CELLECTA

USER MANUAL

CloneTracker™ Barcode Cell Labeling Kits

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Cellecta, Inc.

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1. CloneTracker™ Barcode Cell Labeling Kits

The **CloneTracker™ 5-Barcode and 10-Barcode Cell Labeling Kits** (Cat.#s **BCRPP5-V**, **BCRPP10-V**, and **BCXP3RPM10-V**) provide packaged lentiviral particles for labeling 5 or 10 different cell populations/clones with unique barcodes which differ in sequence but are all the same size. The barcode constructs in these kits also contain a red fluorescent protein marker (TagRFP) and puromycin-resistance selection marker.

The **CloneTracker™ 4-Barcode-Plus Cell Labeling Kits** (Cat.# **BC4P-V**, **BC4PPO-V**) both provide 4 different packaged barcode constructs, each of which differ in both size and sequence and can be differentially amplified by PCR. The barcode constructs in Cat.# **BC4P-V** express both TagRFP fluorescent protein and puromycin-resistance selection marker, but those in Cat.# **BC4PPO-V** express only puromycin-resistance selection marker.

This Manual provides instructions on how to titer and transduce a target cell population with a single lentiviral barcode construct with the purpose of generating a cell population uniformly labeled with a single genomically integrated unique barcode sequence. The differently barcoded cell populations (e.g. with different genotypes) can be mixed together and used for *in vitro* or *in vivo* experiments, for example to monitor proliferation rate of different clones with and without treatment conditions (e.g. drug). The relative proliferation rate of individual barcode-labeled groups of cells can be identified in a mixed population by standard sequencing or, in the case of the CloneTracker Barcode-Plus Cell Labeling Kit, by gel electrophoresis analysis of the amplified barcodes.

The CloneTracker Cell Labeling Kits are designed for labeling only a limited number of different cell populations. For studying clonal dynamics and growth heterogeneity in the complex cell population with mixed representation of different cell types (e.g. different by phenotype or genotype), we recommend Cellecta's **CloneTracker XP Lentiviral Barcode Libraries**. The CloneTracker XP lentiviral libraries comprise 1M, 5M, 10M, or 50M different expressed barcodes and can be effectively used to measure representation of different clones (e.g. stem cells, drug-resistant clones) by Next-Generation Sequencing (NGS) on the Illumina platform. Cells labeled using the BCXP3RPM10 kits can can be mixed together with cells labeled with the CloneTracker XP 5M or 50M Barcode Libraries as they have different unique barcode sequences but can be amplified and sequenced by the same set of primers. Cells labeled using the BCRPP5 or BCRPP10 kits can be mixed together with cells labeled with the CloneTracker 50M Barcode Library (discontinued as of Dec. 2023) as they have different unique barcode sequences but can be amplified and sequenced by the same set of primers.

The experimental design for using barcoded cell populations is mainly defined by your model system and depends on your specific scientific goals. Puromycin-selected barcoded cell populations can be mixed together and analyzed for relative growth characteristics under different conditions in both *in vitro* and *in vivo*. For example, the mixed barcoded cell populations (e.g. with different genotype) can be mixed with other cell types (e.g. stromal cells) and/or treated with different agents (e.g. drugs) or grown under different growth conditions (e.g. hypoxia vs normoxia, attached vs suspension, etc). Alternatively, the single barcoded cell population(s) can be spiked-in to heterogeneous cell populations transduced with the CloneTracker 50M Barcode Library or CloneTracker XP 5M or 50M Barcode Libraries (as mentioned above), and these single barcoded cells could be used as an internal positive/negative control to monitor the growth characteristics of other cell types present in the heterogeneous cell population.

Please read the entire user manual before proceeding with your experiment. Also, please note that, when working with pseudoviral particles, you should follow the recommended guidelines for working with Biosafety Level 2 (BSL-2) materials.

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2. Materials Provided

CloneTracker™ XP 10-Barcode Cell Labeling Kit (Cat.# BCXP3RPM10-V)

Box 1 (Reagents for amplification and sequencing)

Contains reagents for amplification and sequencing of up to 10 genomic DNA samples.

> **Storage: -20°C**

Component	Description	Conc.	Volume
Taq DNA Polymerase	Thermostable DNA Polymerase	50X	300 µl
Taq DNA Polymerase Buffer	Reaction Buffer for PCR	10X	1,500 µl
dNTP Mix	Mix of dATP, dGTP, dCTP and dTTP, 10 mM each	50X	300 µl
PCR-Grade Water	Water for Reaction Mixes	NA	10 ml
First PCR Primer: Forward-XP	First Round Forward PCR Primer (10 uM)	20X	150 µl
First PCR Primer: Reverse-XP	First Round Reverse PCR Primer (10 uM)	20X	150 µl
Second PCR Primer: NFwd-XP	Second Round Forward PCR Primer (10 uM)	20X	240 µl
Second PCR Primer: NRev-XP Index A	Second Round Reverse PCR Primer (10 uM)	20X	20 µl
Positive Control Template DNA	Positive Control template—genomic DNA labeled with one unique, single-copy barcode (0.2 µg/µl)	10X	25 µl

Box 2 (Pre-packaged lentiviral particles)

Panel of 10 packaged barcode labeling constructs; >1×10^{7} TU of each construct.

> **Storage: -80°C**

CloneTracker™ 5-Barcode Cell Labeling Kit (Cat.# BCRPP5-V)

Box 1 (Reagents for amplification and sequencing)

Contains reagents for amplification and sequencing of up to 10 genomic DNA samples.

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Storage: -20°C

Component	Description	Conc.	Volume
Taq DNA Polymerase	Thermostable DNA Polymerase	50X	300 µl
Taq DNA Polymerase Buffer	Reaction Buffer for PCR	10X	1,500 µl
dNTP Mix	Mix of dATP, dGTP, dCTP and dTTP, 10 mM each	50X	300 µl
PCR-Grade Water	Water for Reaction Mixes	NA	10 ml
First PCR Primer Mix	Mix of Forward and Reverse PCR Primers for First PCR (2.5 µM each)	10X	120 µl
Second PCR Primer Mix	Mix of Forward and Reverse PCR Primers for Second PCR (2.5 µM each)	10X	1,500 µl
Positive Control Template DNA	Positive Control template—genomic DNA labeled with one unique, single-copy barcode (0.2 µg/µl)	10X	25 µl

Box 2 (Pre-packaged lentiviral particles)

Panel of 5 packaged barcode labeling constructs; >1×10^7 TU of each construct.

Storage: -80°C

Barcodes are the same as the first 5 barcodes in the 10-Barcode Cell Labeling Kit. Constructs have both fluorescent (RFP) and puromycin resistance markers (pRSI16-U6-bc-UbiC-TagRFP-2A-Puro).

CloneTracker™ 10-Barcode Cell Labeling Kit

(Cat.# BCRPP10-V)

Box 1 (Reagents for amplification and sequencing)

Contains reagents for amplification and sequencing of up to 10 genomic DNA samples.

Storage: -20°C

Component	Description	Conc.	Volume
Taq DNA Polymerase	Thermostable DNA Polymerase	50X	300 µl
Taq DNA Polymerase Buffer	Reaction Buffer for PCR	10X	1,500 µl
dNTP Mix	Mix of dATP, dGTP, dCTP and dTTP, 10 mM each	50X	300 µl
PCR-Grade Water	Water for Reaction Mixes	NA	10 ml
First PCR Primer Mix	Mix of Forward and Reverse PCR Primers for First PCR (2.5 µM each)	10X	120 µl
Second PCR Primer Mix	Mix of Forward and Reverse PCR Primers for Second PCR (2.5 µM each)	10X	1,500 µl

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Positive Control Template DNA	Positive Control template—genomic DNA labeled with one unique, single-copy barcode (0.2 µg/µl)	10X	25 µl

Box 2 (Pre-packaged lentiviral particles)

Panel of 10 packaged barcode labeling constructs; >1×10^7 TU of each construct.

Storage: -80°C

The first 5 barcodes are the same ones in the 5-Barcode Cell Labeling Kit. Constructs have both fluorescent (RFP) and puromycin resistance markers (pRSI16-U6-bc-UbiC-TagRFP-2A-Puro).

CloneTracker™ 4-Barcode-Plus Cell Labeling Kit (Cat.# BC4P-V)

Box 1 (Reagents for amplification and sequencing)

Contains reagents for amplification and sequencing of up to 20 genomic DNA samples.

Storage: -20°C

Component	Description	Conc.	Volume
Taq DNA Polymerase	Thermostable DNA Polymerase	50X	50 µl
Taq DNA Polymerase Buffer	Reaction Buffer for PCR	10X	500 µl
dNTP Mix	Mix of dATP, dGTP, dCTP and dTTP, 10 mM each	50X	50 µl
PCR-Grade Water	Water for Reaction Mixes	NA	2 ml
First PCR Primer Mix	Mix of Forward and Reverse PCR Primers for First PCR (2.5 µM each)	10X	120 µl
Second PCR Primer Mix	Mix of Forward and Reverse PCR Primers for Second PCR (2.5 µM each)	10X	1,500 µl
Barcode 1 PCR Primer Mix	Mix of Forward and Reverse PCR Primers specific for Barcode 1 (2.5 µM each)	10X	120 µl
Barcode 2 PCR Primer Mix	Mix of Forward and Reverse PCR Primers specific for Barcode 2 (2.5 µM each)	10X	120 µl
Barcode 3 PCR Primer Mix	Mix of Forward and Reverse PCR Primers specific for Barcode 3 (2.5 µM each)	10X	120 µl
Barcode 4 PCR Primer Mix	Mix of Forward and Reverse PCR Primers specific for Barcode 4 (2.5 µM each)	10X	120 µl
Positive Control Template DNA	Positive Control template—mix of 4 genomic DNAs (1:1 ratio) each labeled with one of the 4 Barcodes (0.2 µg/µl)	10X	25 µl

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Box 2 (Pre-packaged lentiviral particles)

Panel of 4 packaged barcode labeling constructs; >1×10^7 TU of each construct.

> **Storage: -80°C**
>
> *Barcodes have unique sequences and sizes, and they can be differentially amplified with primers specific to each of the barcodes. Constructs have both fluorescent (RFP) and puromycin resistance markers (pRSI16-U6-bc-UbiC-TagRFP-2A-Puro).*

CloneTracker™ 4-Barcode-Plus Cell Labeling Kit (Puro only) (Cat.# BC4PPO-V)

Box 1 (Reagents for amplification and sequencing)

Contains reagents for amplification and sequencing of up to 20 genomic DNA samples.

> **Storage: -20°C**

Component	Description	Conc.	Volume
Taq DNA Polymerase	Thermostable DNA Polymerase	50X	50 µl
Taq DNA Polymerase Buffer	Reaction Buffer for PCR	10X	500 µl
dNTP Mix	Mix of dATP, dGTP, dCTP and dTTP, 10 mM each	50X	50 µl
PCR-Grade Water	Water for Reaction Mixes	NA	2 ml
First PCR Primer Mix	Mix of Forward and Reverse PCR Primers for First PCR (2.5 µM each)	10X	120 µl
Second PCR Primer Mix	Mix of Forward and Reverse PCR Primers for Second PCR (2.5 µM each)	10X	1,500 µl
Barcode 1 PCR Primer Mix	Mix of Forward and Reverse PCR Primers specific for Barcode 1 (2.5 µM each)	10X	120 µl
Barcode 2 PCR Primer Mix	Mix of Forward and Reverse PCR Primers specific for Barcode 2 (2.5 µM each)	10X	120 µl
Barcode 3 PCR Primer Mix	Mix of Forward and Reverse PCR Primers specific for Barcode 3 (2.5 µM each)	10X	120 µl
Barcode 4 PCR Primer Mix	Mix of Forward and Reverse PCR Primers specific for Barcode 4 (2.5 µM each)	10X	120 µl
Positive Control Template DNA	Positive Control template—mix of 4 genomic DNAs (1:1 ratio) each labeled with one of the 4 Barcodes (0.2 µg/µl)	10X	25 µl

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Box 2 (Pre-packaged lentiviral particles)

Panel of 4 packaged barcode labeling constructs; >1×10^7 TU of each construct.

> **Storage: -80°C**
>
> *Barcodes have unique sequences and sizes, and they can be differentially amplified with primers specific to each of the barcodes. Constructs have a puromycin-resistance marker, but no fluorescent marker (pRSIUP-U6-bc-UbiC-Puro).*

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2.1. Additional Materials for Transduction of Cells

The materials below are based on the transduction protocol in this manual which has been optimized for HEK293 and K-562 cells. Depending on the characteristics of your specific cells, the choice of media or other cell culture specifics may be different.

- LentiTrans™ Transduction Reagent (Cellecta, Cat.# LTDR1)
- Puromycin, 10 mg/ml (Sigma-Aldrich, Cat.# P9620-10ML)
- Dulbecco's Modified Eagle Medium (D-MEM) (1X) (Corning cellgro™, Cat.# 15-013-CV)
- Glutamine (L-Alanyl-L-Glutamine, Dipeptide L-glutamine) (Corning glutagro™, Cat.# 25-015-CI)
- Fetal Bovine Serum (Recommended: Corning, Cat.# 35-010-CV)
- Trypsin-EDTA, 1X (Corning, Cat.# 25-052-CI)
- Tissue Culture Plates and Related Tissue Culture Supplies
- HEPES pH 7.2-7.6, 1M solution (Corning, Cat.# 25-060-CI)
- $MgCl_2$, 1M solution
- Doxycycline, Ready Made Solution, 100 mg/ml (Sigma-Aldrich, Cat.# D3072-1ML) *(Optional, for tet-inducible vectors)*

Other than the specific reagents and instruments listed above, the protocols assume the user has access to standard materials (e.g., polypropylene tubes, pipette tips), equipment (table top centrifuges, pipettes, scales), and common reagents (e.g., TE buffer, ethanol) and buffers used in a typical life science laboratory.

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3. Recommended Pilot Experiments

In order to obtain reliable data from your library screen or individual construct experiment, we suggest appropriate planning beforehand. We recommend the pilot studies below in your cell system of choice (the cell system that will be used in your experiment). Cell-type specific data from these pilot experiments will provide you more confidence in your results.

Using the following procedures, confirm the cell doubling time, antibiotic efficacy on the target cells, and sensitivity of the target cells to the LentiTrans Transduction Reagent.

- Cell Doubling Time
- Calculating a Kill Curve
- Check Toxicity of LentiTrans Transduction Reagent

Visit the Cellecta Vector Information web page for details on our most common sgRNA, shRNA, and barcode lentiviral vectors.

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3.1. Cell Doubling Time

The doubling time is the time it takes your cells to double in number. It is useful to know the doubling time of your cells so that you can plate the appropriate number for transduction with a lentiviral library or construct.

1. Start with cells that have already been growing for a few weeks, rather than using cells that have just been thawed from a frozen state. To calculate the doubling time, trypsinize your cells as if you were going to split them.

2. Count them using a hemacytometer or cell counter, and keep track of the number that you replate onto the cell culture plates. The starting number of cells (at the beginning) is ***Xb***.

3. Propagate the cells as you normally do, replacing media as necessary.

4. The next time they are ready to be split, trypsinize them as usual and count them again using a hemacytometer or cell counter. The number of cells at the end is referred to as ***Xe***.

> **NOTE:** The cells should be in the log phase of growth to calculate doubling time properly, so it is important to not let the cells become confluent.

To calculate the doubling time, use the following formula:

> *Doubling Time* = [***T*** × (ln2)] / [ln (***Xe*** / ***Xb***)]
> where ***T*** = Time in any units

Example

> Let’s say that on Day 0, you count 2 × 10^6 cells. Three (3) days later, you count 16 × 10^6 cells.
>
> ***Xb*** = 2 × 10^6

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> ***T*** = 3 days
> ***Xe*** = 16×10^6
>
> Doubling Time = [3 × (ln2)] / [ln(16,000,000 / 2,000,000)]
> = [3 × (0.69)] / [ln(8)]
> = 2.08 / 2.08 = **1 day**

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3.2. Calculating a Kill Curve

Most of the lentiviral vectors used in making Cellecta's constructs express an antibiotic resistance marker. Regardless of the antibiotic selection marker that the construct expresses, you need to know the concentration of antibiotic that kills non-transduced cells within a given amount of time in order to successfully select transduced cells. We recommend the following methods for obtaining a "Kill Curve".

Puromycin Kill Curve

To create a Puromycin Kill Curve for your target cells, follow the protocol below.

1. Aliquot cells in a 12-well plate, at a density such that they are at 72 hours from confluency.
2. Add puromycin at concentrations of 0, 0.5, 1, 2, 5, and 10 µg/ml in six different wells.
3. Mix and place the cells at 37°C in a CO_2 incubator.
4. Grow cells under standard conditions for 72 hours.
5. Count viable cells, and determine the lowest concentration of drug that kills at least 95% of cells in 3-5 days.

Use this concentration at the puromycin selection step during your experiment.

Blasticidin Kill Curve

To calculate a Blasticidin Kill Curve, follow the same protocol above and use the same concentrations as for the Puromycin Kill Curve.

Hygromycin Kill Curve

If using hygromycin, follow the same protocol as for the Puromycin Kill Curve but use 0, 50, 100, 200, 400, and 800 µg/ml hygromycin in six different wells.

Neomycin Kill Curve

If you are using a construct with a neomycin selection marker, you need to test for resistance to geneticin (G418). Use the same protocol as for the Puromycin Kill Curve, but use a range of concentrations between 400-800 µg/ml.

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3.3. Check Toxicity of LentiTrans Transduction Reagent

The LentiTrans™ Transduction Reagent used during the transduction of target cells with lentivirus contains Polybrene, a transduction enhancement reagent. Some cell lines may be sensitive to this reagent. Before doing large-scale transduction, we recommend checking the toxicity of the LentiTrans Reagent using the procedure below:

1. In six wells of a 12-well plate, grow cells for 24 hours in complete culture medium without the LentiTrans Reagent, and with LentiTrans added at 0.25 µl/ml, 0.5 µl/ml and 1:1000 (1 µl/ml).

2. Replace old medium with LentiTrans-free complete culture medium and grow cells for an additional 72 hours.

3. Check for toxicity by counting viable cells.

For your experiments, use the highest concentration of LentiTrans Reagent that results in less than 10% cell toxicity compared to the no-LentiTrans culture (typically, a 1:1000 is recommended). For a few cell types, you may not be able to use LentiTrans Reagent.

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4. General Lentiviral Transduction Protocol

This section provides the general protocol for transduction of mammalian cells with VSV-G packaged lentiviral particles. This protocol was developed and optimized using HEK293 and K-562 cells, and has been successfully used with many other common cell types. However, each cell is different and, depending on the characteristics of your specific cells, some optimization may be necessary.

Lentiviral transductions are performed by mixing cells and virus in culture media. For both adherent and suspension cells, transductions are initiated in suspension and carried out overnight. Adherent cells are allowed to adhere to substrate during transduction and are transduced at a cell density that allows for 2-3 population doublings before reaching confluence. Suspension cells are typically transduced at a higher density than standard growth density, and then they are diluted to standard growth density 18-24 hours after transduction. Do not let cells become too dense or let the medium become yellow at any point.

Before transduction, seed and expand cells from frozen stocks. Cells should be actively growing.

> ! **CAUTION:** Only open the tube containing the lentiviral particles in the laminar flow hood. Please refer to the **Safety Guidelines** and check with your institution regarding the use of lentiviruses.

Day 0—Inoculate Cells

1. Quickly thaw the lentiviral vector particles in a water bath at 37°C. Transfer the thawed particles to a laminar flow hood, gently mix by rotation, inversion, or gentle vortexing, and keep on ice. Unused viral particles can be aliquoted, refrozen at -80°C, and used again for subsequent experiments. There will be some loss of viral activity (usually 10-20%) with each refreeze.

2. Suspend sufficient cells for transduction in appropriate complete media supplemented with 1 µl/ml LentiTrans™ Transduction Reagent—if cells are sensitive to the LentiTrans Reagent, use a lower concentration or omit it. For HEK293 cells, we usually suspend at a density of 1×10^5 cells/ml in D-MEM supplemented with 10% FBS and LentiTrans Reagent. For K-562 cells, we usually suspend at a density 2×10^6 cells per ml in RPMI/10%FBS supplemented with 20mM HEPES, pH 7.4 and LentiTrans Reagent.

3. Aliquot cells into wells or plates. For small scale transductions or titering assays, cells may be plated into multiwell microtiter plates (e.g., 0.5 ml/well for 24-well plates or 1 ml/well for 12-well plates). To transduce larger numbers of cells, use larger plates and scale up the volume accordingly

4. To each plate, add an appropriate amount of lentivirus. The amount of virus will depend on your viral titer and your experiment. Refer to the Transduction Guidelines or Assay Procedures section of the product manual for application specific recommendations.

5. Close the plate and mix by gentle agitation:
    - For adherent cells, place the plate into the CO_2 incubator and grow cells under standard conditions for 16-24 hours.
    - For suspension cells only, “spinoculate” by wrapping the perimeter with parafilm, placing the plate into the centrifuge with an appropriate balance, and spin the cultures at 1,200 × g at +25°C for 2 hours. Following centrifugation, remove plate(s) from centrifuge, carefully remove parafilm, and place in incubator. After 3 hours, “feed” cells with 0.5 ml additional complete medium per well (no LentiTrans Reagent).

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Day 1—Change Media

Between 16 to 24 hours post-transduction, remove media and replace with fresh complete media without LentiTrans Reagent. For suspension cells, spin down and resuspend cells in complete media at 1-5 × 10^5 cells/ml. Place in incubator and grow for an additional 24-48 hours. Avoid confluency or too high a density of cells during and after transduction. If necessary, replate.

Day 3 or 4—Harvest or Split Cells

At about 72 hours after adding virus, you may expand cells as normal or harvest cells for an assay. To continue growing cells, split the cells 1:4 to 1:8 (or as appropriate, depending on the type of cells) as the culture approaches confluence, and add complete medium. As required by your experiments add antibiotics, other factors (tetracycline), etc., and expand as normal.

NOTE: Typical doxycycline concentration for induction is 0.5 ug/ml. However, some cell lines can be more sensitive than others, so you may want to optimize the concentration.

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5. Amplification and Sequencing of Cell Barcodes

Analysis of barcode representation in the experimental samples first requires amplification of the barcode portion of the integrated lentiviral constructs from sample genomic DNA. Second, the amplified barcoded PCR product needs to be cloned in a TA-cloning vector. Third, subsequent Sanger sequencing of the barcodes present in bacterial clones will allow you to measure representation of the each barcode in the mixed population of founder cells labeled with a different barcodes.

For 10-Barcode Panels with Cat.# BCXP3RPM10-V, if the labeled cell pool(s) have been mixed with cells transduced with the CloneTracker™ XP 5M or 50M Barcoded Library, Next-Gen Sequencing (NGS) of barcodes may be used to quantify the relative number of progeny from each labeled founder population. For using NGS for barcode representation analysis, please refer to the CloneTracker XP Barcoded Library protocol.

For 5- or 10-Barcode Panels with Cat.#s BCRPP5-V or BCRPP10-V, if the labeled cell pool(s) have been mixed with cells transduced with the CloneTracker™ 50M Barcoded Library, Next-Gen Sequencing (NGS) of barcodes may be used to quantify the relative number of progeny from each labeled founder population. For using NGS for barcode representation analysis, please refer to the CloneTracker 50M Barcoded Library protocol.

> **NOTE:** The 4-Barcode-Plus constructs are not compatible and will not be read with CloneTracker™ Barcode Library barcodes during library amplification and NGS.

The barcode amplification procedure below has been developed and optimized for conventional Sanger sequencing.

- Pooled barcodes should be amplified by two rounds of PCR using Taq DNA Polymerase.
- The protocol was optimized using a ThermoFisher Veriti® Thermal Cycler. Use of other PCR enzymes and/or thermal cyclers may require additional optimization.
- For barcoded cell populations with fewer than 1 million cells, we recommend to purify genomic DNA using the QIAGEN QIAamp DNA Micro Kit, according to the manufacturer's instructions. For barcoded cell populations with between 1 million and 10 million cells, you can use the QIAGEN DNeasy Blood and Tissue Kit for genomic DNA isolation.
- In addition to experimental barcoded cell samples, please purify genomic DNA from parental, non-infected cells. This genomic DNA could be used as a negative control in the barcode amplification step.

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5.1. Barcode Amplification

This step enables specific amplification of barcodes from genomic DNA using two rounds of PCR with vector-specific primers flanking the barcode.

1. First Round PCR Step

Prepare the First PCR Master Mix according to the example below. Adjust the amount of Master Mix depending on number of samples, including enough to run the included Positive Control Template DNA (1 µg per test tube) and one negative control (1 µg of genomic DNA sample without barcoded insert).

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Volume	Component
5 µl	200 ng/µl Genomic DNA (1 µg)
5 µl	First PCR Primer Mix *
2 µl	50X dNTP Mix (10 mM each)
5 µl	10X Taq Polymerase Buffer
32 µl	PCR-Grade Water
1 µl	50X Taq Polymerase
50 µl	**Total volume**

** If using CloneTracker XP Barcode Panel constructs, use 2.5 µl of each of the Forward-XP and Reverse-XP primers.*

Perform PCR under the following cycling conditions.

94°C, 1 minute	1 cycle
94°C, 15 seconds, 60°C, 15 seconds, 72°C, 30 seconds	20 cycles
72°C, 2 minutes	1 cycle

2. Second Round PCR Step

Use a 5 µl aliquot from First PCR Step above in the second round of PCR with nested primers:

Volume	Component
5 µl	First Round PCR Product
5 µl	Second PCR Primer Mix **
2 µl	50X dNTP Mix (10 mM each)
5 µl	10X Taq Polymerase Buffer
35 µl	PCR-Grade Water
1 µl	50X Taq Polymerase
50 µl	**Total volume**

** If using CloneTracker XP Barcode Panel constructs, use 2.5 µl of each of the NFwd-XP and NRev-XP Index A primers.*

Perform PCR under the following cycling conditions.

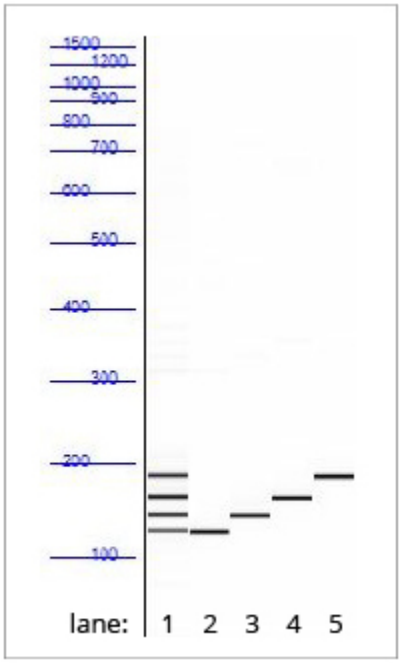
94°C, 1 minute	1 cycle
94°C, 15 seconds, 65°C, 15 seconds, 72°C, 30 seconds	16 cycles
72°C, 2 minutes	1 cycle

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3. PCR Product Analysis

Analyze the PCR products by gel electrophoresis on a 3.5% agarose-1xTAE gel (load 5 µl/lane), an Agilent Bioanalyzer, or an AATI Fragment Analyzer. The gel electrophoresis analysis should reveal a bright band of amplified barcode products (251 bp product for BCRPP5/10-V kits or a set of up to four PCR products with sizes of 121, 141, 161, and 181 bp for the BC4P-V kit) (see Figure below for example results) with intensities similar to the Positive Control Template DNA sample. Run additional cycles if the experimental sample shows very weak (2-3 cycles) or no visible band (5 cycles). Repeat analysis of amplified products on a 3.5% agarose-1xTAE gel in order to ensure similar yields of amplified barcode products for all samples.

For cells barcoded with the Barcode-Plus constructs (Cat.# BC4P-V), the relative intensities of four differently-sized bands (each band specific for one of the unique barcodes) provide an estimate of the number of cells with each barcode in the population (see Figure below). The four PCR products present in the size range of 121-181 bp can be quantified by subtracting the background density of the negative control sample from the density of the bands on the gel using software available on most imaging stations, such as the Bioanalyzer or Fragment Analyzer. The Positive Control sample with a mix of four barcoded genomic DNA samples mixed in equal amounts can be used as a normalization control. If any barcodes exhibit too low an intensity to measure, place the samples back into the thermal cycler and perform an additional 2-4 cycles. However, avoid overcycling which can result in the generation of a longer fragment that corresponds to a fusion double barcode product.



Fragment Analyzer gel image of amplified barcodes in the 4 Barcode-Plus constructs kit (Cat.# BCP4-V). Lane 1 shows all four constructs amplified together, and each of the following lanes shows a specific single barcode amplification.

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5.2. PCR Clean-up Prior to TA Cloning

To remove non-specific PCR products and other factors that may reduce the efficiency of TA cloning, the amplified barcodes may be purified. This may help ensure that a greater number of clones have the correct barcode for sequencing.

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To purify the PCR product, use the QIAquick PCR Purification Kit (QIAGEN) following the manufacturer's protocol. In the last centrifugation step, centrifuge the spin filter at maximum speed for at least 5 minutes to completely dry the membrane thereby avoiding ethanol contamination in the purified PCR product. Separate the purified PCR products by electrophoresis in a preparative 3.5% agarose-1xTAE gel. Cut out the correctly-sized band and extract DNA from the gel using the QIAquick Gel Purification Kit (QIAGEN) and then proceed with cloning the gel-purified PCR product in the TA cloning vector, based on the manufacturer's protocol.

1. Purify and concentrate the PCR products from each sample using the QIAquick PCR Purification Kit following the manufacturer's protocol. Samples should be eluted in a volume of approximately 30 µl.

2. After purifying and concentrating, run each purified PCR product with 10X loading buffer on a 3.5% agarose-1xTAE gel with well sizes that accommodate at least 50 µl.

3. Using a transilluminator and scalpel, excise the narrow band that corresponds to the correct target sgRNA or barcode amplicon size.

> **Caution!** Be sure to use UV safety glasses to protect your eyes when viewing and excising the DNA from the gel on the transilluminator.

4. After excision, purify each PCR product from the gel fragment using a QIAquick Gel Extraction Kit following the manufacturer's protocol. Elute the purified PCR product in 20 µl of elution buffer.

> **Note:** Be sure to centrifuge QIAquick columns at maximum speed for at least 3 minutes before eluting DNA to avoid ethanol contamination in the purified PCR product.

5. Quantify extracted DNA in each sample using A260nm OD measurement using NanoDrop® spectrophotometer (or equivalent), and then adjust the concentrations as needed for TA Cloning.

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5.3. Barcode Analysis by Sanger Sequencing

1. TA Cloning

If the PCR product analysis reveals specific amplification of the expected barcoded product, directly clone 1 µl of PCR product in the TA cloning vector, transfect into One Shot™ INVαF' E. coli, and grow transformed cells overnight on ampicillin agar plates based on the manufacturer's protocol. Use the TA-Cloning Kit with pCR 2.1 Vector and One Shot INVαF' Chemically Competent E. coli (ThermoFisher, Cat.# K200001)

> **Note:** If there seems to be excessive non-specific PCR products, you can purify the specific barcodes before TA cloning. See **Appendix C – PCR Clean-up Prior to TA Cloning**.

2. Barcoded Clone Amplification

To analyze the representation of different barcodes in TA clones, pick 96 clones (or as many as you would like to sequence) and grow these clones in a 96-well plate in 100 µl of LB media with 100 µg/ml of ampicillin in a 37°C shaker for two hours.

1. Prepare the PCR master mix using the protocol below for 96 samples (or scale as needed for the number of clones you picked). For amplification of the TA-cloned inserts, use the T7 and T3 primers included with the TA cloning kit:

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Volume	Component
250 µl	10 mM each of T7 and T3 primers
50 µl	50X dNTP Mix (10mM each)
250 µl	10X Taq Polymerase Buffer
1,900 µl	PCR-Grade Water
50 µl	50X Taq Polymerase
2,500 µl	**Total Volume**

2. Aliquot 25 µl of the Master Mix in a 96-well plate and add a 1 µl aliquot of each bacterial clone culture (from 96-well plate with LB media) to each well with PCR Master Mix. Run PCR under the following cycling conditions:

94°C, 2 minutes	1 cycle
94°C, 30 seconds, 72°C, 20 seconds	25 cycles
72°C, 2 minutes	1 cycle

3. Sanger Sequencing

Analyze the representation of different barcodes in the 96 clones by Sanger sequencing using the M13 Forward and Reverse sequencing primers (optionally, the T7 or T3 primers that were used for amplification may also be used for sequencing). Compare sequencing results to reference barcode sequences (see Product Insert). If necessary (e.g., if some barcodes are present with only 1-2 copies per 96 clones), analyze more clones for better statistics.

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6. Technical Support

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7. Safety Guidelines

The HIV-based lentivector system is designed to maximize its biosafety features, which include:

- A deletion in the enhancer of the U3 region of 3'ΔLTR ensures self-inactivation of the lentiviral construct after transduction and integration into genomic DNA of the target cells.
- The tat-transactivator domain has been removed from the upstream 5'-LTR in the lentivector to disable transcription from the 5'-LTR.
- The three proteins (gag, pol, env) are necessary for replication and packaging of the lentiviral vector. When packaging lentiviral particles, the genes encoding these three packaging proteins are separately introduced and expressed in the packaging cells using separate ordinary plasmids that lack packaging signals and share no significant homology to the lentivector. As a result, none of the HIV-1 genes (gag, pol, env) will be present in the packaged lentiviral genome.
- None of the HIV-1 genes (gag, pol, env) will be present in the packaged lentiviral genome, as they are expressed from packaging plasmids lacking packaging signal—therefore, the lentiviral particles generated are replication-incompetent.
- Lentiviral particles will carry only a copy of your expression construct.

Despite the above safety features, use of replication-incompetent lentiviral particles falls within NIH Biosafety Level 2 criteria. For a description of laboratory biosafety level criteria, consult the Centers for Disease Control Office of Health and Safety Web site at:

> https://www.cdc.gov/labs/bmbl/

It is also important to check with the health and safety guidelines at your institution regarding the use of lentiviruses and follow standard microbiological practices, which include:

- Wear gloves and lab coat at all times when conducting the procedure.
- Always work with lentiviral particles in a Class II laminar flow hood.
- All procedures are performed carefully to minimize the creation of splashes or aerosols.
- Work surfaces are decontaminated at least once a day and after any spill of viable material.
- All cultures, stocks, and other regulated wastes are decontaminated before disposal by an approved decontamination method such as autoclaving. Materials to be decontaminated outside of the immediate laboratory area are to be placed in a durable, leakproof, properly marked (biohazard, infectious waste) container and sealed for transportation from the laboratory.

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8. Contact Us

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