CELLECTA

USER MANUAL

DriverMap™ Targeted Expression Profiling Kits

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Cellecta, Inc.

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1. DriverMap™ Targeted Expression Profiling Assay

The DriverMap™ Targeted Gene Expression Profiling Assay combines highly multiplexed RT-PCR amplification with the depth and precision of Next-Generation Sequencing (NGS) to quantitatively measure gene expression of 1000+ target genes in a single assay. An easy-to-run, one-tube, single-day assay quantifies gene transcript levels directly from total RNA isolated from cells, tissue or blood, or directly from cellular extract (down to a single cell level) using the DirectCell™ Protocol. No mRNA enrichment, or rRNA, mitochondrial, beta-globin depletion, or other processing is required. The single-tube assay provides robust, quantitative, and reproducible measurements of each expressed gene over as much as 5-orders of magnitude differences in expression level.

The development of the DriverMap Assay involved extensive optimization and experimental validation of tens of thousands of primer sets to identify a pool of primers that could be combined in a single multiplex RT-PCR reaction to amplify representative transcript sequences (i.e., conserved transcript regions common to different mRNA isoforms from the same gene) from each of ~19,000 protein-coding genes in the human genome. The amplified sequences are then quantified using NGS on the Illumina platform.

DriverMap Kits provide optimal quantitative expression data within 10^4 – 10^5-fold dynamic range from 10-50 ng of total RNA and can be used effectively with as little as 10 pg of RNA. When using less than 1 ng of total RNA, however, measurements for low abundant transcripts may be less reproducible.

For information on the services we can provide, please visit the DriverMap Expression Profiling Service web page.

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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2. Materials Required

The following sections detail the materials provided with the DriverMap Assay Kits, and additional materials needed to carry out the Assay. This manual is designed to be used with the following DriverMap Assay Kits:

Product	Catalog #	Amount
DriverMap™ Human Genome-Wide Expression Profiling Kit, Version 2, 24 reactions	DM2-HGW	1 kit (24 reactions)
DriverMap™ Human Genome-Wide Expression Profiling Kit, Version 2, 96 reactions	DM2-HGW-96	1 kit (96 reactions)
DriverMap™ Human Cell Marker Panel	DMF-HCM-96	1 kit (96 reactions)
DriverMap™ Human Hallmark Signatures Panel	DMF-HHM-96	1 kit (96 reactions)
DriverMap™ Human LINCSx Panel	DMF-HLX-96	1 kit (96 reactions)
DriverMap™ Human Pan-Cancer Panel	DMF-HPC-96	1 kit (96 reactions)
DriverMap™ Human TransFactor Signatures Panel	DMF-HTF-96	1 kit (96 reactions)
DriverMap™ Mouse Targeted Panel	DMF-MPLH-96	1 kit (96 reactions)

The DriverMap Assay Kits should be stored at -20°C until ready to use.

Please note, there are some variations with the assay, depending on the type of samples and amount of material. Depending on the specifics protocol variation you are running, some of the additional materials may not be required.

Last modified: 7 December 2018

2.1. Kit Components

DriverMap Targeted Expression Profiling Kits contain enough reagents for the preparation of NGS samples starting from total RNA from blood, tissue, cells, or other biological samples, including Positive Control RNA.

Box 1: Kit Components

Component	Description	Cap Color	Conc.	Volume (24 rxn)*	Volume (96 rxn)
Positive Control RNA	Total RNA from multiple cell lines, 50 ng/µl	white	10X	5 µl	5 µl
Random Primers	Random primer mix for cDNA synthesis, 50 µM	yellow	10X	30 µl	120 µl
PCR-Grade Water	RNase/DNase-free water for reaction mixes	white	NA	3 ml	12 ml
dNTP Mix	Mix of dATP, dGTP, dCTP and dTTP, 10 mM	white	20X –	95 µl	375 µl

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	each		50X		
RT-EXT Buffer	cDNA synthesis and Gene-Specific primer extension reaction buffer	green	5X	200 µl	780 µl
Reverse Transcriptase	Multiplex-optimized M-MLV Reverse Transcriptase, 200 U/µl	green	20X	15 µl	55 µl
PCR Buffer	Reaction Buffer for 1st and 2nd PCR steps (*note: tube cap says "DNA"*)	red	5X	650 µl	2,500 µl
DNA Polymerase	Multiplex-optimized thermostable DNA polymerase, 2 U/µl	red	100X	45 µl	165 µl
Forward GS Primer Pool**	Mix of Forward Gene-Specific Primers	highlight yellow	10X	60 µl	225 µl
Reverse GS Primer Pool**	Mix of Reverse Gene-Specific Primers	highlight yellow	10X	85 µl	330 µl
Primer Removal Enzyme	Enzyme Mix for primer removal	orange	150X	15 µl	55 µl
Anchor Primer Mix	Forward/Reverse Primers for 1st PCR, 2.5 mM each	yellow	10X	280 µl	1,050 µl
Forward SeqDNA NGS Primer	Forward NGS Primer for cDNA sequencing	blue	100 µM	30 µl	90 µl
Reverse SeqDNA NGS Primer	Reverse NGS Primer for cDNA sequencing	blue	100 µM	30 µl	90 µl
Forward SeqIND NGS Primer	Forward NGS Primer for Index sequencing	blue	100 µM	30 µl	90 µl
Reverse SeqIND NGS Primer	Reverse NGS Primer for Index sequencing	blue	100 µM	30 µl	90 µl

**The 24 reaction Assay Kit is only available for the DriverMap Human Genome-Wide Assay.*

***The Forward and Reverse GS Primer Pools are specific for each kit. Please see the Product Insert for the Kit to identify which components correspond to the Primer Pools.*

Box 2: Kit Components

Component	Description	Conc.	Volume (24 reactions)*	Volume (96 reactions)
Forward & Reverse Index Primer Set	Forward/Reverse Primer Pairs for **PCR with Indexed Primers**, pre-aliquoted in a 96-well plate	10X	5 µl each (24 wells)*	5 µl each (96 wells)

**The 24 reaction Assay Kit is only available for the DriverMap Human Genome-Wide Assay.*

Kit contents should be stored at -20°C. Shelf life is 6 months.

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2.2. Additional Required Materials

Additional Reagents and Apparatus for Sample Preparation:

- For General RNA Preparation *

Name	Recommended Manufacturer	Catalog #	Purpose
AllPrep RNA/DNA Micro Kit	QIAGEN	80284	RNA isolation from cells or tissues
RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE	Thermo Fisher	AM1995	RNA isolation from FFPE tissues
RNA 6000 Pico Kit	Agilent	5067-1511	RNA quality control calculation
High Sensitivity RNA Analysis Kit	Agilent	DNF-472-1000	RNA quality control calculation

- For DirectCell DriverMap Analysis *

Name	Recommended Manufacturer	Catalog #	Purpose
TurboCapture 96 or 384 mRNA Kit	QIAGEN	72251/ 72271	Direct use with cell lysate
Buffer TCL, 2× (8.5 ml)	QIAGEN	1031586	Cell lysis

Additional Reagents and Apparatus for cDNA Synthesis and Amplification:

Name	Recommended Manufacturer	Catalog #	Purpose
Agencourt® AMPure® XP (Magnetic Beads)	Beckman-Coulter	A63881	PCR product purification
Dynabeads® MPC®-S (Magnetic Particle Concentrator) for 1.5 ml Test Tubes	Thermo Fisher	A13346	PCR product purification
Semi-skirted 96-well PCR plates	Eppendorf	951020303	RT-PCR amplification
Clear Adhesive Film for 96-well plates	Bio-Rad	MSB1001	PCR plate seal
High Sensitivity DNA Kit	Agilent	5067-4626	PCR product QC/ quantification
High Sensitivity NGS Fragment Analysis Kit (1 bp – 6000 bp)	Agilent	DNF-474-1000	PCR product QC/ quantification
Qubit® dsDNA HS (High Sensitivity) Assay Kit	Thermo Fisher	Q32851	Quantify Indexed Libraries for NGS
NextSeq® 500/550 High Output Kit v2 (75 cycles) or HiSeq®/MiSeq® equivalent	Illumina	FC-404-2005 (NextSeq)	Next-Gen Sequencing

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Required Instrumentation:

The protocols were optimized using the specific instruments specified below. Some modifications and optimization to the protocol may be necessary if using different models or instruments from other manufacturers.

Description	Model	Manufacturer
Spectrophotometer (NanoDrop or equivalent)	NanoDrop® ND-2000	Thermo Fisher
Thermal Cycler (Veriti 96-Well or equivalent)	Veriti® 96-Well	Applied Biosystems
Instrument or Gel System to Analyze DNA Fragmentation	2100 Bioanalyzer®	Agilent
	Fragment Analyzer™	Agilent
	Standard gel imaging system	many suppliers
DNA Quantification	Qubit 3.0 Fluorometer	Thermo Fisher
Next-Generation Sequencer	NextSeq, HiSeq, or MiSeq	Illumina

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. Sample Preparation

Use the protocols in this section to prepare RNA from cells, tissue, or blood.

You can use the standard RNA Preparation approach to purify RNA from a range of biological samples. Alternatively, for cells grown or sorted in 96/384-well plates, you can use the DirectCell Protocol to capture mRNA from cells lysed directly in oligo-dT microtiter plates. The DriverMap protocol can then be run directly in these same plates.

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3.1. RNA Preparation

Isolate, Quantify and Perform QC of Total RNA

We recommend using the QIAGEN AllPrep DNA/RNA Micro Kit (Cat.# 80284) for total RNA isolation from cells or tissues. For FFPE tissue, we recommend Thermo Fisher's RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Cat.# AM1975).

Extracted RNA should be DNA-free. With the QIAGEN AllPrep DNA/RNA Micro Kit, a separate DNase treatment is not necessary since the extract is passed through a genomic DNA eliminator column. If using the RecoverAll Isolation Kit for FFPE tissue samples or another Total RNA isolation method, a separate DNase treatment of RNA samples is strongly recommended before starting the procedure. Follow the DNase treatment instructions in the manufacturer's RNA isolation kit manual.

> **NOTE: For small amounts of total RNA** where a standard DNase treatment and purification may result in significant sample loss, we recommend using double-stranded DNAse (**ArcticZymes, PCR Decontamination Kit, Cat.# 80400-100**) following the manufacturer's protocol.

Quantify total RNA with the Thermo Fisher NanoDrop (or equivalent), and confirm the integrity of RNA in each sample prior to starting the assay by one of the following methods:

- Determine the RIN number using the Agilent Bioanalyzer and Agilent RNA 6000 Pico Kit (Cat.# 5067-1511)
- Determine the RIN number using the Agilent Fragment Analyzer and High Sensitivity RNA Analysis Kit (Cat.# DNF-472-1000)
- Using a gel imager, calculate the 28S:18S rRNA ratio after running the RNA samples on an agarose gel.

The RIN number for your RNA samples should be no less than 5. If you are using FFPE RNA samples, you should additionally check the samples to ensure that a significant level (at least 50%) of RNA fragments are larger than 300 nt.

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3.2. DirectCell™ Oligo-dT Capture Protocol

DirectCell™ Oligo-dT Capture Protocol

This modification to conventional DriverMap protocol is designed to perform high-throughput expression profiling directly from cells grown in or sorted into 96- or 384-well plates. The DriverMap protocol can then be run directly in these same plates without transfer. The protocol allows you to streamline the mRNA purification, cDNA synthesis, and multiplex PCR steps in a single multiwell plate without any intermediate purification steps. In the first steps, the cells cultivated in a multiwell plate are

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lysed and transferred to an oligo-dT plate (TurboCapture 96 or 384 multi-well plates from Qiagen). The mRNA is then allowed to hybridize to the immobilized oligo dT, the cell lysate with DNA, rRNA, and other impurities are washed away, and the isolated mRNA is used directly in the cDNA synthesis step.

The procedure below has been run with a few common cell lines with 1 to several thousand cells per well. Some steps, such as lysis time, may need to be optimized for particular cell types.

Step 1: Cell Lysis

> Note: For suspension cells, spin down the cells at 1,000g for 5 min before starting the protocol.

1. Remove media from attached cells grown in multiwell plate (e.g. 5-10 × 10^3 cells/96 plate well or 1-2 × 10^3 cells/384 plate well).

2. Add 25-ul per well (96-well plate) or 10-ul per well (384-well plate) of TCL lysis buffer (with 1% (v/v) beta-mercaptoethanol) to lyse the cells. Incubate the plate for 5 min at room temperature.

Seal the plate and store at -20°C. A freeze/thaw cycle can help optimize cell lysis and RNA yield. Keep frozen until ready to proceed with the binding protocol.

Step 2. Binding mRNA to oligo-dT plates (TurboCapture plates)

1. Transfer 10-ul of cell lysate from each well of lysed cells to a 96- or 384-well TurboCapture plate.

2. Incubate the TurboCapture plate at room temperature for 60 min in orbital shaker (100-300 rpm).

3. Wash the TurboCapture plate three times using 100 ul (96-well plate) or 30 ul (384-well plate) TCW buffer per well. After the third wash briefly centrifuge the plate and remove all residual TCW buffer.

4. Add 6 ul of PCR-grade water and proceed with the standard DriverMap protocol for cDNA Synthesis and Amplification directly in TurboCapture plate. Synthesis of cDNA, second strand primer extension, and PCR with the DriverMap primer pools can all be run directly in the TurboCapture plate.

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4. cDNA Synthesis and Amplification

Cellecta's DriverMap™ Targeted Expression Profiling Kits provide reagents for targeted RNA expression profiling, directly from total RNA samples, or mRNA captured in oligo-dT TurboCapture microtiter plates using the DirectCell Protocol. No preliminary poly(A) selection or ribosomal/mitochondrial/globin RNA depletion is required.

The protocol utilizes a mix of empirically validated RT-PCR primers optimized for a single-tube assay protocol that prepares NGS libraries compatible with Illumina Next-Gen Sequencing (NGS) systems (NextSeq, HiSeq, or MiSeq). With the DriverMap Assay, RNA samples can be processed for sequencing in approximately 8 hours time with just around 2 hours hands-on time.

Please follow these general guidelines when running the DriverMap Assay:

- Keep enzyme components on ice during use. All other components, including primer pools, may be placed at room temperature. Thaw components, gently vortex, and spin down before use. Please be sure to dissolve any precipitate if visible.

- Use Good Laboratory Practices to minimize cross-contamination of products. If possible, perform the first part of the procedure (from cDNA synthesis through First PCR setup) in a location set aside for RNA work, and use a set of equipment, pipettes, test tubes, and other consumables dedicated for work involving RNA. Then, run the PCR amplification reactions in a separate lab or area designated for PCR. Always change pipette tips for adding components to new samples.

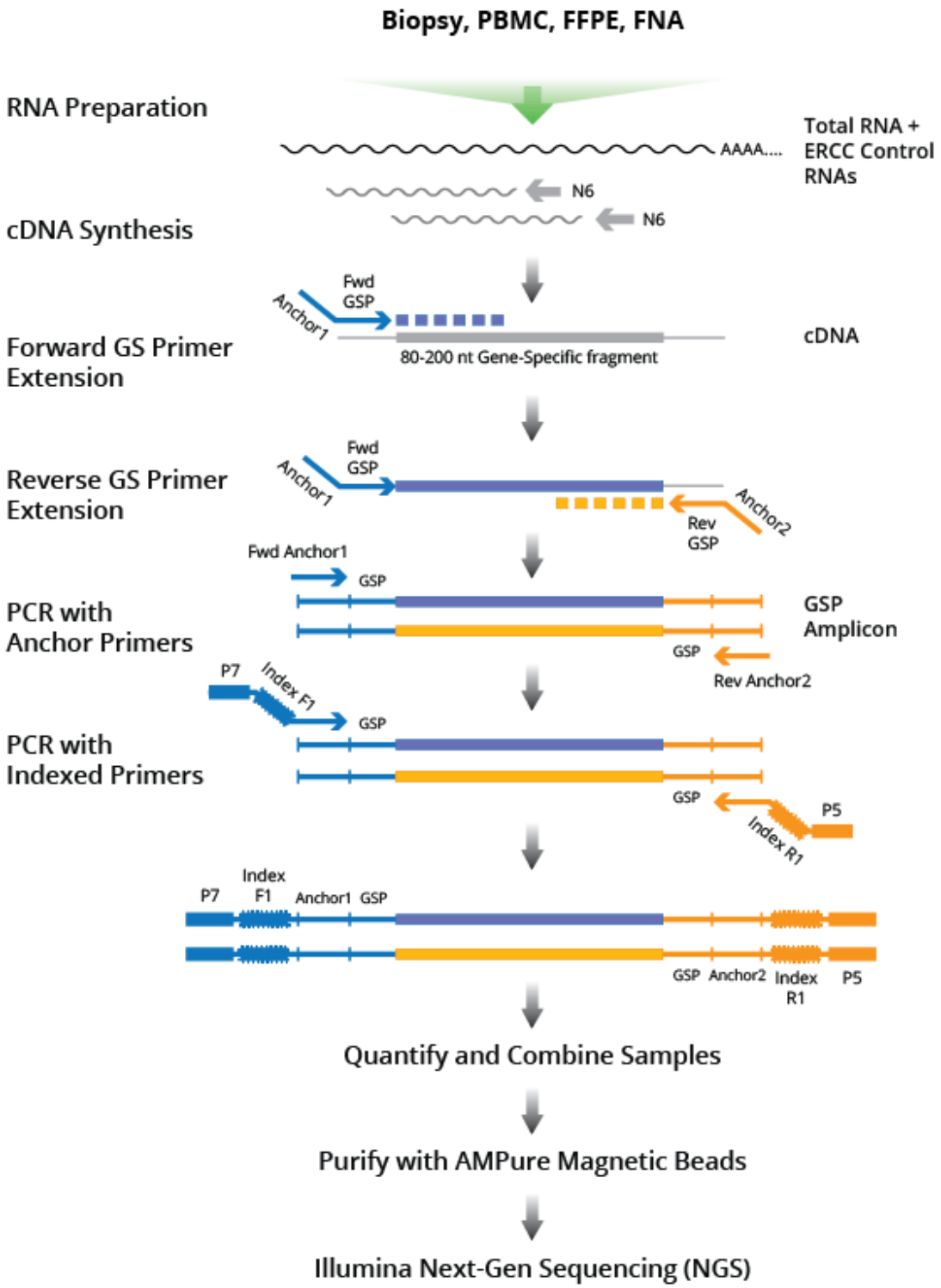
- To minimize the hands-on time and mistakes in liquid deposition, we recommend to run the assay in a 96-well plate using and 8-channel (or 12-channel) pipet. After adding all the necessary reagents, seal the plate well with Clear Adhesive Film and use a Compression Pad to minimize evaporation from experimental samples. Only use each Clear Adhesive Film once. Do not reuse them.

- Do not reuse clear adhesive films to seal 96-well plates.

- Pipet viscous enzyme solutions slowly. After adding them to the reaction mix, ensure complete mixing by vortexing or pipetting up and down several times.

- For several steps in the procedure, we recommend making a Master Mix by combining adequate amounts of the key reagents into a single tube, and then aliquoting the appropriate portion from this combined mix into each sample. This approach helps ensure a consistent amount of each reagent is added to all samples. For these Master Mix reactions, first calculate the volume of each component you need for all the reactions you are running. Then, add 5% to each volume to cover pipetting variance, and pool the appropriate volumes of reagents together into a single tube or well. Mix the pooled reagents, then pipette the required portion into each sample. If you are using a multichannel pipette, aliquot the Master Mix in a tube strip first, then pipette from this strip into each sample.

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Outline of DriverMap Multiplex RT-PCR-NGS assay for expression profiling of up to 20,000 genes in a single test-tube without intermediate purification steps.

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4.1. cDNA Synthesis

In this step, complementary DNA (cDNA) is generated from total RNA. All reactions must be set up in 96-well plate format

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(standard, or separate tubes/strips) using approximately the same adjusted starting amount of RNA across all reactions.

For best results, we suggest starting with 10-50 ng of high quality total RNA (or 50-500 ng of FFPE RNA). Reliable results with as little as 10 pg of high quality RNA are possible for most genes, but some transcripts expressed at low levels may not be accurately measurable. Starting RNA sample volumes must be equal or less than 6 µl.

If you used the **DirectCell™ Oligo-dT Capture Protocol**, the mRNA captured from cell lysates in oligo-dT plates and resuspended in 6 µl can be used directly for cDNA Synthesis. Go directly to Step 2 in the procedure below, and then add the RT Master Mix to the TurboCapture oligo-dT plate (QIAGEN).

For high quality RNA, do not use more than 100 ng in the Reverse Transcription (RT) reaction. Too much RNA can cause non-linear target amplification.

We highly recommend reserving at least one well for the Positive Control RNA at a concentration as close as possible to that of your experimental samples. If running several experiments, it can be useful to include the same amount of Positive Control RNA with each sample run. This will help with troubleshooting, data analysis, and normalization across different sample runs.

If processing fewer than 96 reactions, fill empty wells with water to minimize evaporation. The figures below show example reaction arrangements for 22 or 94 experimental samples plus the two controls for the 24-reaction and 96-reaction kits, respectively.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C				1	2	3	4	5	6			
D				7	8	9	10	11	12			
E				13	14	15	16	17	18			
F				19	20	21	22	C+	C-			
G												
H												

Example arrangement of the reactions for 22 experimental samples plus the two controls, using the Human Genome-Wide 24-sample kit

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	1	2	3	4	5	6	7	8	9	10	11	12
A	1	2	3	4	5	6	7	8	9	10	11	12
B	13	14	15	16	17	18	19	20	21	22	23	24
C	25	26	27	28	29	30	31	32	33	34	35	36
D	37	38	39	40	41	42	43	44	45	46	47	48
E	49	50	51	52	53	54	55	56	57	58	59	60
F	61	62	63	64	65	66	67	68	69	70	71	72
G	73	74	75	76	77	78	79	80	81	82	83	84
H	85	86	87	88	89	90	91	92	93	94	95	96

Example arrangement of the reactions for 94 experimental samples plus the two controls, using a 96-reaction kit

1. Aliquot an equal amount of each starting total RNA in the wells of a 96-well plate and adjust the volume of each sample to 6 µl with PCR-Grade Water as shown in the table.

Component	Volume per sample, µl
Total RNA (0.01 – 50 ng)	1 – 6*
PCR-Grade Water, to 6 µl final volume	0 – 5
Total	**6**

2. Prepare an RT Master Mix as described below for all samples and controls plus 5% extra volume of all components as a safeguard against pipetting error:

RT Master Mix Component	**Volume per sample, µl**
RT-EXT Buffer	2
dNTP Mix	0.5
Random Primers	1
PCR-Grade Water	1
Reverse Transcriptase	0.5
Total	**5**

3. Add 5 µl of RT Master Mix to each well, and mix contents by pipetting 3 times. Seal the plate with adhesive film, and spin down to collect droplets. Load the plate in the thermal cycler, and run the following program to synthesize cDNA:

Temperature	Time
50°C	30 min
95°C	5 min
4°C	∞

Stopping Point in procedure: cDNA samples may be stored at 4°C overnight or at –20°C for longer periods.

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4.2. Forward Gene-Specific Primer Extension

In this step, the pool of Forward Gene-Specific Primers with adjoining Anchor 1 sequences (see **Procedure Overview** generates sense strands of the target amplicons using the cDNA generated in the previous step as a template.

1. Prepare the Forward Gene-Specific Primer Extension Master Mix as follows for all samples plus 5% extra volume of all components as a safeguard against pipetting error:

Forward GS Primer Extension Master Mix Component	**Volume per sample, µl**
RT-EXT Buffer	2
PCR-Grade Water	5.8
Forward GS Primer Pool*	2
DNA Polymerase	0.2
Total	**10**

> **Note:** There are different Forward GS Primer and Reverse GS Primer pools for each DriverMap Assay. Please refer to the Product Insert for your specific kit to identify the appropriate component in the Kit.

2. Gently vortex the Master Mix, and spin down briefly to collect droplets. Spin down the cDNA plate, remove the seal, then add 10 µl of the Forward GS Primer Extension Master Mix to each reaction well of the plate:

Component	**Volume, µl**
cDNA	10
Forward GS Primer Extension Master Mix	10
Total	**20**

3. Mix contents by pipetting 3 times, seal the plate with a new adhesive film, and spin down to collect droplets.

> **Note:** A new adhesive film is required in order to avoid cross-contamination.

4. Load the plate in the thermal cycler, and run the following program:

Temperature	**Time**
98°C	1 min
64°C	30 min
4°C	∞

5. Prepare Primer Removal Master Mix as follows for all samples plus 5% extra volume of all components as a safeguard against pipetting error:

Primer Removal Master Mix Component	**Volume per sample, µl**
RT-EXT Buffer	2.5
PCR-Grade Water	9.5
Primer Removal Enzyme	0.5
Total	**12.5**

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> **Note:** Sufficient Primer Removal Master Mix is prepared above to use at this point in the procedure (5 µl), plus after two other later steps: the Reverse Gene-Specific Primer Extension (5 µl) and when you Quantify and Combine Samples for NGS (2 µl).

6. Spin down, remove the seal from the plate, then add 5 µl of the Primer Removal Master Mix to each reaction well of the plate (keeping the remainder for follow-up steps listed above):

Component	Volume, µl
Forward GSP Extension products (above)	20
Primer Removal Master Mix (prepared above)	5
Total	**25**

7. Mix contents by pipetting 3 times, seal the plate, and spin down to collect droplets.

8. Load the plate in the thermal cycler, and run the following program:

Temperature	Time
37°C	30 min
95°C	5 min
4°C	∞

Immediately proceed to the next step **(Reverse Gene-Specific Primer Extension)**

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4.3. Reverse Gene-Specific Primer Extension

In this step, the pool of Reverse Gene-Specific Primers uses the template generated in the **Forward Gene-Specific Primer Extension** step to generate antisense target amplicon strands flanked by both Anchor 1 and Anchor 2 sequences.

1. Prepare the Reverse Gene-Specific Primer Extension Master Mix for all samples plus 5% extra volume of all components as a safeguard against pipetting error:

Reverse GS Primer Extension Master Mix Component	**Volume, µl**
RT-EXT Buffer	1
dNTP Mix	0.25
PCR-Grade Water	0.65
Reverse GS Primer Pool*	3
DNA Polymerase	0.1
Total	**5**

> **Note:** There are different Forward GS Primer and Reverse GS Primer pools for each DriverMap Assay. Please refer to the Product Insert for your specific kit to identify the appropriate component in the Kit.

2. Gently vortex the Master Mix, and spin down briefly to collect droplets. Spin down the Primer Extension plate, remove the seal, then add 5 µl of the Reverse GS Primer Extension Master Mix to each reaction well of the plate:

Component	Volume, µl
Forward GS Extension product (from previous step)	25

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Reverse GS Extension Master Mix (prepared above)	5
Total	**30**

3. Mix contents by pipetting 3 times, seal the plate with a new adhesive film, and spin down to collect droplets.

> **Note:** A new adhesive film is required in order to avoid cross-contamination.

4. Load the plate in the thermal cycler, and run the following program:

Temperature	Time
98°C	1 min
64°C	30 min
4°C	∞

5. Spin down, remove the seal from the plate, and add 5 µl of the Primer Removal Master Mix (prepared in **Forward Gene-Specific Primer Extension** step) to each reaction well of the plate (keeping the remainder for the **Quantify and Combine Samples for NGS** step):

Component	Volume, µl
Reverse GSP Extension products (above)	30
Primer Removal Master Mix (prepared in **Forward Gene-Specific Primer Extension**	5
Total	**35**

6. Mix contents by pipetting 3 times, seal the plate, and spin down to collect droplets.

7. Load the plate in the thermal cycler, and run the following program:

Temperature	Time
37°C	30 min
95°C	5 min
4°C	∞

Immediately proceed to the next step **(PCR with Anchor Primers)**

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4.4. PCR with Anchor Primers

This step utilizes Anchor PCR primers to amplify the target cDNA fragments flanked with the Anchor 1 and Anchor 2 sequences generated during the previous **Reverse Gene-Specific Primer Extension** step.

1. Prepare the Anchor PCR Master Mix as shown below for all samples and controls plus 5% extra volume of all components as a safeguard against pipetting error:

Anchor PCR Master Mix Component	Volume per sample, µl
Anchor Primer Mix	10
PCR Buffer	13
dNTP Mix	1.3

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PCR-Grade Water	40
DNA Polymerase	0.7
Total	**65**

2. Gently vortex Master Mix, and spin down briefly to collect droplets. Spin down the Reverse GS Primer Extension plate, remove the seal, then add 65 µl of Anchor PCR Master Mix to each reaction well:

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Component	Volume per sample, µl
Reverse Gene-Specific Primer Extension DNA (previous step)	35
Anchor PCR Master Mix (prepared above)	65
Total	**100**

3. Seal the plate with new adhesive film and spin down to collect droplets.

4. Load the plate in the thermal cycler. Run the following program, using the recommended number* of PCR cycles:

Temperature	Time	Cycles
98°C	30 sec	1
98°C	10 sec	14-26 * see table below
72°C	20 sec	
72°C	30 sec	1
4°C	∞	1

- To avoid bias in gene expression levels due to overcycling, ***refer to the table below*** which provides the recommended number of PCR cycles based on the starting amount of RNA used for cDNA Synthesis. The recommended number of cycles may vary depending on the specific DriverMap panel used.

Starting RNA	Amount (ng)	(1) hGW V2 (2) hHallmark (3) Mouse Targeted	(1) hPan-Cancer (2) hTransFactor (3) hLINCSx	(1) hCell Marker
Total RNA RIN > 5 (from cells or fresh tissue)	0.01	26	27	28
	0.05	24	25	26
	0.2	22	23	24
	0.8	20	21	22
	3	18	19	20
	12	16	17	18
	50	14	15	16
FFPE RNA	50	20	21	22
	300	16	17	18

> **NOTE:** For mRNA samples prepared using the **DirectCell™ Oligo-dT Capture Protocol**, we recommend running at least 16 cycles when starting with thousands of cells per well, up to ~23 cycles for single cells.

Stopping Point in procedure: PCR products may be stored at 4°C overnight.

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4.5. PCR with Indexed Primers

This step adds a unique index combination to each DNA template sample amplified in the previous **PCR with Anchor Primers** step as well as universal flanking P5 and P7 sequences needed for cluster formation on the Illumina NGS flow cell.

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The Index PCR Plate in the kit contains a unique combination of Forward and Reverse index primers in each well. The primers have been dried onto the bottom of each well and will resuspend when the PCR reaction mix with the sample is added. One well should be used for each sample being sequenced. See **Appendix C. Forward and Reverse Index Combinations** for the sequences of each Forward and Reverse Index combination.

For the 24 sample kit, just use the first 3 columns of well for the samples. If you are processing less than 96 samples, you can use scissors to cut the desired number of wells from the index plate (e.g., cut 6 colums of wells for 48 samples, or 1 row for 12 samples), then store the rest of the plate for later use. Alternatively, you can set up duplicate or triplicate NGS runs for each samples by setting up each of the Index PCR reactions in 2 or 3 wells.

> **NOTE:** If you just run samples on a cut-out portion of the plate, be sure to reference the well location relative to the whole plate when entering the sample names at each plate index into the **DriverMap Data Alignment Software** (see the **Extract Sequencing Read Data** section),

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	2	3	4	5	6	7	8	9	10	11	12
B	13	14	15	16	17	18	19	20	21	22	23	24
C	25	26	27	28	29	30	31	32	33	34	35	36
D	37	38	39	40	41	42	43	44	45	46	47	48
E	49	50	51	52	53	54	55	56	57	58	59	60
F	61	62	63	64	65	66	67	68	69	70	71	72
G	73	74	75	76	77	78	79	80	81	82	83	84
H	85	86	87	88	89	90	91	92	93	94	95	96

Arrangement of the index pairs for 96 indexed samples.

1. Prepare enough of the Index PCR Master Mix, following the formulation below for a single sample, for all samples and controls plus 5% extra volume of all components as a safeguard against pipetting error:

Index PCR Master Mix Component	**Volume per sample, µl**
PCR Buffer	10
dNTP Mix	1
PCR-Grade Water	38.5
DNA Polymerase	0.5
Total	**50**

> **Note:** If you are using a multichannel pipette, aliquot the master mix in a tube strip to minimize pipetting steps.

2. Gently vortex the Index PCR Master Mix, and spin down briefly to collect droplets. Spin the Index PCR Plate and remove the plate seal from the plate. Set up the Index Primer PCR Reactions as follows:

3. Aliquot 50 µl of the Index PCR Master Mix into appropriate wells of the 24 or 96-well Index PCR Plate. To avoid index-to-index contamination, add Index PCR Master Mix using a new tip for each well.

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4. Spin down, then remove the seal from the Anchor Primer PCR plate (plate from **PCR with Anchor Primers** step). Add 2 µl of Anchor Primer PCR product to each of the Index Primer PCR reactions on the Index PCR Plate. To avoid mistakes, ensure that samples in the Anchor Primer PCR plate are arranged in the same format as the Index PCR Primer pair mixes in the Index PCR Plate (e.g. Sample 1A is aliquoted to well 1A, etc). We recommend that you record the Sample name and well number (e.g., Sample 1 in well 1A) for all the samples, including the positive control. This will help minimize mistakes in the NGS deconvolution step.

Component	Volume per sample, µl
Anchor PCR Product (from previous step)	2
Index PCR Master Mix (above)	50
Total	**52**

5. Seal the plate with new adhesive film and spin down to collect droplets.

6. Load the plate in the thermal cycler, and run the following program:

Temperature	Time	Cycles
98°C	30 sec	1
98°C	10 sec	7
72°C	20 sec	
72°C	30 sec	1
4°C	∞	1

Stopping Point in procedure: PCR products may be stored at 4°C overnight.

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5. NGS Prep and Sequencing

After amplification, the samples may be stored at 4°C until ready to sequence. The product from the **PCR with Indexed Primers** step contains the P5 and P7 sequences for Next-Gen Sequencing (NGS) on Illumina instruments. The protocols in this section provide instructions to normalize the amount of each PCR product to obtain similar reads for each sample, to clean up samples before loading onto the instrument, and guidelines for sequencing and data analysis.

Last modified: 14 November 2018

5.1. Quantify and Combine Samples for NGS

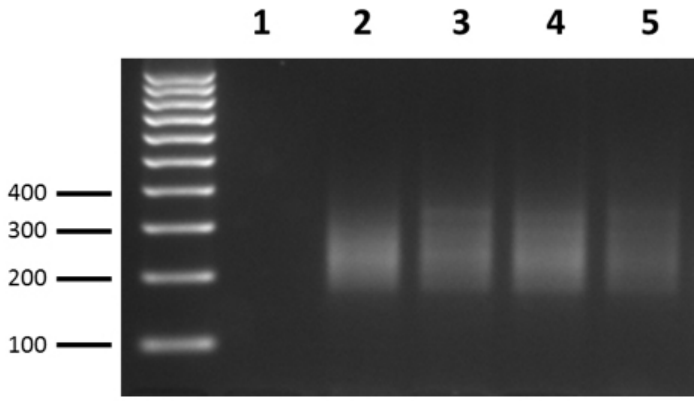
In this step, the yield of products from the Index Primer PCR Reaction—the Amplified NGS Indexed Libraries of transcripts from each sample—are measured and then pooled in equimolar amounts for sequencing.

Quantify the Amplified Indexed Libraries

1. Analyze the Amplified NGS Indexed Libraries using one of the following methods:

    - Analyze 1 µl of each of the Amplified Indexed Libraries on either an Agilent Bioanalyzer with the Agilent High Sensitivity DNA Kit (Cat.# 5067-4626) or Agilent Fragment Analyzer using the Agilent High Sensitivity NGS Analysis Kit (Cat.# DNF-473-1000) using the manufacturer's protocol.

    - Separate 5 µl of Amplified Indexed Libraries on a 3% agarose-TAE gel and analyze size distribution of NGS probes by UV transilluminator. The smear should be in the 180-450 bp range. See below for expected results of amplified libraries generated from good quality RNA samples.



Gel Electrophoresis of Amplified Indexed Libraries. Gel image of amplicons after Index Primer PCR Reaction. Lane 1: Negative control without RNA; Lane 2: Positive Control RNA; Lanes 3-5: Libraries prepared from total RNA of different sources.

2. Quantify the PCR products present in the size range of 180-450 bp by using image analysis software (available with most imaging systems) to subtract the background density of the negative control sample from the density of the PCR smears on the gel, or by using the software on the Agilent Bioanalyzer or Agilent Fragment Analyzer.

3. The yields of the Amplified Indexed Libraries from the experimental RNA samples should all be roughly the same within 2-3 fold levels, and should be similar to the Positive Control RNA sample. If a majority or all of the experimental RNA samples have significantly less PCR product than the Positive Control RNA sample (i.e., >5-10-fold difference),

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you can return all of the experimental RNA samples and the Negative Control sample to the thermal cycler (see Note below) and run them for 2-3 additional cycles. After cycling, quantify the products again relative to the Positive Control RNA. This should not need to be done more than once, and more than 3 additional cycles is not recommended.

> **Note:** Cycle all RNA samples together. Adjustments for small differences in the yield between samples will be made when the samples are combined for the sequencing step following this quantification procedure. Do not include the Positive Control RNA sample in additional cycles. Remove the positive control sample from the plate and keep it as a reference to assess the quantity of your PCR samples.

Remove Excess PCR Primers and Combine Samples

4. Remove excess primers from the completed PCR reactions by adding 2 µl of Primer Removal Master Mix (prepared in the **Forward Gene-Specific Primer Extension** step) to each of the Amplified Indexed Libraries and the Negative Control sample, then incubate at 37°C for 30 minutes.

5. To ensure accurate quantification for sequencing, you should repeat the quantification procedure of the Amplified Indexed Libraries and the Positive Control RNA again. Quantifying PCR products after removal of PCR primers is more accurate than analysis before clean up.

6. After primer removal and re-quantification, use the yield assessment of the Amplified Libraries in the **PCR with Indexed Primers** step as a basis to combine equimolar amounts of each of the Amplified Index Libraries, including the positive control RNA, into a single pool for NGS. For example, if the 180-400 bp amount of Library 1 is twice that of Library 2, then mix 5 µl of Library 1 with 10 µl of Library 2 for a 1:2 ratio. The number of pools and number of samples combined into each pool should be determined based on which samples will be run together in the same lane or flow cell for sequencing. To minimize sample-to-sample sequencing variations, combine and load similar numbers of samples onto each flow cell.

For the Genome-Wide DriverMap Assay (which targets 19,000 genes), refer to the table below for guidelines on how many samples may be combined together for different instruments and read depths. For less complex DriverMap Targeted Kits targeting fewer genes, the number of samples per flow cell can be increased. Generally, you should aim for 500-1000 reads per gene target. For example, for or a DriverMap Assay targeting 4,000 genes, 2-4 million reads per sample (i.e., 4,000 genes times 500 reads to 1,000 reads) is adequate.

Instrument	Reads per flow cell	Number of samples for multiplexing per flow cell	Reads per gene
MiSeq Series	25 million	5	5M reads/sample or 500 reads/gene average
NextSeq Series	400 million	48	8M reads/sample or 1000 reads/gene average
HiSeq Series	2 billion, 8 lanes	192 (96 per lane, 2 lanes)	10M reads/sample or 1000 reads/gene average

Last modified: 4 October 2022

5.2. Purification of the Amplified Indexed Libraries

The purpose of this step is to remove any residual primers and reagents from the pooled Amplified Indexed Libraries so that the preparations are ready for NGS.

1. Add 1.8x volume of Agencourt® AMPure® XP Reagent (at room temperature) to pooled Amplified Indexed Libraries

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and Negative Control sample mix in an Eppendorf tube, and pipet up and down 5 times to thoroughly mix the bead suspension with the pooled Amplified Indexed Libraries.

2. Incubate the mixture for 2 minutes at room temperature.

3. Place tube in the Thermo Fisher Dynabeads® MPC®-S Magnetic Stand for 1 minute or until the solution is clear. Carefully remove and discard the supernatant without disturbing the pellet.

4. Add 500 µl of freshly prepared 80% ethanol to the tube, and wash the beads by pipetting.

5. Place the tube in the Magnetic Stand for 1 minute or until the solution is clear. Carefully remove and discard the supernatant without disturbing the pellet.

6. Repeat steps 4 and 5 for a second wash.

7. Briefly centrifuge tubes at low speed and place the tubes in the Magnetic Stand. Use a 20-µl pipette to remove the residual ethanol droplets from the tube, and air-dry the beads at room temperature for 2 minutes.

8. Add 25 µl of fresh PCR-Grade Water to the pellet to disperse the beads, and let stand for 1 minute.

9. Place the tube on the Magnetic Stand for 1 minute. Transfer 20 µl of the supernatant to a new Eppendorf tube.

10. Measure the concentration of both pooled Amplified Indexed Libraries sample and Negative Control sample using the Qubit® dsDNA High Sensitivity Assay. Subtract the concentration reading (ng/µl) of the Negative Control sample from the pooled Amplified Indexed Libraries reading.

11. Dilute the pooled Amplified Indexed Libraries probe sample to 1.8 ng/µl, which corresponds to a concentration of 10nM for the following NGS step.

Last modified: 5 December 2018

5.3. Next-Gen Sequencing (NGS)

The Amplified NGS Index Libraries made from each total RNA sample should be run on an Illumina sequencer following the manufacturer's instructions. Generally, we recommend setting up the sequencing reactions to generate 8-10 million reads per sample (read depth per sample). This depth works out to produce 500-1000 reads, on average, per gene—of course, low represented genes will have much fewer reads than highly represented ones.

The procedure below is based on our experience with the NextSeq 500 using the 75-cycle NextSeq 500/550 High Output Kit v2 (Illumina Cat.# FC-404-2005).

The multiplexing level may be modified to meet your experimental needs. For example, multiplex fewer samples together to generate more sequencing reads per sample (i.e., more depth) for increased and more sensitive detection of genes present across a broader dynamic range of expression levels, or, if you are mostly interested in highly expressed genes, you can sequence more samples together which will be less expensive.

Follow the standard Illumina procedures for Cluster Generation starting with 10 nM of the purified PCR sample.

1. Add 6 µl of each of the custom sequencing primers into the appropriate wells of the Illumina reagent cartridge, as

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follows:

- Reverse SeqDNA NGS Primer (Read 1 Sequencing Primer) into well #20
- Forward SeqDNA NGS Primer (Read 2 Sequencing Primer) into well #21
- Reverse SeqIND NGS Primer (Index 1 Sequencing Primer) into well #22
- Forward SeqIND NGS Primer (Index 2 Sequencing Primer) into well #22

> **Note:** We do not recommend adding primers to the “Custom” wells of the reagent cartridge. We add our in-house custom sequencing primers into the Illumina premixed primer wells in order to take advantage of the PhiX internal control. Addition of our in-house sequencing primers does not affect the performance of the flow cell.

2. Perform the NGS run using 75-nt paired-end reads (or 150-nt if longer reads are desired for direct alignment to the genome) on the NextSeq, HiSeq or MiSeq. Proceed to cluster amplification using the appropriate Illumina Paired-End Cluster Generation Kit; refer to the manufacturer’s instructions for this step. The optimal seeding concentration for cluster amplification of indexed libraries is approximately 1.8 pM. Use the following program for the sequence run:

Program	Custom Primer	Cycles
Read 1:	Reverse SeqDNA	38
Index 1:	Reverse SeqIND	6
Index 2:	Forward SeqIND	6
Read 2:	Forward SeqDNA	38

> **Note:** The program has a total of 88 cycles. There are enough reagents in the 75-cycle kit (Illumina Cat.# FC-404-2005) to run at least 90 cycles.

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6. Data Processing and Analysis

The protocols in this section describe how to extract the sequencing read numbers for each gene target from the Illumina FASTQ file, and then normalize these reads across the samples to assess expression levels of the gene targets. Cellecta provides **DriverMap Data Alignment Software** to demultiplex the Illumina FASTQ file (*.fasta file) and provide the number of reads of each amplicon for each genes. The read numbers, once normalized, correlate directly to the expression level of the gene.

Install DriverMap Data Alignment Software

You should have received a link to download the **Data Alignment Program** when you received your DriverMap Assay Kit. If you did not, or cannot find email with the link, please contact Cellecta and let us know the lot number of the DriverMap Assay Kit you are using.

1. Click on the link and copy the Zip file for the ***DriverMap Alignment Software*** into a convenient directory of your choice on your computer.

2. Unzip the DriverMap Alignment Software. The Program and supporting files will appear in the directory.

3. To run the Program, simply open the ***executable file*** .

> **Note:** The DriverMap Data Alignment Software is compatible any 64-bit Windows system running Windows 7 or higher.

Last modified: 27 November 2018

6.1. Extract Sequencing Read Data

To extract reads from the Illumina sequencer FASTQ format files, you first need to enter your sample information into the ***Sample Description file***. This template file already contains all the indexing information based on the index plate positions. You just need to add the sample names, save the file as tab-delimited text, then start the program.

Convert Sequencing Data to FASTQ Format

To use the DriverMap Sample Extraction Software, the Illumina sequencing data much be converted to FASTQ format. To do this, run Illumina's bcl2fastq program on a computer running Red Hat Enterprise Linux 6 or CentOS 6 (see Illumina for full installation requirements).

> **Note:** Do not use the bcl2fastq Program to demultiplex the run data. The DriverMap Data Alignment Software will demultiplex the run when it does the alignment. If you demultiplex the run with bcl2fastq Program you will generate a separate FASTQ file for each indexed sample and would need to run the DriverMap Alignment Program separately to align each one.

1. Use the following command line to convert the Illumina bcl2fastq *intensity reads* to FASTQ sequence data:

```
$ bcl2fastq --runfolder-dir $folder --create-fastq-for-index-reads --ignore-missing-bcls --
minimum-trimmed-read-length 0 --mask-short-adapter-reads 0
```

- Make sure to use the "***—create-fastq-for-index-reads***" option to generate both the I1 and I2 FASTQ files, needed for

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Cellecta's DriverMap alignment software.

- The "***$folder***" parameter is the path to the folder containing data for the NGS run. At the end of the bcl2fastq conversion, you should have find in the *$folder* a list of ***non-demultiplexed*** FASTQ files similar to the following set of files that will be read by the Alignment Software:

```
filename_S0_L001_I1_001.fastq.gz
filename_S0_L001_I2_001.fastq.gz
filename_S0_L001_R1_001.fastq.gz
filename_S0_L001_R2_001.fastq.gz
filename_S0_L002_I1_001.fastq.gz
filename_S0_L002_I2_001.fastq.gz
```

> **Note:** The SampleSheet.csv file should not be in the destination folder for the FASTQ files. The bcl2fasq files and FASTQ files need to be in separate folders to ensure the Alignment Program runs correctly.

Set-up the Sample Description File

1. Open the template file "**DriverMap-Sample-Description-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 Form*** has four columns. Enter the experimental sample names in the second column based on their positions in the index plate (as noted in the first column). We recommend using standard alphanumeric characters. If you would like to include sample numbers as well as descriptions for the samples, just add these to the sample names. The software will only extract sequencing data corresponding to rows with sample names. The program will ignore rows with the sample field left blank. Sequences corresponding to indexes of fields without names will not be extracted.

> **Note:** If you used only a fraction of the wells in the index plate (e.g., to run less than 96 samples), be sure to enter the sample information into the field that references the well location relative to the whole plate. In other words, if you cut the plate in half and ran columns 1-6 before and then, in this experiment ran the remaining 6 columns (columns 7-12), you need to enter the sample names in the field that denotes the well positions for the second part of the plate (i.e., starting with 7A, 7B, 7C,….12G, 12H).

> **Note:** If you choose to generate individual demultiplexed FASTQ files for each sample (see below), the plate coordinates (column 1) will be used as the file names for the individual FASTQ files.

> **Note:** If you do not have Microsoft Excel on your computer, you can enter the information directly into the included "**sample-description.txt**" file using another suitable application. Be careful to make sure the table is not altered in any way and the indexes are not changed.

3. Click the button to save the sample table in ***tab-text format***. Choose an appropriate name for the file as desired.

Run the Data Alignment Program

To run the program, you simply need to select the DriverMap Assay Kit you are using, the Sample Description File (.txt format as saved above), and the FASTQ file. This is done simply by selecting the appropriate files using the program interface.

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1. Select the DriverMap Assay Kit used in the experiment. The Program defaults to the Human DriverMap Genome-Wide Assay Kit. If the data was generated using this assay, then leave the selection as-is, otherwise, select the file corresponding to the DriverMap Assay used.

2. Click the button to select the Sample Description text file created above.

3. Select the folder with the FASTQ files for the experiment.

> **Note:** All FASTQ files for the experiment must be decompressed and in the same folder. The Program does not accept compressed (.fastq.gz) files. Decompress them to .fastq. The Program identifies each file using the Illumina NextSeq naming conventions: four files per lane, labeled *Undetermined_S0_L001_I1_001.fastq*, *Undetermined_S0_L001_I2_001.fastq*, etc.

4. At this point, you have the option to have the program generate separate demultiplexed FASTQ files for each of the individual samples. This will require disk space equal to the size of the FASTQ read files from the sequencer and generating these files increases processing time for the program.

> **Note:** If the option to generate FASTQ files is enabled, ensure there is sufficient local disk space available to store the resulting output. The size of the individual FASTQ files output will be approximately equal to the input FASTQ.

5. There is the option to change the sequence length scored and the Hamming distance used to identify targets. These parameters should only be changed if sequencing was done using other than the standard 75-cycle Illumina sequencing kit. Normally, these parameters should not be altered.

6. Click the ***Start Alignment*** button to begin scoring the sequence data. Processing may take an hour or more. The program displays a progress indicator with information regarding the number of records processed while running. When complete, a message will appear on the screen.

> **Note:** Clicking the **_Exit _** button or closing the program window will end the program prematurely. A warning message will appear to alert you that the program will close.

The Program outputs two tab-delimited text files. These files can be uploaded into Excel or other spreadsheet software. Both files include the name of the ***Sample Description File*** with the extension shown below:

- The **_“sample-description-filename”__Statistics.txt** file contains the table of total aligned and unaligned (i.e. background) counts for each sample.
- The **_“sample-description-filename”__Alignment.txt** file has a table of aligned counts for each sample (in columns) and target (in rows).

Optionally, as indicated above, separate FASTQ files can be generated for each of the input samples. These may be used by other analysis software.

All output files will be saved in the same directory as the sample description file.

Last modified: 29 July 2019

6.2. Normalize Sample Reads

Normalization against reference housekeeping genes which are expected to be expressed

consistently across all samples

After data extraction from the FASTQ file, reads for each gene need to be normalized for analysis. We recommend normalizing the read counts for each gene amplicon against endogenous housekeeping genes to enable an accurate comparison of expression levels across the series of samples. Housekeeping genes are expected to be expressed relatively consistently across all biological samples and, therefore, provide a reasonably consistent standard with which to compare counts of the regulated genes of interest between samples. Using the procedure outlined below, the reads can be normalized relative to any set of genes for which expression levels are not expected to change across all the samples. Please refer to Appendix D. Housekeeping Control Genes for the list of the housekeeping genes we recommend using for this analysis.

For housekeeping gene normalization, calculate the average of the geometric means of read counts for all housekeeping genes across all samples and then use this as the reference to normalize each individual sample as follows:

1. For each sample, calculate the geometric mean of the read counts of the housekeeping control genes.

2. Calculate the average of the geometric means across all samples.

3. Divide this average by the geometric mean in each sample to get a sample-specific normalization factor.

4. Multiply all DriverMap gene counts in a sample by that sample's normalization factor.

After normalization measures have been applied, standard statistical tests can be used to analyze the data and identify differentially expressed genes. For normally distributed data, a paired t-test (for two conditions) or one-way ANOVA (three or more conditions) is recommended for this analysis. The Benjamini & Hochberg FDR-controlling procedure (or similar multiple testing approach) is suggested to correct and adjust p-values.

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7. Appendices

A. Tips and Troubleshooting
B. Sequence of Amplicon after Index Primer PCR
C. Forward and Reverse Index Combinations
D. Housekeeping Control Genes

Last modified: 8 August 2019

7.1. Appendix A. Tips and Troubleshooting

Observation	Possible Cause	Recommended Action
Yield of PCR products for DNA samples is low, but is good for Positive Control DNA sample — or — Low yield of PCR products for both experimental and Positive Control DNA samples	Error in quantitation of input DNA, or DNA is degraded	Re-analyze the amount and quality of input DNA using Agilent Bioanalyzer, Agilent Fragment Analyzer, or Thermo Fisher NanoDrop.
	DNA input is too low	Add more DNA, or increase target amplification cycles (Refer to **PCR with Anchor Primers** for cycle numbers recommended based on input amounts).
	RT step is not optimal	Please check for procedural mistakes. Make master mixes where possible. For individual reaction setups, make sure that the correct volume of RT-EXT Buffer and RT enzyme is added into each reaction.
	PCR cycling conditions are not optimal	Increase PCR amplification cycles. Ensure proper dispensing and mixing of viscous components at each step.

Observation	Possible Cause	Recommended Action
Low Cluster Density.	Inefficient purification from low molecular weight DNA products or low yield of PCR products	Re-purify PCR products using different technology (e.g. QIAGEN QIAquick PCR Purification Kit), and/or increase the concentration of pooled PCR products in the cluster generation step.

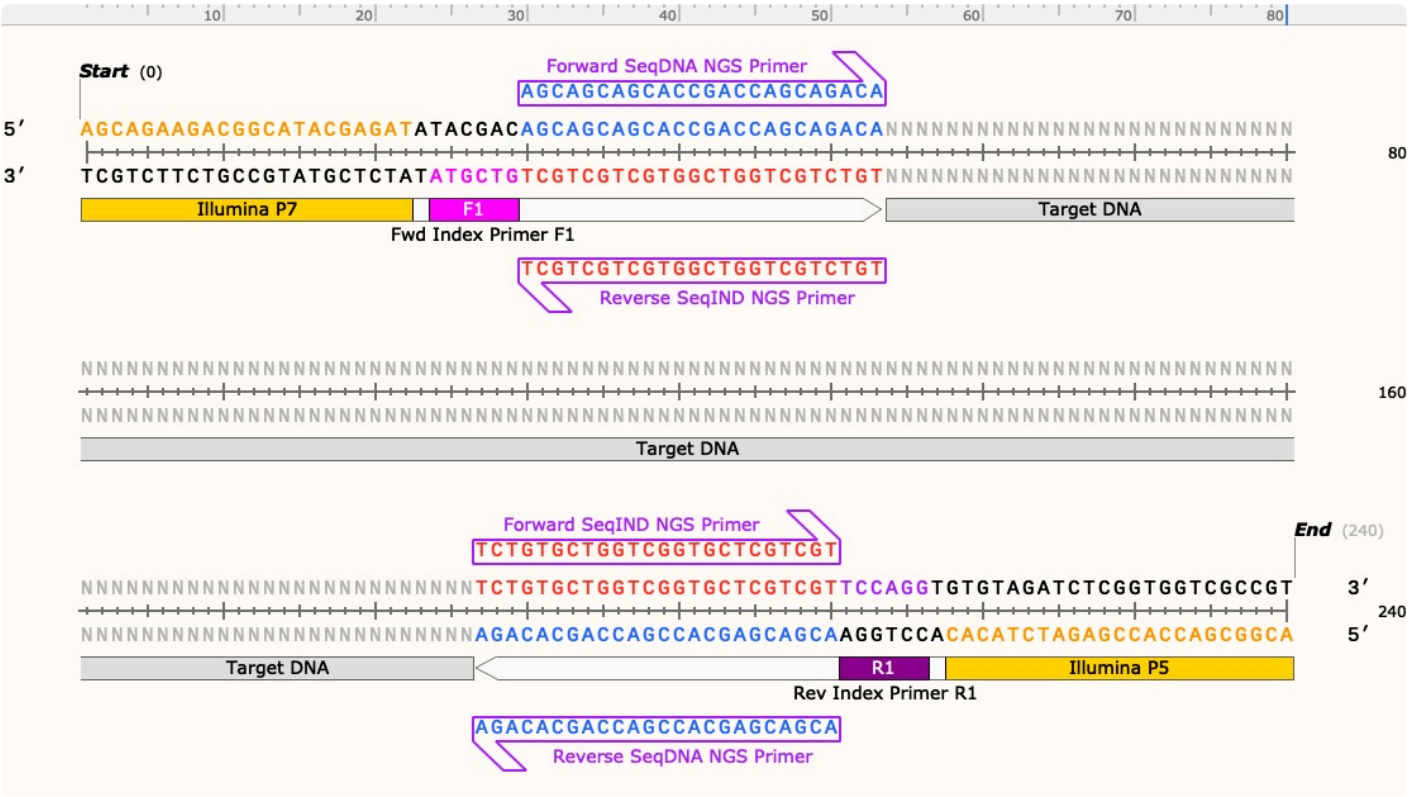
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Observation	Possible Cause	Recommended Action
Yields of PCR products are significantly higher than Positive Control	Incorrect quantitation of input DNA amount	Re-analyze the amount and quality of input DNA using Agilent Bioanalyzer, Agilent Fragment Analyzer, or Thermo Fisher NanoDrop.
	DNA input is too high	Add less DNA or decrease number of PCR cycles in the **PCR with Indexed Primers** step.
Low fluorescence readings on Illumina sequencer — or — Lower than expected number of on-target reads — or — High mutation rate in the Index reads	PCR primers from amplified indexed library quantification step (**Quantify and Combine Samples for NGS**) were not removed	Check that you added Primer Removal Master Mix in the **Quantify and Combine Samples for NGS** step. If it had been added, use a new vial of Primer Removal Reagent and repeat.
Lower than expected number of on-target reads	DNA input too low or PCR cycle number too low	Add more DNA or increase number of PCR cycles in target amplification step (**PCR with Anchor Primers**).
	DNA is degraded	Use highest quality DNA possible. For degraded DNA, increase the number of PCR cycles.
Uneven representation of Indexed Libraries	Inaccurate Indexed Library quantification	Check that you correctly calculate the molar concentration of each indexed library sample.
	Inaccurate Indexed Library mixing	Re-quantify the indexed library samples and mix them in equimolar amounts.
Inconsistent library yields from replicate DNA samples	Sample evaporation in thermal cycler	Seal 96-well plates well with an Adhesive Film Applicator and use a Compression Pad. Fill empty wells with water to minimize evaporation from experimental samples.

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7.2. Appendix B. Sequence of Amplicon after Index Primer PCR

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Start (0)
Forward SeqDNA NGS Primer
AGCAGCAGCACCGACCAGCAGACA
5′ AGCAGAAGACGGCATACGAGATATACGACAGCAGCAGCACCGACCAGCAGACANNNNNNNNNNNNNNNNNNNNNNNNN
3′ TCGTCTTCTGCCGTATGCTCTATATGCTGTCGTCGTCGTGGCTGGTCGTCTGTNNNNNNNNNNNNNNNNNNNNNNNNN
80
Illumina P7
F1
Target DNA
Fwd Index Primer F1
TCGTCGTCGTGGCTGGTCGTCTGT
Reverse SeqIND NGS Primer
NNNNNNNNNNNNNNNNNNNN

7	A7	AGCGTA	TACGCT	CCTGGA	TCCAGG
8	A8	TAGTCG	CGACTA	AAGCCT	AGGCTT
9	A9	TCAAGG	CCTTGA	GCTGAT	ATCAGC
10	A10	AGCGTA	TACGCT	CTTGAC	GTCAAG
11	A11	TAGTCG	CGACTA	TGAAAC	GTTTCA
12	A12	TCAAGG	CCTTGA	ACTTTG	CAAAGT
13	B1	CTGATG	CATCAG	CCTGGA	TCCAGG
14	B2	AGTCGT	ACGACT	AAGCCT	AGGCTT
15	B3	CATAGC	GCTATG	GCTGAT	ATCAGC
16	B4	CTGATG	CATCAG	CTTGAC	GTCAAG
17	B5	AGTCGT	ACGACT	TGAAAC	GTTTCA
18	B6	CATAGC	GCTATG	ACTTTG	CAAAGT
19	B7	GTAGGC	GCCTAC	CCTGGA	TCCAGG
20	B8	GAACTT	AAGTTC	AAGCCT	AGGCTT
21	B9	GGGACT	AGTCCC	GCTGAT	ATCAGC
22	B10	GTAGGC	GCCTAC	CTTGAC	GTCAAG
23	B11	GAACTT	AAGTTC	TGAAAC	GTTTCA
24	B12	GGGACT	AGTCCC	ACTTTG	CAAAGT
25	C1	GCATCA	TGATGC	CCTGGA	TCCAGG
26	C2	TCGCAT	ATGCGA	AAGCCT	AGGCTT
27	C3	TACGAC	GTCGTA	ATCAGC	GCTGAT
28	C4	GCATCA	TGATGC	CTTGAC	GTCAAG
29	C5	TCGCAT	ATGCGA	TGAAAC	GTTTCA
30	C6	TACGAC	GTCGTA	TGGGCA	TGCCCA
31	C7	TAGTCG	CGACTA	CCTGGA	TCCAGG
32	C8	TCAAGG	CCTTGA	AAGCCT	AGGCTT
33	C9	AGCGTA	TACGCT	ATCAGC	GCTGAT
34	C10	TAGTCG	CGACTA	CTTGAC	GTCAAG
35	C11	TCAAGG	CCTTGA	TGAAAC	GTTTCA
36	C12	AGCGTA	TACGCT	TGGGCA	TGCCCA
37	D1	AGTCGT	ACGACT	CCTGGA	TCCAGG
38	D2	CATAGC	GCTATG	AAGCCT	AGGCTT
39	D3	CTGATG	CATCAG	ATCAGC	GCTGAT
40	D4	AGTCGT	ACGACT	CTTGAC	GTCAAG
41	D5	CATAGC	GCTATG	TGAAAC	GTTTCA
42	D6	CTGATG	CATCAG	TGGGCA	TGCCCA
43	D7	GAACTT	AAGTTC	CCTGGA	TCCAGG
44	D8	GGGACT	AGTCCC	AAGCCT	AGGCTT
45	D9	GTAGGC	GCCTAC	ATCAGC	GCTGAT
46	D10	GAACTT	AAGTTC	CTTGAC	GTCAAG

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47	D11	GGGACT	AGTCCC	TGAAAC	GTTTCA
48	D12	GTAGGC	GCCTAC	TGGGCA	TGCCCA
49	E1	TCGCAT	ATGCGA	CCTGGA	TCCAGG
50	E2	TACGAC	GTCGTA	GCTGAT	ATCAGC
51	E3	GCATCA	TGATGC	ATCAGC	GCTGAT
52	E4	TCGCAT	ATGCGA	CTTGAC	GTCAAG
53	E5	TACGAC	GTCGTA	ACTTTG	CAAAGT
54	E6	GCATCA	TGATGC	TGGGCA	TGCCCA
55	E7	TCAAGG	CCTTGA	CCTGGA	TCCAGG
56	E8	AGCGTA	TACGCT	GCTGAT	ATCAGC
57	E9	TAGTCG	CGACTA	ATCAGC	GCTGAT
58	E10	TCAAGG	CCTTGA	CTTGAC	GTCAAG
59	E11	AGCGTA	TACGCT	ACTTTG	CAAAGT
60	E12	TAGTCG	CGACTA	TGGGCA	TGCCCA
61	F1	CATAGC	GCTATG	CCTGGA	TCCAGG
62	F2	CTGATG	CATCAG	GCTGAT	ATCAGC
63	F3	AGTCGT	ACGACT	ATCAGC	GCTGAT
64	F4	CATAGC	GCTATG	CTTGAC	GTCAAG
65	F5	CTGATG	CATCAG	ACTTTG	CAAAGT
66	F6	AGTCGT	ACGACT	TGGGCA	TGCCCA
67	F7	GGGACT	AGTCCC	CCTGGA	TCCAGG
68	F8	GTAGGC	GCCTAC	GCTGAT	ATCAGC
69	F9	GAACTT	AAGTTC	ATCAGC	GCTGAT
70	F10	GGGACT	AGTCCC	CTTGAC	GTCAAG
71	F11	GTAGGC	GCCTAC	ACTTTG	CAAAGT
72	F12	GAACTT	AAGTTC	TGGGCA	TGCCCA
73	G1	TACGAC	GTCGTA	AAGCCT	AGGCTT
74	G2	GCATCA	TGATGC	GCTGAT	ATCAGC
75	G3	TCGCAT	ATGCGA	ATCAGC	GCTGAT
76	G4	TACGAC	GTCGTA	TGAAAC	GTTTCA
77	G5	GCATCA	TGATGC	ACTTTG	CAAAGT
78	G6	TCGCAT	ATGCGA	TGGGCA	TGCCCA
79	G7	AGCGTA	TACGCT	AAGCCT	AGGCTT
80	G8	TAGTCG	CGACTA	GCTGAT	ATCAGC
81	G9	TCAAGG	CCTTGA	ATCAGC	GCTGAT
82	G10	AGCGTA	TACGCT	TGAAAC	GTTTCA
83	G11	TAGTCG	CGACTA	ACTTTG	CAAAGT
84	G12	TCAAGG	CCTTGA	TGGGCA	TGCCCA
85	H1	CTGATG	CATCAG	AAGCCT	AGGCTT
86	H2	AGTCGT	ACGACT	GCTGAT	ATCAGC

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87	H3	CATAGC	GCTATG	ATCAGC	GCTGAT
88	H4	CTGATG	CATCAG	TGAAAC	GTTTCA
89	H5	AGTCGT	ACGACT	ACTTTG	CAAAGT
90	H6	CATAGC	GCTATG	TGGGCA	TGCCCA
91	H7	GTAGGC	GCCTAC	AAGCCT	AGGCTT
92	H8	GAACTT	AAGTTC	GCTGAT	ATCAGC
93	H9	GGGACT	AGTCCC	ATCAGC	GCTGAT
94	H10	GTAGGC	GCCTAC	TGAAAC	GTTTCA
95	H11	GAACTT	AAGTTC	ACTTTG	CAAAGT
96	H12	GGGACT	AGTCCC	TGGGCA	TGCCCA

NOTE: Table shows both direct Index sequences as they appear in the Forward or Reverse Index Primers (see **Appendix B. Sequence of Amplicon after Index Primer PCR**), and adjacent columns show the reverse complement of the Index sequences. Use the corresponding Index sequences, as specified in the specific NGS instrument manual.

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7.4. Appendix D. Housekeeping Control Genes

Below is the list of housekeeping genes we recommend to use for normalization analysis of Human DriverMap Assays, as described in the **Data Processing and Analysis** section. For suggested housekeeping genes to normalize Mouse DriverMap Assays, please refer to the Product Insert provided with the Kit.

Gene Symbol	Entrez Gene ID
ABCF1	23
ACAD9	28976
AGK	55750
ALAS1	211
AMMECR1L	83607
C10orf76	79591
CC2D1B	200014
CNOT10	25904
CNOT4	4850
COG7	91949
DDX50	79009
DHX16	8449
DNAJC14	85406
EDC3	80153
EIF2B4	8890
ERCC3	2071
FCF1	51077
G6PD	2539
GPATCH3	63906
GUSB	2990
HDAC3	8841
HPRT1	3251
MRPS5	64969
MTMR14	64419
NOL7	51406
NUBP1	4682
PIAS1	8554
PIK3R4	30849
POLR2A	5430
PPIA	5478
PRPF38A	84950
RBM45	129831
SAP130	79595

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SDHA	6389
SF3A3	10946
SLC4A1AP	22950
TBP	6908
TLK2	11011
TMUB2	79089
TRIM39	56658
TTC31	64427
TUBB	203068
USP39	10713
VPS33B	26276
ZC3H14	79882
ZKSCAN5	23660
ZNF143	7702
ZNF346	23567
ZNF384	171017
MRM2	29960

Last modified: 5 December 2018

7.5. Appendix E: Sample Data for T/B Marker Assay

Download 500 Panel Sample Data from purified T and B cells

Table of Contents

1. **Gene Sigs-Basic:** Top Genes to Identify T & B Cell Subtypes

2. **Gene Sigs-Data Summarized:** Summarized Data used for Top Gene Sigs Lists

3. **HeatMaps:** Heatmaps comparing T vs B cells

4. **Gene Sigs-Data Raw:** Raw Count Data for T & B Cell Subtyping with AIR 500 Panel

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