

Takara Bio USA, Inc.

PicoPLEX® Gold Single Cell DNA-Seq Kit User Manual

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(112719)

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I. Introduction

A. Overview

The PicoPLEX Gold Single Cell DNA-Seq Kit is designed to generate Illumina® NGS libraries from a small number of cells (1–5) or an equivalent amount of purified genomic DNA. The kit includes reagents for lysing, extracting, and amplifying the genomic DNA from single cells and constructing the amplified DNA into Illumina NGS libraries. The kit features a streamlined workflow consisting of four steps, from input to amplified NGS libraries, that can be completed in less than three hours.

The PicoPLEX Gold Single Cell DNA-Seq Kit is based on our patented PicoPLEX technology for single-cell whole genome amplification (Figure 1). The kit uses high-fidelity DNA polymerases and optimized primers and is suitable for accurate and reproducible profiling of copy number variants (CNVs), single-nucleotide variants (SNVs), indels, and other small structural variants. The kit is compatible with single cells and purified genomic DNA from humans.

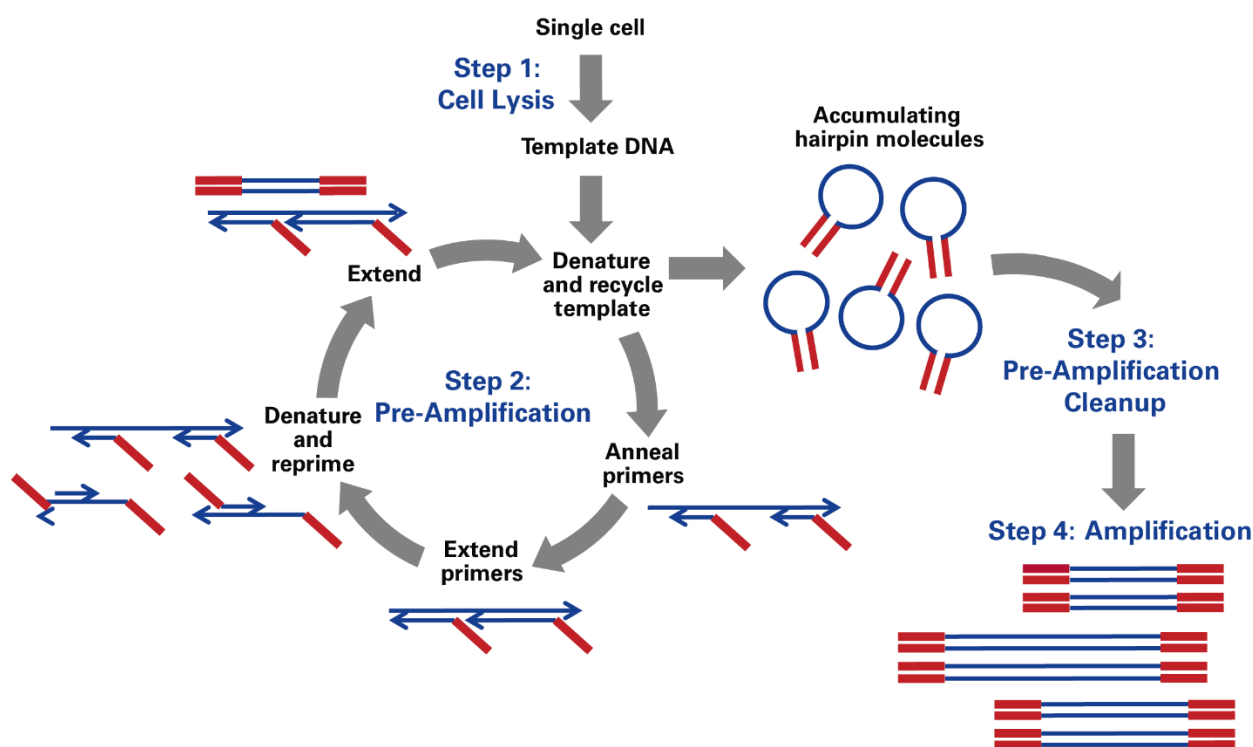


Figure 1. PicoPLEX Gold Single Cell DNA-Seq technology: a four-step reaction that starts with a single cell. Cellular gDNA extracted in Step 1 is used as template for multiple cycles of quasi-random priming and linear amplification followed by exponential library amplification.

B. PicoPLEX Gold Single Cell DNA-Seq Kit Workflow

The PicoPLEX Gold Single Cell DNA-Seq Kit workflow (Figure 2, below) is highly streamlined and consists of the following four steps:

- **Cell Lysis step** for efficient lysis and release of gDNA
- **Pre-Amplification step** for reproducible and consistent priming with multiple cycles of linear amplification of the released DNA
- **Pre-Amplification Cleanup step** for removing excess primers from the pre-amplification step
- **Amplification step** for exponential amplification and Illumina-compatible indexing for NGS

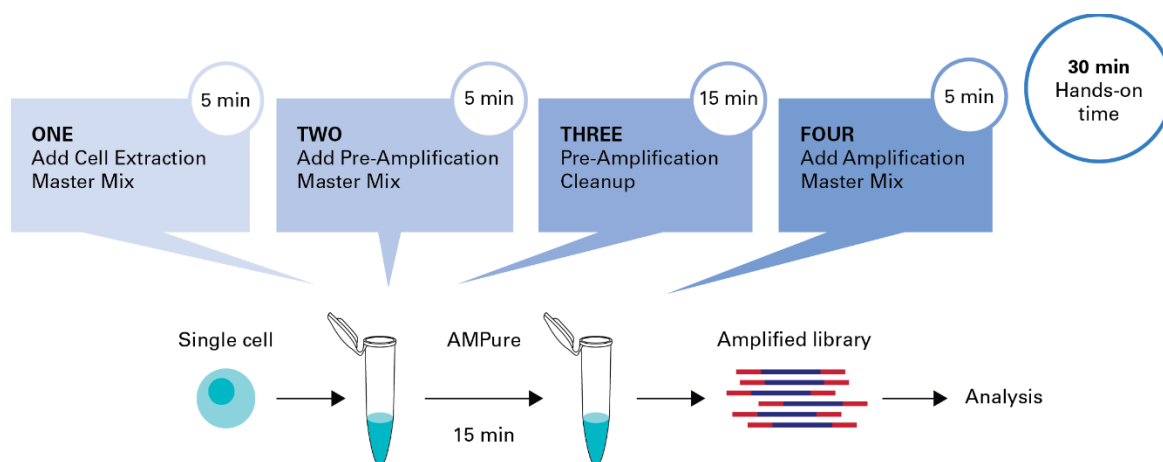


Figure 2. PicoPLEX Gold Single Cell DNA-Seq Kit workflow. The four-step PicoPLEX Gold Single Cell DNA-Seq workflow takes place in two tubes or plates and is completed in less than three hours with just 30 minutes of hands-on time.

II. Components

The PicoPLEX Gold Single Cell DNA-Seq Kit consists of the following components. These components have been specifically designed to work together and are optimized for this particular protocol. Please do not make any substitutions. Make sure to vortex buffers and spin down all components to collect the liquid at the bottom of the tubes before first use. Store all kit components at -20°C .

		R300669	R300670	R300698
		24 rxns	96 rxns	384 rxns
Cap Color	Component Name			
Red	Cell Extraction Buffer	116 μl	461 μl	461 μl x 4
Red	Cell Extraction Enzyme	5 μl	20 μl	20 μl x 4
White	PreAmp Buffer	208 μl	835 μl	835 μl x 4
White	PreAmp Enzyme	31 μl	130 μl	130 μl x 4
Blue	Amplification Buffer	500 μl	1000 μl x 2	1000 μl x 8
Blue	Amplification Enzyme	60 μl	240 μl	240 μl x 4
Clear	Nuclease-Free Water	500 μl	1000 μl	1000 μl x 4

III. Getting Started

A. Required Materials Not Included

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.

- DNA HT Dual Index Kit – 24N (Takara Bio, Cat. No. R400664), DNA HT Dual Index Kit – 96N (Takara Bio, Cat. Nos. R400660–R400663), or DNA Unique Dual Index kits (Takara Bio, Cat. Nos. R400665–R400668)
- Hot-lid PCR thermal cycler (real-time instrument recommended)
- Centrifuge or microcentrifuge
- Vortex mixer
- 96-well nuclease-free thin-wall PCR plates or PCR tubes
- Nuclease-free 1.5-ml tubes

- Optical PCR plate sealing films or caps
- Single-channel pipette: 10 µl, 20 µl, and 200 µl
- Multi-channel pipettes: 20 µl and 200 µl
- Filter pipette tips: 10 µl, 20 µl, 200 µl
- TE Buffer (10 mM Tris, 0.1 mM EDTA, pH8.0).
- Phosphate-buffered saline (1X PBS free of Mg²⁺, Ca²⁺, and BSA)
- 80% ethanol (freshly prepared)
- Single-donor reference DNA (see [Section III.F](#); Positive and Negative Controls)
- Agencourt AMPure XP beads (Beckman Coulter Cat. No. A63880, A63881, A63882)
- Magnetic Beads Separator

NOTES:

- Agencourt AMPure XP beads need to come to room temperature before the container is opened. Therefore, **we strongly recommend aliquoting the beads into 5-ml tubes 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 min). This aliquoting process is also essential for minimizing the chances of bead contamination.
- Immediately prior to use, vortex the beads until they are well dispersed. The color of the liquid should appear homogeneous. Confirm that there is no remaining pellet of beads at the bottom of the tube. Mix well to disperse before adding the beads to your reactions. The beads are viscous, so pipette them slowly.

B. Additional 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. Thermal Cycler Considerations

Thermal cycling and heated lid

Use a thermal cycler equipped with a heated lid that can handle 50 µl reaction volumes. Set the temperature of the heated lid to 100–105°C to avoid sample evaporation during incubation and cycling. Select plates or tubes 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. Evaporation reduces the robustness and reproducibility of the reactions.

Monitoring amplification during the library amplification step

Amplification can be monitored using a real-time thermal cycler with the addition of fluorescent dyes (not provided; [Section III.B](#); Additional Materials) to the reaction (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.

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

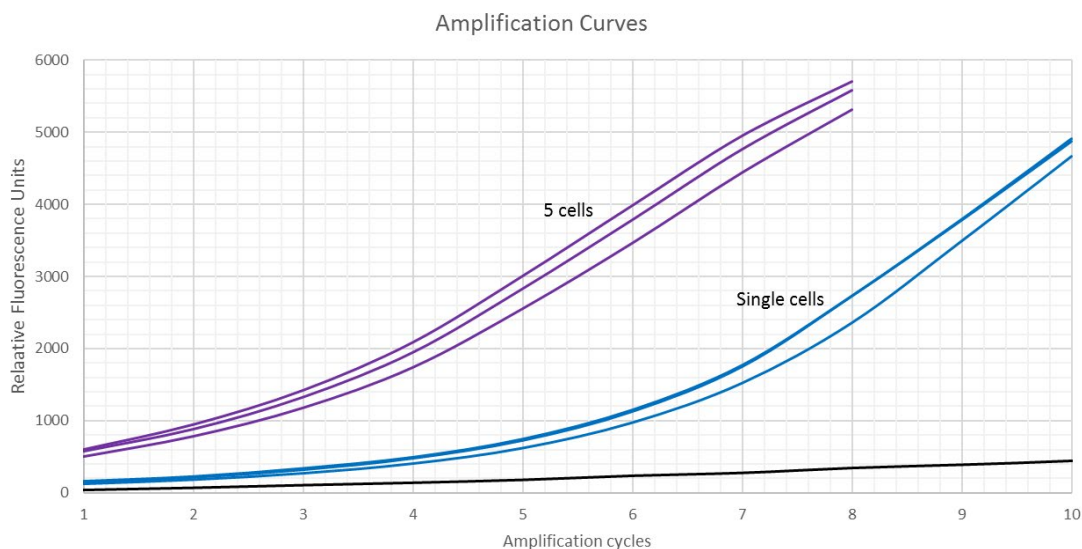


Figure 3. Amplification curves of six libraries prepared with 1 or 5 cells. Real-Time PCR curves for triplicate samples of single cells (blue), and five cells (red) are shown, and non-template control (NTC; black).

D. Input Material Requirements

- One to five (1–5) human cells from sources such as embryos, tumor cells, fixed cells, circulating tumor cells, and cultured clonally expanded cells. Flow-sorted cells stained with surface antibodies are also suitable substrates to generate libraries using PicoPLEX Gold Single Cell DNA-Seq.
- Serial dilutions of gDNA can be prepared as described in [Appendix A](#). Isolated human gDNA from 15 to 30 pg are supported with this kit. Note that a diploid human cell contains ~6 pg 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.

E. Washing the Cells

We recommend washing the cells three times with sterile, nuclease-free 1X PBS buffer (free of Ca^{2+} , Mg^{2+} , and BSA) freshly prepared from a 10X PBS stock before sorting the cell to remove protein carried over from the serum-containing medium. Sorted cells should be stored in sterile, nuclease-free 1X PBS. After washing and sorting, the cells can be processed directly following the PicoPLEX Gold Single Cell DNA-Seq protocol. However, the 1X PBS volume must not exceed 2.5 μl . Alternatively, single cells can be sorted in 5 μl of 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0 and stored frozen at -80°C .

F. Positive and Negative Controls

Including a positive control gDNA (15 pg) and a no-template control (NTC) as a negative control in parallel is recommended to ensure that the reaction proceeds as expected. Serial dilutions of gDNA can be prepared as described in [Appendix A](#).

G. Preparation of Master Mixes

It is recommended to prepare master mixes with appropriate buffers and enzymes at each 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 immediately 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 pipette while avoiding the introduction of excessive air bubbles and briefly centrifuge prior to dispensing into the PCR plate/tubes.

H. Low-Level Multiplexing: Index Tubes

- DNA HT Dual Index Kit - 24N (Takara Bio, Cat. No. R400664) or DNA Unique Dual Index Kit - 24U sets A to D (Takara Bio, Cat. Nos. R400665–R400668) are suitable for low-level multiplexing. Each 48-reaction kit contains 24 tubes with i7 and i5 index primers carrying 8-nt dual index sequences. Please see the Protocol-At-A-Glance for [DNA Unique Dual Index Kit - 24U sets A to D](#) and [DNA HT Dual Index kits](#) for the respective index sequences.
- Select appropriate index combinations that meet Illumina-recommended compatibility requirements. See Index Adapters Pooling Guide from Illumina; Document # 1000000041074 v02 for preparing libraries for Illumina sequencing systems that require balanced index combinations.

NOTE: when creating a sample sheet using Illumina Experiment Manager, improper index combinations will be flagged as invalid.

I. High-Level Multiplexing: Dual-Index Plates

- DNA HT Dual Index Kit - 96N sets A to D (Takara Bio, Cat. Nos. R400660–R400663) are suitable for high-level multiplexing. These kits contain indexed PCR primers carrying the Illumina Nextera® XT v2 index sequences for multiplexing of up to 384 samples. The indexed PCR primers are supplied pre-dispensed in four different barcoded index plates. Please see the Protocol-At-A-Glance for the [DNA HT Dual Index kits](#) for the respective index sequences. For low-plex (2- to 12-plex) pooling guidelines using DNA HT Dual Index Kit 96N Sets A to D, please refer to Illumina's Nextera Low Plex Pooling Guide (Illumina, Publication Document #770-2011-044).
- Prior to use, remove the kit from the freezer and thaw for 10 min on the bench top. Spin in a tabletop centrifuge to collect contents at the bottom of the tubes or wells. Thoroughly wipe the tubes or index plate foil seal with 70% ethanol and allow it to dry.
- After removing indexing reagents of choice, cover any pierced or used index wells with scientific tape (e.g., VWR, Cat. No. 89097-920, General-Purpose Laboratory Labeling Tape, 0.5"). Replace the plastic lid, return the index plate to its sleeve, and store at –20°C.

NOTE: The index plate should not be frozen and thawed more than four times.

J. Safety Information

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 amplification reactions. For more information, please refer to the appropriate Material Safety Data Sheets (MSDS) available online at takarabio.com/manuals.

IV. Protocol

A. Cell Lysis

For this step, you will need the following components: Cell Extraction Buffer (red cap), Cell Extraction Enzyme (red cap).

1. **Samples:** Prepare cells as described in [Section III.E: Washing the Cells](#). Equilibrate cells (1–5) or gDNA (15–30 pg) to a final volume of 5 µl by adding an appropriate amount of TE Buffer (10 mM Tris, 0.1 mM EDTA, pH8.0).
2. **Positive control reaction using reference gDNA:** Assemble reaction using freshly diluted reference gDNA at an input amount of 15 pg (follow instructions provided in [Appendix A: Reference gDNA Dilution](#)). Add 1 µl of a 15 pg/µl dilution to 4 µl of 10 mM Tris pH 8.0, 0.1 mM EDTA.
3. **Negative control reactions/no-template control (NTC):** Assemble NTC with corresponding buffer for each sample to be tested, e.g. 5 µl of 10 mM Tris pH 8.0, 0.1 mM EDTA, pH 8.0 or 2.5 µl of 1X PBS plus 2.5 µl of 10 mM Tris pH 8.0, 0.1 mM EDTA.
4. Prepare **Cell Extraction Master Mix** as described in [Section III.G: Preparation of Master Mixes](#) (Prepare 5% extra, mix several times gently, and keep on ice until used).

4.8 µl	Cell Extraction Buffer (red cap)
0.2 µl	Cell Extraction Enzyme (red cap)
5 µl	Total volume per reaction

5. Add 5 µl of previously prepared samples (Step 1), reference gDNA (Step 2) or NTC (Step 3) to 5 µl of **Cell Extraction Master Mix** (Step 4) to assemble the Lysis Reactions as shown below. The final reaction volume at this stage will be 10 µl.

5 µl	Sample
5 µl	Cell Extraction Master Mix
10 µl	Total volume per Lysis Reaction

6. Seal the PCR plate using an appropriate sealing film or close the tubes tightly. Centrifuge briefly to ensure the entire volume of the reaction is collected at the bottom of each well.
7. Place the plate or tubes in a thermal cycler with a heated lid, and perform the **Lysis Reaction** using the following program:

75° C	10 min
95° C	4 min
4° C	forever

8. At the end of the **Lysis Reaction** (after the thermal cycler reaches 4°C), remove the plate or tube(s) and centrifuge briefly. Proceed immediately to the Pre-Amplification step in the same plate or tubes (Section IV.B; Pre-Amplification).

B. Pre-Amplification

For this step, you will need the following components: PreAmp Buffer (white cap), PreAmp Enzyme (white cap)

1. Prepare a **Pre-Amplification Master Mix** as described in [Section III.G: Preparation of Master Mixes](#) (Prepare 5% extra, mix several times gently, and keep on ice until used).

8.7 µl	PreAmp Buffer (white cap)
1.3 µl	PreAmp Enzyme (white cap)
10 µl	
	Total volume per reaction

2. Remove the seal on the plate or open the tubes, and to each lysis reaction mixture, add 10 µl of **Pre-Amplification Master Mix** to assemble the Pre-Amplification reactions as shown below. The final reaction volume at this stage is 20 µl.

10 µl	Lysis Reaction
10 µl	Pre-Amplification Master Mix
20 µl	
	Total volume per Pre-Amplification Reaction

3. Seal the plate or tubes tightly and centrifuge briefly to collect the contents to the bottom of each well.
4. Return the plate or tubes to the thermal cycler with the heated lid set to 101°C–105°C and perform the **Pre-Amplification Reaction** using the following cycling conditions:

95°C	3 min	
16 cycles:		
95°C	15 sec	}
15°C	50 sec	
25°C	40 sec	
35°C	30 sec	
65°C	40 sec	
75°C	40 sec	
4°C	forever	

STOPPING POINT: Samples may be stored at –20°C at this step.

5. At the end of the **Pre-Amplification Reaction** (after the thermal cycler reaches 4°C), remove the plate or tubes and centrifuge briefly. Proceed to the Pre-Amplification Cleanup step in the same plate or tubes (Section IV.C; Pre-Amplification Cleanup).

C. Pre-Amplification Cleanup

It is important to remove the excess primers from the Pre-Amplification Reaction by performing a 1:1 AMPure XP purification as follows:

1. Prepare fresh 80% ethanol.
2. Equilibrate Agencourt AMPure XP beads to room temperature for 20–30 min before use.
3. Add an equal volume of resuspended beads slurry to each sample.
4. Mix well by pipetting or gently vortexing.
5. Incubate the mixture for 5 min at room temperature.
6. Pellet the beads on a magnetic stand for 2 min and carefully discard the supernatant.
7. Wash the beads by adding 200 µl of freshly prepared 80% ethanol to each pellet. Incubate on the magnetic stand for 1 min, then carefully discard the supernatant without disturbing the beads.
8. Repeat Step 7 for a total of two ethanol washes.
9. Briefly spin down the beads.
10. Incubate on the magnetic stand for 2 min, then carefully discard the supernatant.
11. Elute by resuspending in 20 µl of 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0. Incubate the mixture for 2 min at room temperature.

12. Pellet the beads on the magnetic stand for 2 min. Carefully transfer 20 µl supernatant to a new PCR plate or tube(s) without disturbing the beads.
 13. Following the Pre-Amplification Cleanup, centrifuge briefly and proceed to the **Library Amplification Step** in the same plate or tubes (Section IV.D; Library Amplification).
- STOPPING POINT: Samples may be stored at –20°C at this step.**

D. Library Amplification

For this step, you will need the following components: Amplification Buffer (blue cap), Amplification Enzyme (blue cap), Nuclease-Free Water (clear cap) or fluorescent dyes (if performing real-time monitoring), and Index Plate/Tubes (sold separately).

If monitoring in real-time refer to [Section III.C](#); Thermal Cycler Considerations.

Prepare **EvaGreen/Fluorescein dye mix** as follows:

9 µl	20X EvaGreen Dye in water (Biotium Inc., Cat. No. 31000-T)
1 µl	Fluorescein Calibration Dye 1:500 (Bio-Rad, Cat. No. 170-8780)
10 µl	Total volume

Use 2.5 µl of the mixed dyes per reaction when assembling the Amplification Master Mix.

1. **If not monitoring in real-time**, use 2.5 µl of Nuclease-Free Water (clear cap) when assembling the **Amplification Master Mix**. Prepare the index plate/tubes. Refer to [Section III.H](#); Low-Level Multiplexing: Index Tubes and [Section III.I](#); High-Level Multiplexing: Dual-Index Plates.
 - a. Thaw the index plate/tubes for 10 min on the benchtop.
 - b. Centrifuge the index plate/tubes to collect the contents at the bottom of the wells/tubes.
 - c. If using plates, wipe the foil seal with 70% ethanol and allow it to dry.
2. Prepare the **Amplification Master Mix** as described in [Section III.G](#); Preparation of Master Mixes (Prepare 5% extra, mix several times gently, and keep on ice until used).

20 µl	Amplification Buffer (blue cap)
2.5 µl	Amplification Enzyme (blue cap)
2.5 µl	Fluorescent Dyes or Nuclease-Free Water (clear cap)
25 µl	Total volume per reaction

3. Remove the seal on the Pre-Amplification cleanup PCR plate or open the tubes and add 25 µl of the **Amplification Master Mix** to each well or tube.
4. Add 5 µl the indexing primers to the Pre-Amplification cleanup PCR plate or tubes:
 - a. If using Dual Index Plate, use clean pipette tips to pierce the seal above the desired index, discard tip(s) used for piercing.
 - b. Use a clean pipette tip to collect 5 µl of a unique index and add to the Pre-Amplification cleanup PCR plate already containing the **Amplification Master Mix**.
 - c. Gently mix the contents several times with a pipette.

NOTE: Follow the index plate/tube handling instructions ([Sections III.H and I](#)) to avoid cross-contamination.

- The assembled Amplification reactions should contain three components as shown below. The final reaction volume at this stage will be 50 µl:

20 µl	Pre-Amplification Cleanup
25 µl	Amplification Master Mix
5 µl	Indexing Primers
50 µl	Total volume per reaction

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

NOTE: Use an optical sealing film or caps if a real-time thermal cycler is used.

- Return the plate or tubes to the real-time thermal cycler with the heated lid on and perform the **Amplification Reaction** using the cycling conditions indicated below:

95°C	3 min
<u>4 cycles:</u>	
95°C	30 sec
63°C	25 sec
68°C	1 min
<u>Variable cycles*:</u>	
95°C	30 sec
68°C	1 min**
4°C	forever

*Refer to the table below for the recommended number of amplification cycles.

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

Table 1. Recommended number of amplification cycles based on input material.

Input	Recommended cycles
1 cell	11–12
5 cells	9–10

A diploid human cell contains ~6 pg genomic DNA

- At the end of library amplification, remove the PCR plate or tubes from the thermal cycler and centrifuge briefly to collect the contents at the bottom of each well. At this stage, samples can be processed for library purification immediately or stored at –20°C for later processing.

STOPPING POINT: Samples may be stored at –20°C at this step.

NOTE: The product from the Library Amplification step contains a high amount of Illumina libraries. We suggest using physically distinct work areas and equipment to avoid cross-contamination for the next steps. Use [this website](#) for guidance on how to minimize contamination.

- Refer to the recommendations below for library purification, subsequent quantification, and sequencing.

V. PicoPLEX Gold Single Cell DNA-Seq Library Processing

A. Overview

This section contains guidelines for processing PicoPLEX Gold Single Cell DNA-Seq libraries for Illumina NGS.

At the end of the **Library Amplification Step**, indexed libraries can be purified individually or pooled before AMPure XP purification. For individual purifications, 40 µl of each Library Amplification Reaction can be used. Proceed as described in Section V.B; Library Purification.

If pooling samples is preferred or desired, libraries can be combined as equal volume aliquots containing a unique index combination. Typically, a 10 µl aliquot from each library is adequate, and the remainder of the library can be stored at –20°C. The total volume obtained at the end of pooling will vary depending on the number of libraries pooled. For example, if 12 libraries are pooled, then the final volume of the pool is 120 µl. A 40 µl aliquot of this pooled library is sufficient for AMPure XP purification as described in the next section.

NOTE: When pooling replicates, it is advisable to add unique index combinations to each replicate so that they can be demultiplexed after sequencing.

B. Library Purification

It is important to remove the excess primers from the Amplification step by performing a 1:1 AMPure XP purification as follows:

NOTE: It is important to bring all the samples and reagents to be used to room temperature before starting.

1. Prepare fresh 80% ethanol (enough for Steps 7 and 8 below).
2. Equilibrate Agencourt AMPure XP beads to room temperature for 20–30 min before use.
3. Add an equal volume of resuspended Agencourt AMPure XP bead slurry to each sample (or the pooled libraries).
4. Mix well by pipetting or gently vortexing.
5. Incubate the mixture for 5 min at room temperature.
6. Pellet the beads on a magnetic stand for 2 min and carefully discard the supernatant.
7. Wash the beads by adding 200 µl fresh 80% ethanol to each pellet. Incubate on the magnetic stand for 1 min then carefully discard the supernatant without disturbing the beads.
8. Repeat Step 7 for a total of two ethanol washes.
9. Briefly spin down the beads.
10. Incubate on the magnetic stand for 2 min then carefully discard the supernatant.
11. Elute by resuspending in 20 µl 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0. Incubate the mixture for 2 min at room temperature.
12. Pellet the beads on the magnetic stand for 2 min. Carefully transfer 20 µl supernatant to a new PCR plate or tube(s) without disturbing the beads.
13. The purified library can be stored at –20°C. If storing the library, be sure to measure the concentration before reusing it.

C. Library Quantification and Sequencing Recommendations

NOTE: Prior to sequencing, purified libraries should be quantified using the real-time PCR Library Quantification Kit (Takara Cat. # 638324, 638325) or Qubit dsDNA HS Assay Kit (Thermo Fisher Cat. # Q32851, Q32854) following the manufacturer's instructions. The expected yield from a single-cell library prep is at least 1 µg. See also [Appendix B](#); Library Quantification Considerations.

The PicoPLEX Gold Single Cell DNA-Seq Kit generates libraries ready for cluster amplification and sequencing on Illumina NGS platforms using standard Illumina reagents. Due to low-diversity bases in the Pre-Amplification primers of PicoPLEX Gold Single Cell DNA-Seq chemistry (Figure 1), a higher

effective concentration of PhiX or other high complexity libraries is required for optimal sequencing results. Follow the loading recommendations in Table 2 and Illumina instructions below for optimal clustering and sequencing performance.

Table 2. Loading recommendations

	MiSeq®	NextSeq®
Conversion factor	800 bp	800 bp
Concentration	8–12 pM	0.9–1.4 pM
Recommended PhiX*	15%	20%

*PhiX can be replaced with high complexity libraries.

Visit the Illumina website to retrieve the MiSeq System Denature and Dilute Libraries Guide or the NextSeq System Denature and Dilute Libraries Guide, respectively.

D. Data Analysis

1. Trimming and alignment

The first 14 bases of the reads should be trimmed. These bases originate from the Pre-Amplification primers introduced during the PicoPLEX Gold Single Cell DNA-Seq library preparation (Figure 1). Subsequent alignment can be performed in the standard way, with tools such as BWA-MEM and Bowtie2.

2. Size distribution

Please note, libraries prepared using this kit result in a broad size distribution of library fragments. Prior to sequencing, the clustering process preferentially amplifies shorter fragments. For this reason, the distribution of the insert size observed after alignment of paired-end sequencing is smaller than the input libraries size distribution examined using Agilent Bioanalyzer 2100 (Figure 4).

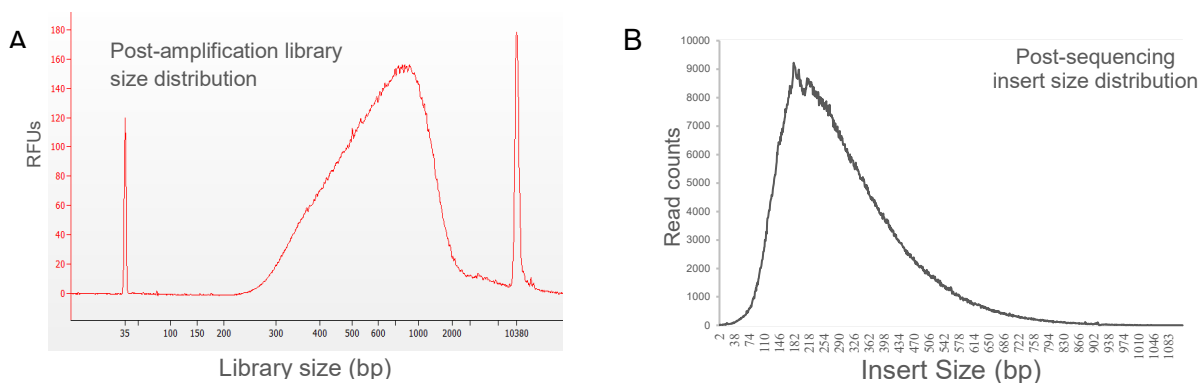


Figure 4. PicoPLEX Gold Single Cell DNA-Seq library and insert size. **Panel A.** Electropherogram results from Agilent Bioanalyzer 2100 (high sensitivity) depicts the size distribution of the library after purification. The library includes Illumina adapters (~130 bp) and has an average size of ~800 bp. **Panel B.** The insert size distribution from paired-end sequencing data was determined using Picard CollectInsertSizeMetrics. Average insert size of ~330 bp is observed.

3. CNV Analysis

For copy number variation (CNV) analyses, a typical workflow is comprised of two steps:

Step 1: Generate bin counts (e.g., reads mapped to 500 kb bins) and

Step 2: Compare bin counts to a ‘reference’ sample.

The reference sample is the control sample, which represents the normal/expected copy number (e.g., a stable euploid cell). Several commercial and open source software, such as CNVseq (Pubmed ID: PMC2667514), can be used to compare bin counts and generate CNV profiles statistically.

Key considerations for generating appropriate reference samples:

The reference samples should be processed using the same chemistry (i.e. PicoPLEX Gold Single Cell DNA-Seq). The input material for the reference sample should either be stable cells (e.g., PBMCs) or sufficient quantities of gDNA (e.g., 15–30 pg gDNA). Finally, we recommend using multiple reference samples and appropriate bin sizes (deeper sequencing enables higher resolution) to generate CNV profiles.

4. SNV Analysis

For single nucleotide variants (SNV), we recommend using a germline variant caller, such as the Genome Analysis Tool Kit (GATK) from the Broad Institute (or equivalent software), that identifies and reports variants in the variant call format (VCF) file. A typical GATK based-variant calling workflow includes the following steps:

Step 1: Trim reads and align to the reference genome.

Step 2: Optionally, remove duplicates. Sort BAM file by genomic coordinates.

Step 3: Process the resulting BAM file using GATK (local re-alignment and base score recalibration, followed by variant calling).

Step 4: Filter variants using appropriate filters. e.g., minimum depth ≥ 10 and VQS ≥ 30 .

Step 5: Optionally, compare VCF to a gold standard reference to examine error rates (allele drop-out and allele drop-in rates).

E. Application Specific Sequencing Requirements

In addition to being high-fidelity, PicoPLEX Gold Single Cell DNA-Seq Kit provides significantly higher genome coverage and a much lower duplication rate.

The kit generates highly reproducible libraries that are ideal for CNV analysis, even at low-pass sequencing. Higher sequencing depth may be required for SNV analysis for applications such as genome-wide karyomapping. The expected genome coverage and the number of SNVs detected for a given depth of sequencing are shown below.

Table 3. Genome coverage of PicoPLEX Gold Single Cell DNA-Seq Kit.

Total Reads	% coverage ($\geq 1X$)	% coverage ($\geq 10X$)	Number of validated SNVs
2.5 million read pairs	~ 13%	~ 0.1%	~ 1,000
8.5 million read pairs	~ 25%	~ 1.0%	~ 25,000
35 million read pairs	~ 50%	~ 10%	~ 270,000

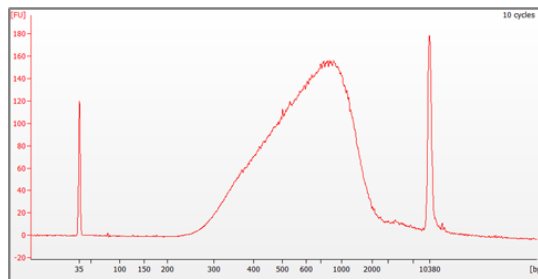
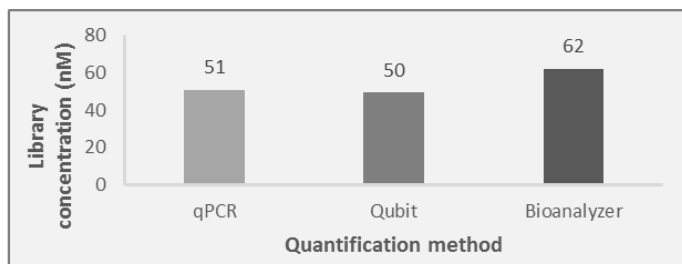
Libraries were generated from single cells (GM12878) using PicoPLEX Gold Single Cell DNA-Seq Kit and sequenced on Illumina NextSeq (2 x 150 cycles). SNVs were called using the GATK-lite (v2.3) and filtered (depth ≥ 10 and phred quality score ≥ 75). SNVs that agree with a gold-standard deep-sequencing dataset using bulk DNA are reported.

Appendix A: Reference DNA Dilution

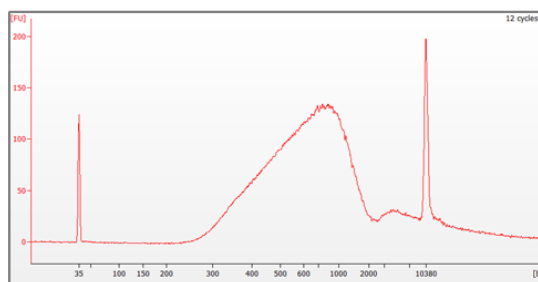
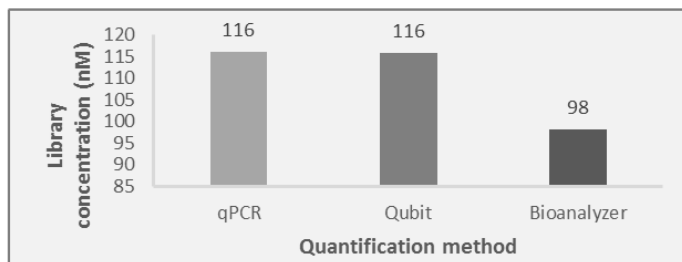
Single-donor human genomic DNA is ideal for use as positive control gDNA (e.g., NA12878, Coriell Institute). Follow the steps below to prepare the working dilutions for the reference genomic DNA.

1. Determine the concentration of the stock gDNA by using Qubit.
2. All dilutions should be carried out using 10 mM Tris pH 8.0, 0.1mM EDTA in low-binding microcentrifuge tubes.
3. Use a minimum volume of 3 μ l to ensure adequate transfer of gDNA from each dilution step to a final concentration of 15 pg/ μ l. At the end of each dilution step, mix the contents gently to avoid mechanical fragmentation. Centrifuge briefly before going to the next dilution step.
4. Always use freshly diluted gDNA for positive control reactions.

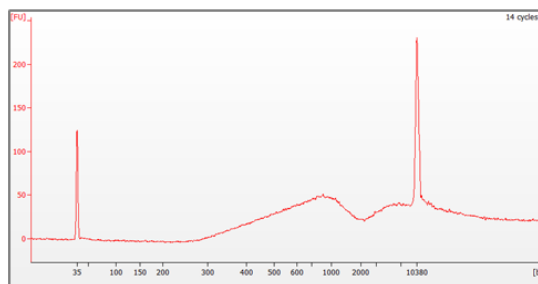
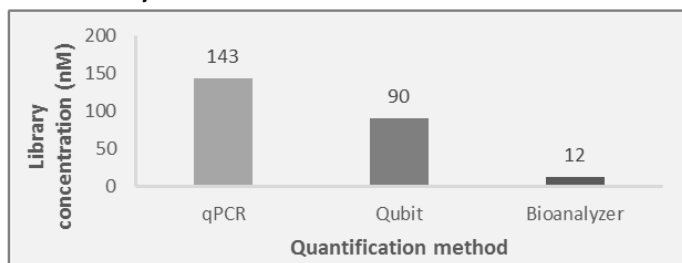
A. 10 cycles



B. 12 cycles



C. 14 cycles



D. 16 cycles

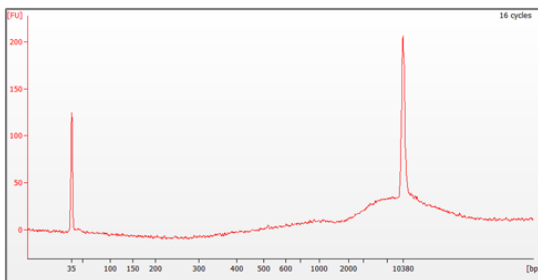
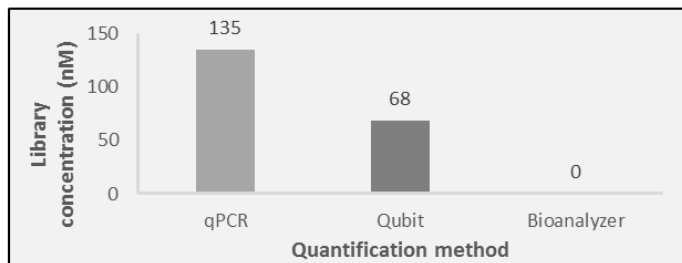


Figure 5. PicoPLEX Gold Single Cell DNA-Seq library quantification. Libraries were generated from 15 pg of gDNA (Coriell NA12878) using PicoPLEX Gold Single Cell DNA-Seq Kit. Libraries were amplified for 10 cycles (A), 12 cycles (B), 14 cycles (C) and 16 cycles (D) before purification. **Panels A–D, Left.** Library concentrations were determined by qPCR Library Quantification Kit (Takara cat # 638324, 638325), Qubit dsDNA HS Assay Kit, using a conversion factor of 800 bp, or Agilent Bioanalyzer 2100 High sensitivity (Cat # 5067-4626). **Panels A–D, Right.** Electropherogram results from Agilent Bioanalyzer 2100 high sensitivity depict the size distribution of the libraries.

Appendix B: Library Quantification Considerations

Three different methods of quantification were used following PicoPLEX Gold Single Cell DNA-Seq library preparation: qPCR, Qubit dsDNA HS, and Agilent Bioanalyzer 2100. Overamplification of the library leads to the formation of partially single-stranded heteroduplex. As depicted in Figure 5 above, only qPCR assays are suitable for the accurate quantification of heteroduplex libraries, as fluorometric assays (Qubit and Bioanalyzer) are double-stranded DNA-specific.

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