CELLECTA

USER MANUAL

NGS Prep Kit for sgRNA, shRNA, and DNA Barcode Libraries

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Cellecta, Inc.

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1. NGS Prep Kit for sgRNA, shRNA, and DNA Barcode Libraries

Background

Cellecta's NGS Prep Kit provides a protocol and reagents for the PCR amplification and NGS sequencing of effector or barcode inserts from genomic DNA isolated from cell populations screened with any of several Cellecta CRISPR sgRNA, dual-sgRNA, shRNA, and barcode libraries, as well as a few similar libraries available from other sources. Sufficient reagents are provided to process 12-48 DNA samples (depending on the amount of DNA/sample) with unique indexing primers to multiplex up to 12 samples run in a single lane. Depending on the specific library used for a screen, several versions of the NGS Prep Kit are available—each containing the appropriate primers for the target library. To multiplex more samples, Supplementary Primer Sets with an additional 12 indexed primers are also available for each Sequencing Kit.

Cat.#	Description
LNGS-101	NGS Prep Kit for shRNA Libraries in pRSI12 (DECIPHER)
LNGS-102	NGS Prep Kit for shRNA Libraries in pRSI16/17 (HGW shRNA) (or pRSI16cb/17cb)
LNGS-120	NGS Prep Kit for sgRNA Libraries in pRSG16/17 (CRISPR KOHGW)
LNGS-130	NGS Prep Kit for sgRNA Libraries in pRSGT16/17
LNGS-200	NGS Prep Kit for Barcode Libraries in pRSI16/17 (CloneTracker™ 50M)
LNGS-300	NGS Prep Kit for Barcode Libraries in pScribe (CloneTracker XP™)
LNGS-350	NGS Prep Kit for CRISPR-Barcode Libraries in pRSGScribe1cb (CloneTracker XP™ CRISPR)
LNGS-360	NGS Prep Kit for sgRNA Libraries in pRSGScribe (no clonal barcodes)
LNGS-510	NGS Prep Kit for CloneSelect V2 Libraries in pCloneSelect1
LNGS-400	NGS Prep Kit for Dual-sgRNA Libraries in pRSL10 (KADHGW/KIDHGW)
LNGS-900	NGS Prep Kit for sgRNA Libraries in LentiGuide (GeCKO, Brunello)
LNGS-905	NGS Prep Kit for sgRNA Libraries in LentiCRISPRv2 (GeCKO, Brunello Kinome)

If you are not sure which kit to use for your library, please contact Cellecta (tech@cellecta.com).

Supplementary Primer Sets

Cat.#	Description
LNGS-101-SP	Supplementary Primer Set for LNGS-101 (12 Additional Index Primers)
LNGS-102-SP	Supplementary Primer Set for LNGS-102 (12 Additional Index Primers)
LNGS-120-SP	Supplementary Primer Set for LNGS-120 (12 Additional Index Primers)
LNGS-130-SP	Supplementary Primer Set for LNGS-130 (12 Additional Index Primers)
LNGS-200-SP	Supplementary Primer Set for LNGS-200 (12 Additional Index Primers)
LNGS-300-SP	Supplementary Primer Set for LNGS-300 (12 Additional Index Primers)
LNGS-350-SP	Supplementary Primer Set for LNGS-350 (12 Additional Index Primers)
LNGS-360-SP	Supplementary Primer Set for LNGS-360 (12 Additional Index Primers)
LNGS-400-SP	Supplementary Primer Set for LNGS-400 (12 Additional Index Primers)
LNGS-510-SP	Supplementary Primer Set for LNGS-510 (12 Additional Index Primers)

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LNGS-900-SP	Supplementary Primer Set for LNGS-900 (12 Additional Index Primers)
LNGS-905-SP	Supplementary Primer Set for LNGS-905 (12 Additional Index Primers)

Please read the entire user manual before proceeding with your experiment.

Click the ? Download as PDF link located at the bottom of the left menu to download the PDF version of this user manual.

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Last modified: 16 September 2024

2. NGS Prep Kit Required Materials

Materials Provided

Additional Materials Required

Related Products and Services from Cellecta

Last modified: 10 March 2017

2.1. Materials Provided

Box 1 Components

Description	Concentration	Quantity
Taq DNA Polymerase	50X	200 µl (2 vials)
Taq DNA Polymerase Buffer	10X	1,200 µl (2 vials)
dNTP Mix	50X (10 mM each)	200 µl
PCR-Grade Water	NA	7,320 µl (4 vials)

Box 2 Components

Description (Generic primer names: see Note below)	Concentration	Quantity
Forward Primer	10 µM	150 µl
Reverse Primer	10 µM	150 µl
NFwd Primer	20X (10 µM)	240 µl
NRev Index Primer A	20X (10 µM)	20 µl
NRev Index Primer B	20X (10 µM)	20 µl
NRev Index Primer C	20X (10 µM)	20 µl
NRev Index Primer D	20X (10 µM)	20 µl
NRev Index Primer E	20X (10 µM)	20 µl
NRev Index Primer F	20X (10 µM)	20 µl
NRev Index Primer G	20X (10 µM)	20 µl
NRev Index Primer H	20X (10 µM)	20 µl
NRev Index Primer I	20X (10 µM)	20 µl
NRev Index Primer J	20X (10 µM)	20 µl
NRev Index Primer K	20X (10 µM)	20 µl
NRev Index Primer L	20X (10 µM)	20 µl
Seq NGS Primer*	100 µM	40 µl
Index NGS Primer	100 µM	40 µl

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* In the NGS Prep Kits for CRISPR-Barcode and Dual-sgRNA Libraries, two unique Seq NGS Primers are included and are required for sequencing.

> **Note:** The generic names for each of the primers are listed above for reference. However, each version of the NGS Prep Kit has primers specific for the vector of the target library. These specific primers names also include a suffix to indicate the vector or library they target (e.g., Forward-G in LNGS-120, Forward-12 in LNGS-101, NRev-16 in LNGS-102, etc.). This Manual uses the generic primer name when, for example, it is describing which one to include in a reaction. For each generic primer reference, you should use the appropriate fully named specific primer included in your kit.

Storage Conditions:

Store kits and all components at -20°C until ready for use.

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2.2. Additional Materials Required

- QIAquick PCR Purification Kit (QIAGEN, Cat.# 28106)
- QIAquick Gel Extraction Kit (QIAGEN, Cat.# 28706)
- PhiX Control (Illumina, Cat.# FC-110-3001)
- NGS Reagent Kits, for NextSeq and HiSeq (Illumina):

Platform	Kit Type	Illumina Cat.#	Description
NextSeq® 500/550 Single Read (SR) or Paired End (PE)	Sequencing & Cluster Gen.	20024906	NextSeq 500 v2.5 Kit (75 cycles, High-Output)
HiSeq® 2000/2500 **Single Read (SR)**	Sequencing	GD-401-3001	TruSeq SBS Kit v3 – HS (50 cycles)
	Cluster Gen.	FC-401-3002	TruSeq SR Cluster Kit v3-cBot-HS
HiSeq® 2000/2500 **Paired End (PE)**	Sequencing	GD-401-3001	TruSeq SBS Kit v3 – HS (50 cycles)
	Cluster Gen.	PE-401-3001	TruSeq PE Cluster Kit v3-cBot-HS
HiSeq® 3000/4000 **Single Read (SR)**	Sequencing	FC-410-1001	HiSeq 3000/4000 SBS Kit (50 cycles)
	Cluster Gen.	GD-410-1001	HiSeq 3000/4000 SR Cluster Kit
HiSeq® 3000/4000 **Paired End (PE)**	Sequencing	FC-410-1001	HiSeq 3000/4000 SBS Kit (50 cycles)
	Cluster Gen.	PE-410-1001	HiSeq 3000/4000 PE Cluster Kit
NextSeq® 1000/2000 Single Read (SR) or Paired End (PE)	Sequencing & Cluster Gen.	20100983	NextSeq 1000/2000 P1 XLEAP-SBS Reagent Kit (100 Cycles)
NextSeq® 1000/2000 Single Read (SR) or Paired End (PE)	Sequencing & Cluster Gen.	20100987	NextSeq 1000/2000 P2 XLEAP-SBS Reagent Kit (100 Cycles)
NextSeq® 1000/2000 Single Read (SR) or Paired End (PE)	Sequencing & Cluster Gen.	20100990	NextSeq 1000/2000 P3 XLEAP-SBS Reagent Kit (100 Cycles)
NextSeq® 1000/2000 Single Read (SR) or Paired End (PE)	Sequencing & Cluster Gen.	20100995	NextSeq 1000/2000 P4 XLEAP-SBS Reagent Kit (50 Cycles)
NextSeq® 1000/2000	Sequencing	20100994	NextSeq 1000/2000 P4 XLEAP-SBS

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Single Read (SR) or Paired End (PE)	& Cluster Gen.		Reagent Kit (100 Cycles)
NextSeq 1000/2000	Illumina Primers	20112856	XLEAP-SBS NextSeq 1000/2000 Read & Index Primers

> **Note:** See Illumina website for information on kits for other Illumina instruments (e.g. NovaSeq, MiSeq).

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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2.3. Related Products and Services from Cellecta

Related Services

- NGS of Samples from Genetic Screens
- Lentiviral Packaging Services

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3. Procedure Overview

Quantifying the representation levels of sgRNA, shRNA, or barcode sequences in experimental samples derived from genetic screens using pooled effector libraries first requires isolation of the genomic DNA. After DNA isolation, effector or barcode sequences are amplified by PCR (First Round PCR) from purified genomic DNA using library-specific primers. A Second Round of PCR amplification of the effector and barcode sequences is then performed with “nested” primers to introduce indexes and generate template compatible with Illumina NGS. Finally, the representation of sgRNA or barcode sequence in each sample are quantified by counting the number of specific reads generated by NGS on an Illumina Instrument (e.g., NextSeq, NovaSeq, HiSeq, or MiSeq).

> **Note:** The protocol was optimized using a Life Technologies Veriti® Thermal Cycler. Use of other PCR enzymes and/or thermal cyclers may require additional optimization.

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3.1. Genomic DNA Extraction

There are several protocols that can be used for DNA isolation. However, to accurately measure the relative fraction of each sgRNA or barcode present in a specific cell population, it is important to isolate the whole amount of genomic DNA from the cells derived from a genetic screen or barcoded sample. Purification of genomic DNA from just a fraction of cells at a particular time point or treatment condition in a screen, may not provide full representation the guides, hairpins, or barcodes present in the screened sample. As a result, the choice of DNA isolation protocol should be based on the number of cells in your samples.

We recommend the following protocols:

- For genomic DNA isolated from 10 million or more cells—which are typical from standard “dropout viability” CRISPR and RNAi screens with larger libraries—we highly recommend using the Large-Scale Genomic DNA Extraction Protocol. With this procedure, you usually obtain 50-100 µg of genomic DNA per 10 million cells. This amount of genomic DNA will overload most column-based DNA isolation kits and compromise yields. Conventional Genomic DNA Extraction is not column based and avoids DNA loss which can distort representation of the guides or barcodes in the population.

- For small and medium-sized populations of cells (from small libraries screens, positive selection screens where most of the cells are killed off, or FACS-based enrichment screens), we recommend using the following QIAGEN kits:
  - From 1 million to 10 million cells: Use the QIAGEN DNeasy Blood and Tissue Kit (QIAGEN, Cat.# 69504)
  - For fewer than 1 million cells: Use the QIAGEN QIAamp DNA Micro Kit (QIAGEN, Cat.# 56304)

- After purification, you should resuspend your DNA at a concentration of ca. 1-2 µg/µl in water or Tris buffer (**Important:** Do not resuspend DNA in a buffer that contains EDTA. It will inhibit the PCR reaction.). DNA samples can be stored at +4°C for a few weeks or at -20°C for an extended period of time.

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3.1.1. Large-Scale Genomic DNA Extraction Protocol

> **NOTE:** Use of disposable tubes is highly recommended in order to avoid contamination.

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1. Resuspend cell pellet in 5 ml QIAGEN Buffer P1 (with RNase A) in a 15 ml polypropylene (phenol:chloroform resistant), Falcon screw-cap centrifuge tube (12,000 RCF rated).

2. Add 0.25 ml 10% SDS, mix, and incubate 5 minutes at room temperature.

3. Using an ultrasonic homogenizer, sonicate to shear DNA into 10-100 kb sized fragments. To prevent cross-contamination, thoroughly wash the ultrasound head with running water and dry with clean paper towels between samples.

4. Add 10 µl of Proteinase K, mix, and incubate 15 minutes at room temperature.

5. Add 5 ml Phenol:Chloroform:Isoamyl Alcohol solution, vortex hard, and spin down 60 min, 20°C at 8,000 rpm in JA-14 or equivalent rotor (Beckman).

6. At this point, there should be approximately 5 ml of clear upper phase. Transfer 4 ml of upper phase to a new 15 ml disposable screw cap tube (same as in Step 1).

7. Add 0.5 ml of 3M Sodium Acetate and 4 ml isopropanol, mix well, then spin down 30 min, 20°C at 8,000 rpm in a JA-14 or equivalent rotor.

> **NOTE:** To produce a more visible pellet that is compacted at the bottom of the tube, it is recommended to incubate overnight at room temperature before centrifugation.

8. Discard supernatant, add 10 ml of 70% ethanol, and spin down 5 min, 20°C at 8,000 rpm in a JA-14 or equivalent rotor.

9. Discard supernatant and air-dry pellet.

10. Dissolve DNA pellet in an appropriate volume of dH_2O to a concentration of ~2 mg/ml.

11. Incubate 30 minutes at 80°C before spectrophotometer reading.

> **NOTE:** Expected yield is about 10 µg genomic DNA per 1 million cells.

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3.2. Amplification of sgRNA or Barcode Sequences from Genomic DNA

The protocol is based on two rounds of PCR to amplify the integrated sgRNA, shRNA barcode sequences, or clonal barcodes from the genomic DNA. Also, the second round of PCR includes primers that add the sample-specific indexes and sequences (P5 and P7) complementary to primers immobilized in Illumina flow cells. With the PCR reactions, the optimization of cycles is required to avoid over-cycling. Using an optimal cycle number is necessary to generate a sufficient amount of amplicons to sequence while maintaining the relative representation of each sgRNA or barcode in the cell population.

- As an amplification/sequencing positive control, we recommend that you use the original shRNA/sgRNA/barcode plasmid library (1 ng) used in the screen. This positive control can be used as a baseline in NGS data analysis to measure the enrichment or depletion of effector constructs in the experimental samples. If you do not have the plasmid library, we suggest either using any construct in the same vector to serve as a positive control for PCR or the

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packaged library to serve as an amplification/sequencing positive control as described in the following bullet.

- Although it is typically not necessary, you may use the packaged effector library as a positive control. In order to use the packaged library as a positive control, you can start with a volume containing sufficient viral particles equal to at least a few hundred-fold the complexity of the library. For example, for a 50,000-construct library, you should use a volume containing 10-25 million transduction units (TU). Also, before starting PCR, you will need to reverse transcribe the viral RNA sequence using a reverse primer that targets the sense (upper) strand of the insert site at a location where it will generate a full transcript of the target sgRNA/barcode region, including the PCR primer sites. For most Cellecta libraries containing the UbiC promoter, the RevUbiC1 primer (5'-AGGCAGCGCTCGCCGTGAGGA-3') adjacent to the cloning site can be synthesized and used for the reverse transcription step.

First Round PCR

The goal of the first PCR is to amplify shRNA barcodes, sgRNA inserts, or barcode library sequences from genomic DNA isolated from each experimental cell population. The amount of starting DNA can vary depending on the type of screen. For samples with up to 50 µg of genomic DNA, run a single 100 µl reaction for the each sample. For samples with more than 50 µg of DNA, we recommend dividing up the run into multiple 100 µl PCR reactions, each with a maximum of 50 µg DNA.

1. For each sample, prepare a PCR mix according to the table below, where ***x*** is the volume of genomic DNA (typically 50 µl at 1 µg/µl). Many samples will have more than 50 µg of DNA. For these samples, you will need to scale up the master reaction below as needed for each sample, then divide the reaction into multiple tubes with 100 µl in each. For example, if you have 180 µg of DNA in one sample, you should prepare a 400 µl master mix, then aliquot 100 µl in four PCR tubes for amplification (180 µg / 50 µg = 3.6, so round to 4 reactions).

Volume	Component
x µl	Genomic DNA (up to 50 µg)
3 µl	Forward 1st Round PCR Primer
3 µl	Reverse 1st Round PCR Primer
2 µl	dNTP Mix
10 µl	Taq Polymerase Buffer
80 – ***x*** µl	PCR-Grade Water
2 µl	Taq Polymerase
100 µl	**Total volume**

2. Mix gently, centrifuge briefly to collect droplets, divide samples with more than 50 µg of genomic DNA into 100 µl PCR reactions as necessary.

3. Perform PCR using the following cycling conditions.

95°C, 2 minutes	1 cycle
95°C, 30 seconds, 65°C, 30 seconds, 68°C, 2 minutes	16-18 cycles*
68°C, 2 minutes	1 cycle

> * **Note:** Depending on your library, the specific number of cycles may need some optimization. For barcode libraries (and shRNA libraries with barcodes), 16 cycles is typically sufficient. For sgRNA libraries and dual-sgRNA libraries, 18 cycles is recommended. If you are using less than 50 µg of genomic DNA in a 100 µl PCR reaction, increase the number of cycles (e.g., for 25 µg, use one extra cycle). However, please note that we do not recommend analyzing the First Round PCR product by gel or equivalent (Bioanalyzer, etc.). You may not see a band at all or just a light band at most. You

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> should evaluate the PCR product only after running both First and Second Round PCR.

Second Round PCR

The Second Round of PCR is required in order to significantly reduce genomic DNA carryover into the NGS step. Additionally, the Second Round PCR primers add the P5 and P7 sequences that are complementary to the immobilized primers in the NGS Illumina Flow Cells.

- For Cellecta NGS Prep Kits and Supplementary Index Primer Sets, the NRev Index Primers (up to 24) each contain a different index sequence which will be used to deconvolute the sequencing results for each sample mixed together in the same Flow Cell or sequencing lane (i.e., multiplex sequencing). It is important, therefore, to use a different NRev Index Primer (each signified by a different letter) to amplify each sample that will be run together in the same Flow Cell or lane.

For the Second Round of PCR, start with an aliquot of 5 µl of the First-Round PCR reaction for each sample.

1. If you are starting from more than 50 µg of genomic DNA, it is important to combine all individual reactions for each sample together into a single tube.

2. Perform the Second Round of nested PCR using a 5 µl aliquot of the First Round PCR product for each sample:

Volume	Component
5 µl	First Round PCR Product
5 µl	NFwd Primer
5 µl	NRev (Index) Primer*
2 µl	dNTP Mix
10 µl	Taq Polymerase Buffer
71 µl	PCR-Grade Water
2 µl	Taq Polymerase
100 µl	**Total volume**

> * **Note:** Each sample to be sequenced together in a single Flow Cell or lane needs to be amplified with a different NRev Index Primer in the Cellecta NGS Prep Kit. Be sure to use a differently lettered NRev Index for each sample that will be loaded on the same Flow Cell or lane.

3. Perform PCR under the following cycling conditions.

95°C, 2 minutes	1 cycle
95°C, 30 seconds, 65°C, 30 seconds, 68°C, 2 minutes	12 -16 cycles**
68°C, 2 minutes	1 cycle

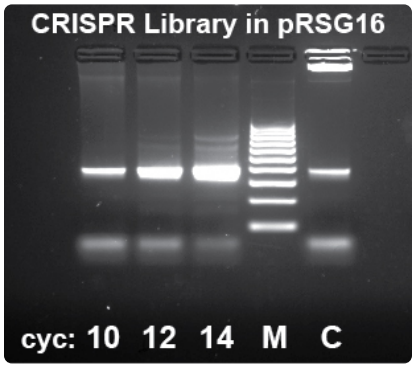
> ** **Note:** Additional cycles may be needed for samples as described below.

4. Analyze the amplified products from the Second Round of PCR. This analysis may be done on an Agilent® 2100 Bioanalyzer® instrument with the Agilent® High Sensitivity DNA Kit (Cat.# 5067-4626) or a Fragment Analyzer (Advanced Analytical) instrument using Standard Sensitivity NGS Analysis Kit (Cat.# DNF-473-1000) using the manufacturer's protocol, if you have access to this equipment. If not, then we recommend the following procedure

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using agarose gel electrophoresis to analyze the samples:

- Run all samples including positive control on 3.5% agarose-1xTAE gel. Mix 5 µl of aliquot from the each sample with 2x loading buffer (e.g. 10% sucrose with Bromophenol Blue tracking dye).
- Analyze the intensity of the bands after electrophoresis (i.e., Bromophenol Blue tracking dye about 80% down the gel). The typical results of agarose gel electrophoresis analysis are shown in the figure below. The expected size of amplified barcode, shRNA barcode, and sgRNA products is typically between 125 to 400 bp or dual-sgRNA product around 750bp and is provided in the NGS Cassette Design information specific for your library or NGS Prep Kit Product Certificate. If you did not receive Cellecta's Order Information email with links to this information, please contact us at tech@cellecta.com.



CRISPR Human Genome 80K Knockout Library NGS PCR product analysis by gel electrophoresis. In this example, 10 cycles is the recommended cycle number for the Second Round PCR step.



DECIPHER Library NGS PCR product analysis by gel electrophoresis

5. Quantify the amount of the amplified product for each sample using software tools provided with instrument or gel imager. If all samples show similar yield of amplified products (+/- 2-fold) no addition cycling is required and you should proceed to NGS Samples Purification. If there is a greater than 2-fold variance, you should put the samples with very weak or missing bands back into the PCR instrument for additional cycles. For example, if the amount of PCR product in the sample is 4, 8, or 16-fold less than in other samples, run that sample for an additional 2, 3, or 4 PCR cycles, respectively. For samples in the +/- 5-cycle range, don't run additional cycles.
    - If you run additional cycles on any samples, quantify the reaction products again by using one of the instruments mentioned above or gel electrophoresis to ensure that all samples have correctly amplified products.
    - Avoid overcycling! Try to use the minimum number of cycles which allows quantitation of the bands. Importantly, for dual-sgRNA libraries, overcycling induces a significant level of recombination between different dual-sgRNA molecules. Overcycling also often generates additional higher molecular weight bands. These bands do not normally interfere with NGS unless they are very strong. Also, overcycling has the potential to compress the representation of the targeted sequences if the reaction exceeds the exponential phase. Therefore, it is desirable to minimize the cycle number. If there are excessive higher molecular weight bands, you can rerun Second Round PCR for that sample using another 2 µl of DNA from the First Round PCR and a

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fewer number of cycles.

DNA samples can be stored for several days at +4°C at this stage or move directly onto NGS Sample Purification

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3.3. NGS Sample Purification

To maximize sequencing of the targeted sgRNA or barcodes and minimize background sequences, we recommend purifying the amplified PCR products by preparative gel electrophoresis.

1. Based on quantitative analysis of PCR product yield for each of the samples, combine the PCR products from each sample at the same amount. For example, for samples with yields of correct PCR product of 20 ng/µl and 60 ng/µl, combine 6 µl and 2 µl of PCR reaction, respectively.

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

2. After purifying and concentrating, run each purified combined 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 scalpel and a 365 nm (UV-A) lamp, excise the narrow band that corresponds to the correct target sgRNA or barcode amplicon size. DO NOT use a 302/312 nm (UV-B) lamp!

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 the combined 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 the combined sample by A260nm OD measurement using a NanoDrop spectrophotometer (or equivalent), and then adjust the concentrations in all samples to 10 nM. For example, if the amplicon size is 200bp, the 10 nM concentration corresponds to 1.32 ng/µl based on A260 OD measurement. For an amplicon size of 750bp (dual-sgRNA product), a 10 nM concentration corresponds to 4.95 ng/µl.

DNA samples can be stored at -20°C at this stage.

Last modified: 3 April 2025

3.4. Sequencing sgRNA Inserts or Barcodes

The Second Round nested PCR primers contain sequences (P5 and P7) complementary to the sequences of the immobilized primers necessary for generating amplification clusters on any of Illumina's NGS Instruments. In addition, if you are using a Cellecta NGS Prep Kit, the NRev Index Primers add sample-specific index sequences which allow you to combine and multiplex up to 24 samples on a single flow cell or lane. The primers were designed with the NextSeq instrument in mind, but they are compatible with other Illumina instruments as well.

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IMPORTANT: For certain libraries, such as sgRNA and dual-sgRNA, the Cellecta 6-nt Index should be read using Read 2, **not** in the Illumina Index 1. Please check the Product Certificate for the appropriate LNGS Kit for more information.

The number of sequencing cycles (read length) required depends on the sgRNA or barcode length. Please refer to your library information (NGS Cassette Diagram for the specific Cellecta library or the Product Certificate for the NGS Prep Kit).

Guidelines for preparing samples for sequencing:

- Combine together equal amounts (each adjusted to 10 nM) of experimental and positive control samples to be sequenced together. Importantly, each sample in the combined pool needs to have a unique index sequence.

We recommend that you DO NOT combine Cellecta samples with samples of different origin on a single flow cell or lane. It may cause sequencing errors.

> **Note:** The number of samples which can be sequenced in one lane or flow cell is based on the complexity of the pooled library and expected total number of reads generated with the Illumina instrument. You will need to determine how many target reads you want per sample based on your experiment.

- Prepare the samples for NGS by following the Illumina “Denature and Dilute Libraries Guide” specific for your system.
  - NextSeq 500: https://support.illumina.com/downloads/nextseq-500-denaturing-diluting-libraries-15048776.html
  - HiSeq: https://support.illumina.com/downloads/hiseq-denature-dilute-libraries-guide-15050107.html

- Spike in 5-15% of the PhiX Control to the pooled indexed sample to increase the nucleotide diversity.

- Mix Cellecta’s **Seq NGS Primer** with the Illumina primer mix (for the NextSeq, spike in the primer at the appropriate cartridge position—usually at Read 1). For indexing multiple samples in a flow cell or lane using a Cellecta NGS Prep Kit, also add the **Index NGS Primer** directly to the appropriate well (i.e., spike in either with the Illumina index primer or the reverse sequencing primer; see Note below). Each primer should have a final concentration of 300 nM for the NextSeq, or 500 nM for the HiSeq. We recommend measuring the final volume of the primer mix in each well to ensure accurate spiked-in primer concentrations. For primer spike-in information on other Illumina instruments, please refer to this Illumina guide: https://knowledge.illumina.com/library-preparation/general/library-preparation-general-reference_material-list/000001542

> **Note:** Depending on the way the amplification and sequencing primers were designed, the position of the Seq NGS and Index NGS primers may vary. Refer to the supplementary information (Product Certificate or NGS Cassette Diagram file) provided for the specifics on how to set up the NGS Primers for your specific library.

- Specify the appropriate number of cycles to sequence the complete sgRNA or barcode region (please refer to Product Certificate or NGS Cassette Diagram file), and specify the indicated number of cycles to read the 6-base Index sequence if using a Cellecta NGS Prep Kit.

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3.5. Demultiplexing and Aligning Sequencing Reads

Cellecta provides NGS Demultiplexing and Alignment Software for most of its libraries. The program demultiplexes, aligns, and scores Illumina platform sequence data generated from samples of Cellecta libraries prepared using the primers in Cellecta NGS Prep Kits (Cat.#s LNGS-100 through LNGS-999). Below are the instructions for use of this Software.

If you are not using the Cellecta software, please see the section **Other Options Instead of Cellecta's Alignment Software** at the bottom of this page.

Program Requirements and Installation

- The program runs on 64-bit Windows, such as Windows 7 or higher.
- If the option to generate FASTQ files is enabled, sufficient local disk space to store the resulting output is required; this will approximately equal the combined disk space of the input FASTQ files.
- For premade libraries, you may download the appropriate Library Configuration File(s) from the Cellecta website product pages.
- If you cannot find the Library Configuration File(s) for your library, please contact Cellecta Technical Support at tech@cellecta.com. Please note that Library Configuration Files or links to files are also provided in "Cellecta Order Information" emails sent to customers upon shipment of libraries. For premade libraries, please visit the appropriate product page to download the Library Configuration File(s) under Alignment Software Supporting Files in the Product Information section.
- To obtain the latest version of the software, click on the original link provided in the "Cellecta Order Information" email at the time of shipment. Please contact us at tech@cellecta.com if you do not have this link. Please note that you may need updated Library Configuration File(s) if you have older "Library Design Files" required with earlier versions of the software (shipped before 2022).

Using the Alignment Software

The program requires the following input files:

1. The Illumina **FASTQ file** (.fastq or .fastq.gz) with the raw NGS data.
2. A **Sample Description File** with data on which samples were sequenced and what indexes were used for each sample in tab-delimited TEXT (.txt or .tsv) or FASTA (.fa or .fna) format.
3. The appropriate **Library Configuration File(s)** in JSON format (.json), which contain(s) the list(s) of target sequences and deconvolution instructions for the Cellecta Library or other compatible library (e.g. Brunello, GeCKO).

The program outputs two files:

1. A table of aligned counts for each sample (in columns) and target (in rows).
2. A table of total aligned, unaligned, and ambiguous counts for each sample to assess the quality of the sequencing.

- "Unaligned" sequences are those for which no sample or target could be identified.
- "Ambiguous" sequences are those for which multiple samples or targets could be identified; they are not expected to occur with most libraries, as library sequences are chosen to be unambiguous within the Hamming distance used.

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In addition to the files above, there is an option to have the program output an individual FASTQ file for each sample when it demultiplexes the FASTQ file for the sequencing run.

Key Steps for Use of the Software:

1. Obtain the FASTQ format files from the Illumina instrument. The file names should follow Illumina's naming convention.
2. If the samples have not been demultiplexed already, fill out the "Sample Description Input Form" and save the Sample Description File as .txt.
3. Enter the locations of the FASTQ NGS Files, Sample Description File (if needed), and Library Configuration File(s) into the Program interface.

> ! *DO NOT PROVIDE a Sample Description File if the FASTQ files have been demultiplexed already.*

Setting up the Sample Description File

****Skip this section if your FASTQ files have been demultiplexed already.****

1. Open the template file **NGS-Sample-Description-Input-Form.xlsm**. You will enter your sample names into this template, and then save the file with a name descriptive of your experiment.

> **Note:** Please make sure to Enable Macros in Excel, if prompted.

2. The ***Sample Description Input Form*** has 3 columns:

- Column 1 lists the primer designation for each barcode.
- Column 2 is reserved for the user to enter the sample descriptions.
- Column 3 indicates which DNA index sequence will be used to demultiplex the samples.

3. Enter the sample descriptions in column 2. Fill in a name for each sample at the appropriate location based on the primer designation used to PCR amplify the specific sample, and the DNA index sequence associated with that sample.

> **Note:** Do not skip a description for any sample. No sequencing data will be extracted for samples lacking descriptions. Avoid duplicate descriptions—duplications will be highlighted in red.

4. Save the Sample table in tab-delimited text format by either using the "Click to save as Sample Description File" button or manually saving the table in tab delimited (.txt) text format.

Running the Cellecta Alignment Software

1. Double-click the "Cellecta Alignment Software" link to open the program.

2. Select the samples. Click the button to navigate the file system and select the text-format Sample Description File corresponding to the experiment that was prepared above.

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> ! **DO NOT SELECT** a Sample Description File if the FASTQ files have been demultiplexed already.

3. Select the library sequences for alignment with the Library Configuration File(s). Click the button to navigate the file system and select the appropriate Library Configuration File(s). Some program versions require more than one Library Configuration File (depending on the design of the library being sequenced).

4. Select the folder with the FASTQ NGS data files. Click the button to navigate the file system and select the folder containing the FASTQ files from the sequencer. The program will report how many distinct lanes it detected in that location. If the FASTQ files are compressed in GZIP format (have the ".gz" extension), it is not necessary to de-compress them.

> **Notes:**
>
> - The program identifies FASTQ files by looking for file names following the **Illumina Naming Convention**, for example, **Data\Intensities\BaseCalls\SampleName_S1_L001_R1_001.fastq.gz**. Do not alter the Illumina-assigned file names.

5. Click the checkbox to save the individual demultiplexed FASTQ files. The program will run more slowly if the individual FASTQ files option is selected. Also, ensure that there is adequate disk space (i.e., greater than the size of the input FASTQ files) to output these files.

6. Begin the deconvolution/alignment run. Click the "RUN" button at the bottom of the tab, and choose the name and location of your output file in the file navigator window. If you are saving the demultiplexed files, they will be written to the same location, so please ensure that sufficient disk space is available.

- After pressing *return*, a new window will pop up describing the run. When the run is completed the "DONE" button at the bottom will become active. This may take several hours.

> **Note:** Additional runs can be started while waiting for the software to finish. To start an additional run, navigate to the main window and click any of the three buttons to select FASTQ files, samples, or library. The "RUN" button will re-activate and a new process can be initiated.

> **Note:** For CRISPR sgRNA Library samples prepared with Cellecta's NGS Prep Kit for sgRNA Libraries (Cat.# LNGS-120), Cellecta's Alignment Software can also be used to demultiplex the Illumina data to make individual FASTQ files for each sample without additional analysis.

Other Options Instead of Cellecta's Alignment Software

If you are not using Cellecta's Alignment software, you will need to demultiplex the run to into separate FASTQ (*.bcl files) files. This can be done using the Illumina BaseSpace Hub or, alternatively, demultiplexing and fastq file generation may be done faster using a UNIX server and the Illumina bcl2fastq software. Using the bcl2fastq software also avoids the rather slow step of downloading FASTQ files from BaseSpace. You can obtain the bcl2fastq program at this link. Information on how to use this software is available at the following sites:

- The Bioinformatics I/O Blog on the University of Glasgow's Centre for Viral Research Site (for instance, see blog articles How to generate a Sample Sheet from sample/index data in BaseSpace and How to demultiplex Illumina data and generate fastq files using bcl2fastq).

- The BaseSpace Sequence Hub Help Center from Illumina. In particular, see How to download data from Run.

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4. Troubleshooting

No PCR Product

No Barcodes Present in NGS Results

NGS Data Includes Significant Percentage of Reads from a Single Sequence

Incorrect Band Pattern on Agilent Bioanalyzer

Last modified: 16 March 2017

4.1. No PCR Product

Possible reasons:

1. Incorrect primers or bad reagents used
2. Missing reagents
3. Low transduction of target cells
4. Poor gDNA purification prep contains PCR inhibitors
5. PCR conditions are not optimal

Solutions:

- Include 1 ng of plasmid library DNA as a positive control. If it produces the correct amplification product, the problem lies either with absent or low numbers of sgRNA (e.g., low MOI or problems with the transduction efficiency) or impurities in genomic DNA that block sgRNA amplification. Dilute the genomic DNA 2-5 fold and repeat the amplification step in several test tubes. If the positive control does not produce the correct product, confirm use of the correct primers and reagents.

- Verify that primer sequences are correct.

- If you don’t see a band for any of the samples, we suggest increasing the number of PCR cycles as follows:
    - Run the First Round PCR at 18, 20, and 22 cycles and retrieve 10 µl aliquots at each cycle number. For example, run the first PCR and retrieve 10 µl aliquots at 18 cycles, cycle 2 more times to 20 cycles and retrieve 10 µl, and cycle a couple more times to 22 cycles. Don’t run these on a gel because you are unlikely to see a band—and you don’t want to overcycle them until a band appears.
    - Use 5 µl of each cycle-number aliquot collected during the first PCR. Run the Second Round PCR on each of the first PCR reactions, and then check for a clean visible band. Start with 14 cycles for the second PCR; if you don’t see a band, cycle 3 more times to 18; if no band, 3 more to 21, and then 24.
    - If you see at least a faint band by 25 cycles second PCR, then cycle a couple more times to produce a clean band.
    - If you don’t see any band by 25 cycles of the second PCR, then you can go back to the first PCR and try adding a few more cycles to 26 and 28, and then run the second PCR again up to a max of 25 cycles. If you don’t see any band by then, there is a problem with the samples.
    - if no band is observed with ~40 combined cycles between the two PCR reactions, it is very likely there is likely a more significant problem with the DNA samples or PCR set up.

Last modified: 27 September 2024

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4.2. No sgRNA/Barcodes Present in NGS Results

Possible reason #1:

Incorrect First or Second Round PCR Primers or Sequencing Primer used.

Solution:

Ensure that you use the correct First and Second Round PCR Primers and Cellecta Sequencing Primer for your library. Check the Library Cassette Diagram provided with the kit.

Possible reason #2:

Incorrect NGS reagent kit used.

Solution:

Ensure that you or your NGS Core Facility uses the proper Illumina NGS reagent kit. For details, please check the kit descriptions in the Additional Materials Required section.

Last modified: 10 January 2019

4.3. NGS Data Includes Significant Percentage of Reads from a Single Sequence

Possible reason #1:

Cells used in screen may carry foreign DNA which contain NGS primer binding sites.

Solution:

Do not use cells for your screen which contain foreign DNA with NGS binding sites.

Last modified: 10 May 2018

4.4. Incorrect Band Pattern on Agilent Bioanalyzer

Possible reasons:

The Agilent Bioanalyzer is not a good tool to analyze these preps. The sizing is not as accurate as a 3% agarose gel for small fragments, and the preps tend to have a significant amount of genomic DNA which overloads the tiny Agilent capillary.

Solutions:

Use a 3% agarose gel to analyze your PCR products.

Last modified: 16 March 2017

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5. Technical Support

If the Troubleshooting section has not helped, please provide answers to the questions below (where applicable) before contacting us.

Library Used:

1. Which library did you use?
2. What is the lot number?

Packaging the Library:

1. What was the lentiviral titer, and what was the total number of TU packaged?
2. How was the virus concentrated? (if applicable)

Transducing Target Cells:

1. What MOI did you use to transduce your target cells?
2. What target cells did you use?
3. How many replicates did you use? (i.e., duplicate, triplicate, etc.)
4. Did you use puromycin after transduction, and at what concentration?
5. For how long did you use puromycin on the cells?

Library Screen:

1. Could you briefly explain your experiment?
2. How many infected cells were used?

Sample Preparation & NGS:

1. Describe the protocol you used to amplify the barcodes.
2. What NGS system and which Illumina Kit did you use?
3. How much PCR product was used for NGS?
4. How many sequences were read per sample?

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For the latest technical news and updates, visit Cellecta's blog at: https://cellecta.com/blogs/news

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Find the Genes that Matter

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