Single Cell WGA Kit. An aliquot of product was diluted to 1 ng/µl and 1 µl of this diluted sample was loaded on a Bioanalyzer using a high sensitivity DNA chip (Agilent Technologies, Inc.). Electropherogram results above show a broad size range distribution.

Appendix A: Reference DNA Dilution

A. Overview

Single donor human genomic DNA is ideal for use as positive control DNA (e.g., Human Genomic DNA male, 1 mg/ml, Zyagen, Cat. No. GH-180M; Human Genomic DNA, female, 1 mg/ml, Zyagen, Cat. No. GH-180F; Control DNA Male 2800M, 10 µg/ml, Promega, Cat. No. DD7101). Follow the steps below to prepare the working dilutions for the reference genomic DNA. At the end of each dilution step, mix the contents gently and centrifuge briefly before going to the next dilution step. Always use freshly diluted DNA for positive control reactions.

B. Protocol

All reference DNA dilutions are carried out using low EDTA TE buffer, pH 8.0 (10 mM Tris pH 8.0, 0.1 mM EDTA) in 500 µl low-binding microcentrifuge tubes.

1. Prepare a working stock solution of 1000 pg/µl, by appropriately diluting an aliquot of the original stock DNA.
2. Pipet 14 µl of TE buffer (low EDTA) into a microcentrifuge tube and add 6 µl of the 1000 pg/µl reference DNA working stock solution from Step 1 to achieve a final concentration of 300 pg/µl.
3. Pipet 36 µl of TE buffer (low EDTA) into a second microcentrifuge tube, and add 4 µl of the 300 pg/µl DNA stock solution from Step 2 to achieve a final concentration of 30 pg/µl.
4. Pipet 18 µl of TE buffer (low EDTA) into a third microcentrifuge tube, and add 6 µl of 30 pg/µl stock solution from Step 2 to achieve a final concentration of 7.5 pg/µl.
5. To prepare the final concentrations:
   - **60 pg of reference DNA input:** Use 2 µl of the 30 pg/µl DNA from Step 3
   - **15 pg of reference DNA input:** Use 2 µl of the 7.5 pg/µl DNA from Step 4.

Appendix B: Troubleshooting Guide

Table III. Troubleshooting Guide for the SMARTer PicoPLEX Single Cell WGA Kits.

Problem	Potential Cause	Suggested Solutions
Single-cell amplification curve looks like no-cell control amplification curve or does not produce amplified product	Sample tube or well did not contain a cell	Confirm that the cell collection method reproducibly results in a single cell per tube or well
	Improper sample preparation	Follow instructions above
	Improper purification or quantification	Follow instructions above
Single-cell amplification curve reaches “plateau” phase earlier than 15-pg control DNA reaction	More than one cell in sample	Confirm that the cell-collection method reproducibly results in a single cell per tube or well
	Single-cell sample is contaminated with extraneous DNA	Use fresh, BSA-free PBS
No-cell control amplification curve appears early or produces yield similar to single-cell reaction products	Control solution is contaminated with DNA	Use fresh control solution
	Work area is contaminated with DNA	Clean work area thoroughly and use PCR-dedicated plastics and pipettes
	Kit has become contaminated with DNA	Use a fresh kit

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