Takara Bio USA, Inc.

PicoPLEX® DNA-seq Kit User Manual

Cat. No. R300381
(061220)

Takara Bio USA, Inc.
1290 Terra Bella Avenue, Mountain View, CA 94043, USA
U.S. Technical Support: technical_support@takarabio.com

United States/Canada	Asia Pacific	Europe	Japan
800.662.2566	+1.650.919.7300	+33.(0)1.3904.6880	+81.(0)77.565.6999

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I. Introduction

Next Generation Sequencing (NGS) enables precise and impartial analysis of the genome for many applications including detecting chromosomal aneuploidies, copy number variations (CNV), insertions, and deletions. These are especially relevant in pre-implantation genetic screening (PGS) in IVF clinics and cancer genetics.

In each of these applications the starting material is limited due to the use of single cells, and requires reproducible amplification of the genome as a prerequisite for the downstream analysis by NGS. The PicoPLEX DNA-seq Kit has been developed specifically to meet this growing need and facilitate preparation of Illumina®-compatible NGS libraries from single cells.

PicoPLEX DNA-seq libraries are used to characterize unique genomic signatures that include chromosomal aneuploidies and copy number variations in PGS *in Vitro* Fertilization (IVF) clinics as well as in cancer research with single tumor cells (e.g., CTCs).

A. Overview

The PicoPLEX DNA-seq Kit is designed to generate Illumina NGS-ready libraries in a single tube from a single cell in less than 2.5 hr. The kit includes all necessary reagents for extracting DNA from a single cell and preparing amplified NGS-ready libraries. Dual indexes (barcodes) included in the kit avoid potential sample misidentifications due to barcode switching. The resulting library is ready for Illumina NGS instruments using standard Illumina sequencing reagents and protocols.

The PicoPLEX DNA-seq Kit offers robust and reproducible amplification of DNA from a single cell for limited coverage sequencing analysis and is not intended for high-coverage, deep sequencing such as *de novo* sequencing and/or whole genome sequencing.

B. Principle

PicoPLEX DNA-seq 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 library construction (Figure 1, below). The PicoPLEX DNA-seq Kit follows the same three-step workflow of the PicoPLEX WGA Kit. In the first step, a single cell or up to 10 cells are efficiently lysed to release gDNA (note that DNA may also be used in this step). In the second step, proprietary quasi-random primers bind to selective sites on the gDNA and, through a linear amplification, create a highly reproducible library. In the third step, the library is further amplified exponentially with primers containing unique dual barcodes suitable for Illumina NGS.

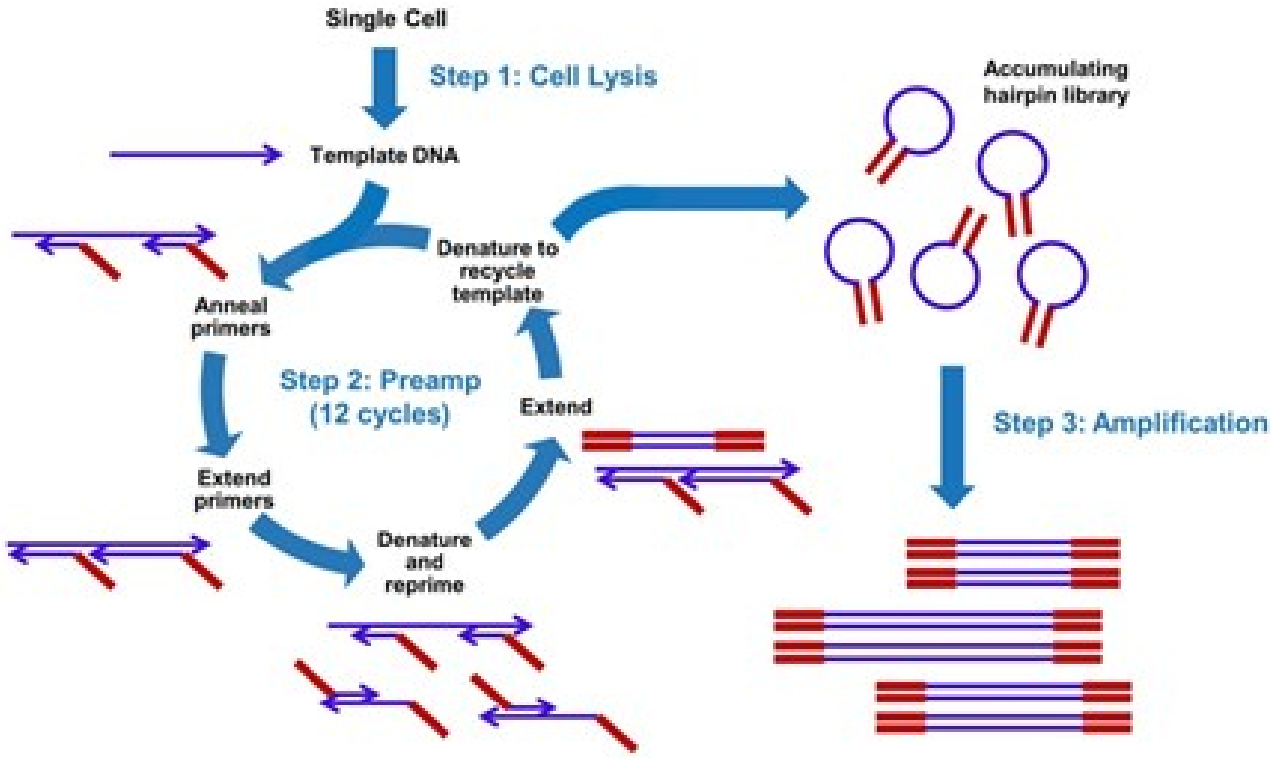


Figure 1. PicoPLEX DNA-seq technology. A three-step, single-tube reaction that starts with a single cell. Cellular gDNA extracted in Step 1 is used as template for multiple cycles of quasi-random priming and linear amplification followed by exponential library amplification.

C. PicoPLEX DNA-seq Workflow

The PicoPLEX DNA-seq 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 and Illumina-compatible dual indexing for NGS.

The three-step PicoPLEX DNA-seq workflow takes place in the same tube or plate and is completed in less than 3 hours.

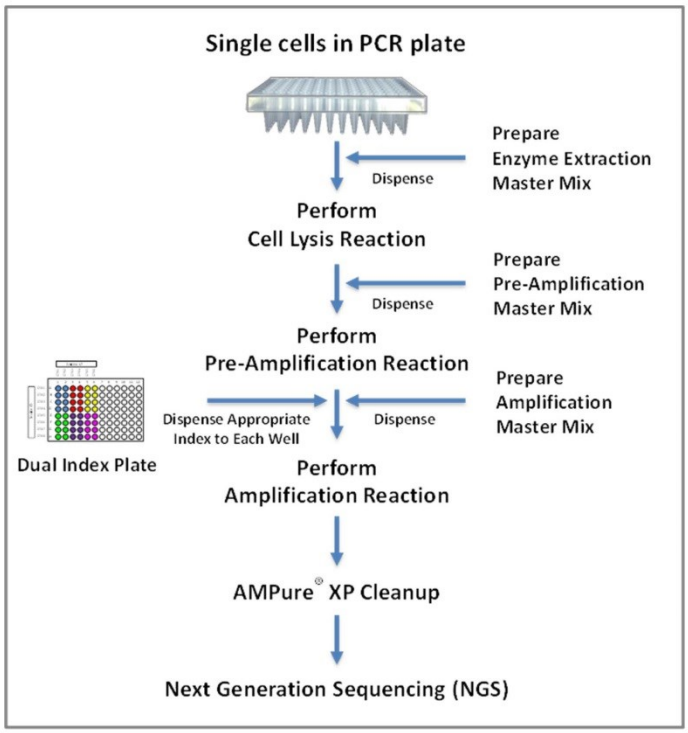


Figure 2. PicoPLEX DNA-seq sequencing workflow overview. Steps involved in PicoPLEX library preparation starting from a single cell for Illumina NGS. Note that tubes may replace the PCR plate.

II. List of Components

The PicoPLEX 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.** 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. Store all kit components at –20ºC.

Table 1. PicoPLEX DNA-seq Kit list components.

Cap Color	Component Name	Component Volume
Green	Cell Extraction Buffer	250 µl
Violet	Extraction Enzyme Dilution Buffer	240 µl
Yellow	Cell Extraction Enzyme	10 µl
Red	Pre-Amp Buffer	230.4 µl
White	Pre-Amp Enzyme	9.6 µl
Orange	Amplification Buffer	1200 µl
Blue	Amplification Enzyme	24 µl
Clear	Nuclease-Free Water	1176 µl
	Dual Index Plate	
	Quick Protocol	

IMPORTANT: The PicoPLEX DNA-seq 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 recommended)
- Centrifuge
- 96-well nuclease-free thin-wall PCR plates or PCR tubes
- 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)
- 80% ethanol: freshly made for each experiment
- Single-donor reference DNA (positive control)
- Agencourt AMPure XP beads
- Magnetic Separator - PCR Strip (Takara Bio, Cat. No. 635011)
- Nuclease-free water

NOTES:

– Agencourt AMPure XP beads need to come to room temperature before the container is opened. Therefore, we strongly recommend aliquoting the beads into 1.5-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. 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. 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 subsequent amplification reactions. For more information please refer to the appropriate Material Safety Data Sheets (MSDS) available online at takarabio.com.

D. 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 headed lid to 100–105ºC to avoid sample evaporation during incubation and cycling.

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 with the kit) 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. In the absence of real-time monitoring, library amplification can be analyzed by gel or by analysis of an aliquot of the library using the Agilent Bioanalyzer (see Library Quantification, Section V.D).

Depending on the real-time instrument used, select an appropriate calibration dye and mix with EvaGreen detection dye mix (see Amplification Protocol, Step 1). For some real-time instruments calibration dye may not be needed; please refer to the real-time thermal cycler instrument's user manual.

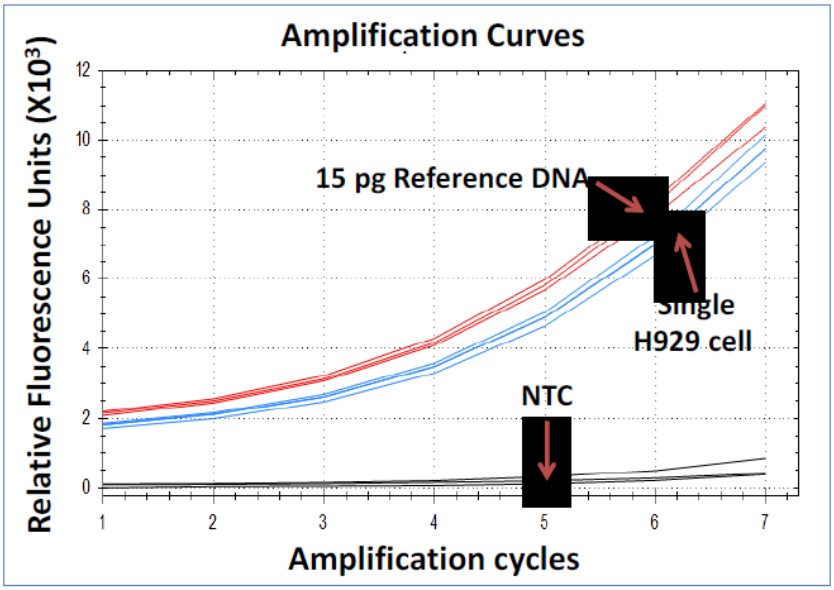


Figure 3. Real-time analysis of library amplification using PicoPLEX DNA-seq. A typical real-time amplification analysis of libraries prepared with the PicoPLEX DNA-seq Kit using three single H929 cells (blue) or three reference DNA samples, 15 pg (red), relative to NTC (grey). Results obtained using CFX96 Touch Real-Time PCR Detection System with EvaGreen and fluorescein as the dyes.

Selecting PCR plates/tubes

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.

E. Starting Material

Cells

Single mammalian cells (1–10 cells) from a broad range of sources can be used. Examples of cells for reliably detecting chromosomal aneuploidies by NGS include blastomeres and trophectoderms; CTCs; cultured cells; clonally expanded cells; micro-manipulated single cells and flow-sorted single cells.

Genomic DNA

In place of whole cells, small amounts (less than 6 pg to 60 pg) of purified genomic DNA can be used as starting material for library preparation. Purified eukaryotic, prokaryotic, fungal, or viral DNA can also be used as starting material.

F. 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 with the kit. Cell fixation 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 PicoPLEX DNA-seq Kit is that it provides robust and reproducible library construction for NGS 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 PicoPLEX DNA-seq Protocol.

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. Always prepare fresh dilutions of gDNA to use as the positive control. 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. Dual Index Plate

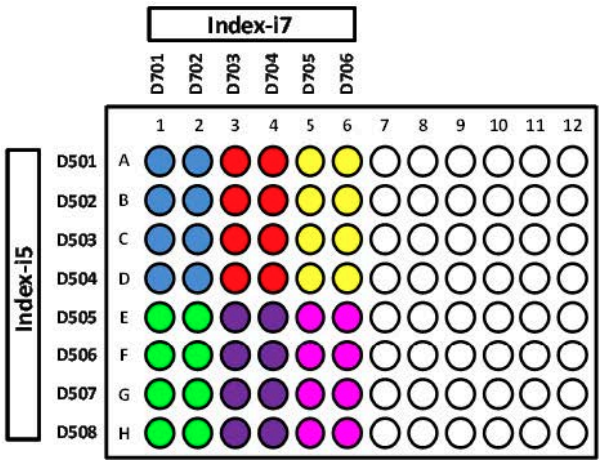
The PicoPLEX DNA-seq Kit is designed for high-throughput applications and includes a 96-well single-use Dual Index Plate containing 48 unique dual indexes. Each of 48 wells contains a combination of i5 and i7 indexes prepared with 8-nt Illumina sequences to avoid potential sample misidentifications due to barcode switching (Figure 4). Each well contains sufficient volume of the dual-index pair for a single use. The plate is sealed with foil that can be pierced with a pipet tip to collect the required amount of the dual-index pair to assemble the reactions.

It is recommended to design your experiment to use the entire plate of barcodes to avoid contamination problems. However, the Dual Index Plate can also be used for low-level multiplexing of a small number of samples. The plate should not be used more than 4 times. If all 48 index pairs are not used at the same time, it is important to seal the opened wells with laboratory labeling tape to avoid cross contamination. Please refer to the index plate handling instructions in Appendix B.

It is also very important to select appropriate dual index combinations such that they are unique and meet Illumina-recommended compatibility requirements. Please refer to Illumina's technical manuals (TruSeq® Sample Preparation Pooling Guide, Illumina Part # 15042173 Rev A, 2013) for additional information. Barcode combinations may also be evaluated using Illumina Experiment Manager Software for compatibility.

NOTES:

- The Illumina dual-index oligonucleotides contained in this kit cannot be substituted with index oligonucleotides from any other sources.
- Avoid repeated freezing and thawing of the plate. No more than 4 freeze/thaw cycles are recommended.



Index-i5	Sequence	Index-i7	Sequence
D501	TATAGCCT	D701	ATTACTCG
D502	ATAGAGGC	D702	TCCGGAGA
D503	CCTATCCT	D703	CGCTCATT
D504	GGCTCTGA	D704	GAGATTCC
D505	AGGCGAAG	D705	ATTCAGAA
D506	TAATCTTA	D706	GAATTCGT
D507	CAGGACGT		
D508	GTACTGAC		

Figure 4. Dual-index plate map with well locations. Single-use index plate representing well locations (left) of 48 dual indexes, with appropriate i7 (columns) and i5 (rows) index combinations; the sequences of respective indexes are shown in the table (right). Wells A7–H12 are empty.

I. Preparation of Master Mixes

It is recommended to prepare a master mix 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 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.

IV. Protocols

A. Protocol: Cell-Lysis Step

For this protocol, you will need the following components: Cell Extraction Buffer (green cap), Extraction Enzyme Dilution Buffer (violet cap), Cell Extraction Enzyme (yellow cap).

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. **Test samples:** Equilibrate cells (1 to 10) or DNA (6–60 pg) to a final volume of 5 µl by adding an appropriate amount of Cell Extraction Buffer (CEB).

   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 CEB.

2. **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 CEB to each tube to bring the final volume of each reaction to 5 µl.

3. **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 CEB.

4. Prepare **Cell Extraction Master Mix** as described below for the chosen number of reactions plus 5% extra, mix gently several times, and keep on ice until used.

4.8 µl	Extraction Enzyme Dilution Buffer (violet cap)
0.2 µl	Cell Extraction Enzyme (yellow cap)
5 µl	Total volume per reaction

5. To each 5 µl of equilibrated sample from steps 1–3 above, containing (a) cell(s) (b) reference gDNA, or (c) NTC(s), add 5 µl of **Cell Extraction Master Mix** to assemble the Lysis Reactions as shown below. Do not touch the cell or DNA sample with the pipet tip. 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 tube(s) tightly. Centrifuge briefly to ensure the entire volume of the reaction is collected at the bottom of each well.

7. Place the plate or tube(s) in a thermal cycler with heated lid on, and perform the **Lysis Reaction** using the following program:

75°C	10 min
95°C	4 min
22°C	forever

8. At the end of the **Lysis Reaction** (after the cycler reaches 22ºC), remove the plate or tube(s) and centrifuge briefly. Proceed to the Pre-Amplification step (Section IV.B, below).

B. Protocol: Pre-Amplification Step

For this protocol, you will need the following components: Pre-Amp Buffer (red cap), Pre-Amp Enzyme (white cap)

1. Prepare a **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 Buffer (red cap)
0.2 µl	Pre-Amp Enzyme (white cap)
5 µl	Total volume per reaction

2. Remove the seal on the plate or open the tube(s), and to each lysis reaction mixture, add 5 µl of **Pre-Amplification Master Mix** 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 per Pre-Amplification Reaction

3. Seal the plate or tube(s) tightly and centrifuge briefly to collect the contents to the bottom of each well.
4. Return the plate or tube(s) to the thermal cycler with the heated lid on and perform the Pre-Amplification Reaction using the following cycling conditions:

95°C	2 min
12 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

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

C. Protocol: Library Amplification Step

For this protocol, you will need the following components: Amplification Buffer (orange cap), Amplification Enzyme (blue cap), Nuclease-Free Water (clear cap), fluorescent dyes (if performing real-time monitoring), Dual Index Plate

NOTE: It is critical to handle the Dual Index Plate following the instructions to ensure that there is no barcode cross contamination. If the entire Dual Index Plate will not be used, please refer to Appendix B for handling procedures. No more than 4 freeze/thaw cycles are recommended for the Dual Index Plate.

- **If monitoring in real-time:** Prepare 20X EvaGreen/Fluorescein dye mix (or dye recommended for your thermal cycler):
  - Mix 90 µl of 20X EvaGreen dye (20X EvaGreen dye in water; Biotium Inc., Cat. No. 31000-T) with 10 µl of 1:500 dilution of Fluorescein (Fluorescein Calibration Dye; Bio-Rad Laboratories, Cat. No. 170-8780).
  - Use 2.5 µl of this mix per reaction.
- **If not monitoring in real-time:** Replace dyes with water in the Master Mix (Step 3).

1. Prepare the Dual Index Plate
   a. Thaw the Dual Index Plate for 10 min on the benchtop.
   b. Spin the Dual Index Plate in a tabletop centrifuge to ensure its contents are at the bottom of the well.
   c. Thoroughly wipe the foil seal with 70% ethanol and allow it to dry.
2. Prepare the **Amplification Master Mix** as described below for the chosen number of reactions plus 5% extra, mix gently several times, and keep on ice until used.

25 µl	Amplification Buffer (orange cap)
0.5 µl	Amplification Enzyme (blue cap)
2.5 µl	Fluorescent Dyes (Step 1.a)*
2 µl or 4.5 µl	Nuclease-Free Water (clear cap)**
30 µl	Total volume per reaction

*Optional, for real-time reaction monitoring.
**The amount of Nuclease-Free Water added per reaction will vary depending on the inclusion of fluorescent dye.

3. Remove the seal on the PCR plate or open the tube(s), and add 30 µl of the Amplification Master Mix to each well.
4. Add the indexes to the PCR plate or tube(s):
   a. Using clean pipet tips pierce the seal above the target index of the Dual Index Plate; discard tip(s) used for piercing.
   b. Use a clean pipet tip to collect 5 µl of a specific index and add to the reaction.
   c. Gently mix the contents several times with a pipettor. The final reaction volume at this stage will be 50 µl:

15 µl	From Pre-Amplification Reaction
30 µl	Amplification Master Mix
5 µl	Index Oligonucleotide
50 µl	Total volume per reaction

NOTE: Follow the index plate handling instructions (Appendix B) to avoid cross contamination.

5. 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.

6. 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:

95°C	4 min
4 cycles:	
95°C	20 sec
63°C	25 sec
72°C	40 sec
7 cycles*:	
95°C	20 sec
72°C	55 sec
4°C	forever

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

7. At the end of library 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. At this stage, samples can be processed for library purification immediately or stored frozen at –20 °C for later processing.

V. PicoPLEX DNA-seq Library Processing for Illumina Next Generation Sequencing

A. Overview

This section contains guidelines for processing PicoPLEX DNA-seq libraries for Illumina NGS. In some cases, recommended protocols are listed (Section V.C, Library Purification by AMPure XP beads) while in others, general guidelines are given. For more information, please contact our technical support staff at technical_support@takarabio.com.

Libraries prepared from each sample will contain the sample-specific index combinations that were selected at the time of the amplification. An equal volume of each individual library containing their unique index combinations are pooled into one tube for further processing. This library pool is purified to remove unincorporated primers and other reagents. Once purified, the library is quantified prior to NGS. The library pooling process, purification protocol, quantification protocols and recommendations for sequencing are described in the following sections of the PicoPLEX DNA-seq protocol.

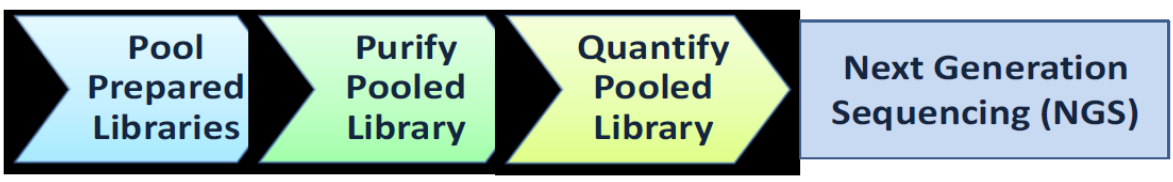


Figure 5. Workflow for processing PicoPLEX DNA-seq amplified libraries for Illumina NGS.

B. Library Pooling for Purification

Pool the samples by combining equal volume aliquots of each PicoPLEX DNA-seq library, each containing a unique dual barcode 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; if 48 libraries are pooled, then the volume is 480 µl. A 100-µl aliquot of this pooled library is sufficient for AMPure XP purification purposes.

C. Protocol: Library Purification by AMPure XP Beads

AMPure XP is the preferred method of library purification due to its ability to preserve sequence complexity. Do not use QIAquick cleanup or other silica-based filters for purification as this will result in incomplete removal of primers.

The ratio of AMPure XP beads to library DNA will determine the size-selection characteristics of the library. The ratio is also application dependent. For most NGS-based CNV detection purposes, a good starting point is mixing the beads and the sample(s) at a 1:1 ratio. For more information please refer to the vendor's recommendations on AMPure XP protocols for DNA purification.

Library Purification Reagents (supplied by the user)
AMPure XP beads
Magnetic rack for 1.5-ml centrifuge tubes
80% Ethanol
TE Buffer

AMPure XP Protocol

NOTE: It is important to bring all the samples and reagents to be used to room temperature. In the meantime, prepare fresh 80% ethanol (enough for Steps 4 and 6 below).

1. Re-suspend the AMPure XP reagent by gentle vortexing until no visible pellet is present at the bottom of the container.
2. In a 1.5-ml tube, mix 100 µl of AMPure XP reagent with a 100-µl aliquot of the pooled library ensuring a 1:1 (v/v) ratio. Gently mix by pipette 10 times to achieve a homogeneous solution and incubate the tube at room temperature for 5 min.
3. Pulse-spin the sample(s) on a benchtop centrifuge and place the tube in a magnetic stand. Wait for 2 min or until the beads are completely bound to the side of the tube(s) and the solution is clear.
4. With the tube(s) in the magnetic stand and without disturbing the pellet use a pipette to aspirate off and discard the supernatant. Add 300 µl of freshly prepared 80% ethanol to the pellet.
5. With the tube(s) in the magnetic stand, rotate each tube 90 degrees and wait until all the beads come to a halt (DO NOT INVERT TUBE-RACK). Repeat this step three more times.
6. With the tube(s) in the magnetic stand and without disturbing the pellet, use a pipette to aspirate off and discard the supernatant. Add 300 µl of freshly prepared 80% ethanol to the pellet.

7. With the tube(s) in the magnetic stand and without disturbing the pellet, turn each tube 90 degrees and wait until all the beads come to a halt (DO NOT INVERT TUBE-RACK). Repeat this step three more times.
8. With the tube(s) in the magnetic stand and without disturbing the pellet, use a pipette to aspirate off and discard the supernatant.
9. Pulse-spin the sample(s) using a low-speed, benchtop centrifuge, place into a magnetic stand, and wait for 2 min or until the beads are completely bound to the side of the tube(s).
10. With the tube(s) in the magnetic stand and without disturbing the pellet, use a pipette to aspirate off and discard any residual ethanol without disturbing the pellet.
11. Leaving the cap open, incubate the sample(s) in a heating block at 37ºC for 2–3 min or until the pellet is dry. DO NOT OVERDRY THE PELLET(S).
12. Elute the DNA by resuspending the beads with 50 µl of 1X TE buffer, pH 8.0.
13. Pulse-spin the sample(s) using a low-speed, benchtop centrifuge and place it into a magnetic stand and let the beads bind to the side of the tube(s) completely (for ~2 min) until the solution is clear.
14. While keeping the sample(s) in the magnetic stand, without disturbing the pellet, transfer the supernatant with a pipette into a new tube. If not used immediately, the purified library can be stored at –20ºC.

D. Library Quantification

There are several approaches available for library quantification, including real-time PCR, UV absorption, fluorescence detection, and sizing and quantification using the Agilent Bioanalyzer. It is important to understand the benefits and limitations of each approach. Real-time PCR-based approaches quantify the library molecules that carry the Illumina adapter sequences on both ends, and therefore reflect the quantity of the clustering competent library molecules. This approach assumes a relatively uniform size of sheared or fragmented starting gDNA inserts used for library construction. On the other hand, 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. These methods do not discriminate adapter presence (an indication of clustering competence) and offer no information about the size of the library molecules. The Agilent Bioanalyzer system provides sizing and quantitation information about the library analyzed, but not about the clustering competency.

To quantify PicoPLEX DNA-seq libraries by real-time qPCR, select the appropriate instrument-specific Illumina library quantification kit. Pooled, purified libraries are diluted and used as the template for quantification. It is recommended to use 300 bp as the size for calculating the library concentration.

To quantify PicoPLEX DNA-seq libraries by using the BioAnalzyer (Figure 6, below), remove an aliquot of each library and dilute 1:15. Load a 1-µl aliquot of this diluted sample onto a Bioanalyzer high sensitivity DNA chip (Agilent Technologies, Inc; Cat. No. 5067-4626).

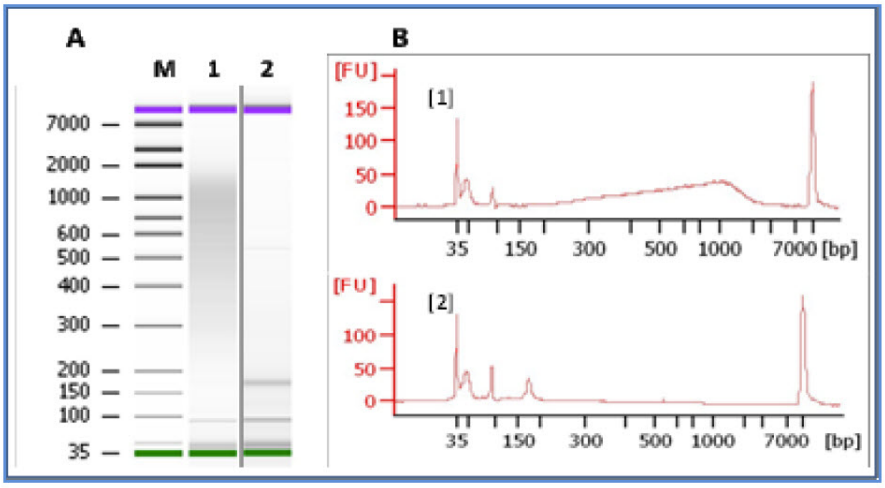


Figure 6. Bioanalyzer analysis of libraries prepared using PicoPLEX DNA-seq. Libraries were prepared using 15 pg of reference DNA or NTC with the PicoPLEX DNA-seq Kit. An aliquot of each library was diluted at 1:15 in TE buffer and 1 µl of this diluted sample was loaded on a Bioanalyzer using a high sensitivity DNA chip (Agilent Technologies, Inc.). Electropherogram results (Panels A and B) showing a broad size range distribution. M) Markers 1) Library prepared using 15 pg reference gDNA, 2) NTC.

E. Sequencing Recommendations

The PicoPLEX DNA-seq Kit generates libraries which are ready for cluster amplification and sequencing on Illumina NGS platforms using standard Illumina reagents and protocols for multiplexed libraries. Libraries prepared using the PicoPLEX DNA-seq Kit result in a broad size distribution of library fragments (Figure 6, above), typically ranging from ~300 to 1000 bp total size (~200 to 900 bp insert size). It is important to note that when libraries consist of a broad range of fragments, the clustering process preferentially amplifies shorter fragments as the longer fragments tend to cluster less efficiently.

To achieve optimal cluster density on the Illumina flow cell it is important to adjust the DNA concentration used for clustering based on these preferences. With libraries made using the PicoPLEX DNA-seq Kit, loading 16 pM of a library with an average size of 300 bp is a good starting point for calculating the amount to use with the Illumina MiSeq, v3. It is very important to add at least 5% PhiX DNA to the library prior to loading on the flow cell to achieve necessary diversity.

F. Analysis of Sequencing Reads

The first 11 cycles of each read will contain quasi-random bases introduced during PicoPLEX DNA-seq library preparation. For sequence alignment, either trim the initial 14 bases from each read or begin calibration and data collection at base position 15.

Appendix A: Reference DNA Dilution

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.

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

1. Prepare a working stock solution of 1000 pg/µl, by appropriately diluting a 2–3 µl 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: Dual Index Plate

The PicoPLEX DNA-seq Kit is designed for high-throughput applications and includes a 96-well single use Dual Index Plate containing 48 different dual indexes. Each of 48 wells contains a combination of i5 and i7 indexes prepared with Illumina 8-nt sequences to avoid potential sample misidentifications due to barcode switching (Figure 7, below). The Illumina dual index oligonucleotides contained in this kit cannot be substituted with index oligonucleotides from any other sources.

The plate is intended for high-throughput applications; each well contains sufficient volume of the dual-index pair for a single use. The plate is sealed with foil that can be pierced with a multichannel pipet tip to collect the required amount of the dual-index pair to assemble the reactions.

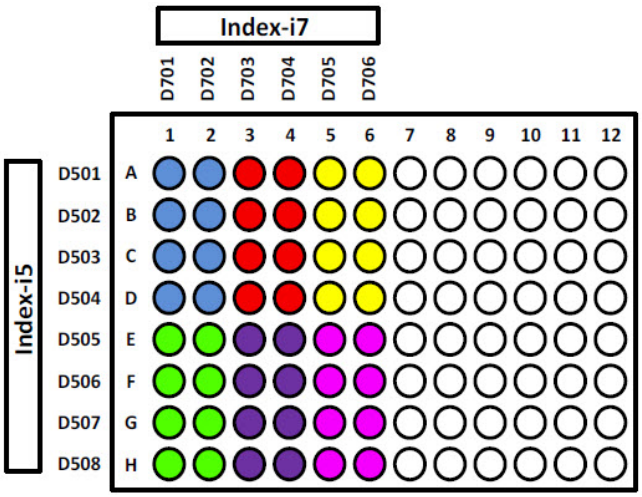
It is recommended that your experiment be designed to use the entire plate of barcodes to avoid contamination problems. However, the Dual Index Plate can also be used for low-level multiplexing of a small number of samples. The plate should not be used more than 4 times. If all 48 index pairs are not used at the same time, it is important to seal the opened wells with a laboratory labeling tape to avoid cross contamination.

It is very important to select appropriate dual-index combinations such that they are unique and meet Illumina's recommended compatibility requirements. Please refer to Illumina's technical manuals (TruSeq Sample Preparation Pooling Guide, Illumina Part No. 15042173 Rev A, 2013) for additional information. Barcode combinations may also be evaluated using Illumina Experiment Manager Software for compatibility.

Handling for Low-Throughput Applications

NOTE: If the entire Dual Index Plate is not used, it is critical to follow the instructions below to avoid cross contamination.

1. After removing indexes of choice, cover any pierced or used index wells with scientific tape such as General Scientific Tape 0.5” (VWR, Cat. No. 89097-920).
2. Thoroughly wipe the seal with 70% ethanol and allow it to dry completely.
3. Replace the plastic lid, return the Dual Index Plate to its sleeve and store at –20ºC.



Index-i5	Sequence	Index-i7	Sequence
D501	TATAGCCT	D701	ATTACTCG
D502	ATAGAGGC	D702	TCCGGAGA
D503	CCTATCCT	D703	CGCTCATT
D504	GGCTCTGA	D704	GAGATTCC
D505	AGGCGAAG	D705	ATTCAGAA
D506	TAATCTTA	D706	GAATTCGT
D507	CAGGACGT		
D508	GTACTGAC		

Figure 7. Dual index plate map with well locations. Single-use index plate representing well locations (left) of 48 dual indexes, with appropriate i7 (columns) and i5 (rows) index combinations; the sequences of respective indexes are shown in the table (right). Wells A7–H12 are blank wells and do not contain any index oligonucleotides.

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