Journal of Visualized Experiments

Predictive Immune Modeling of Solid Tumors using the ImmunoPrism Assay

--Manuscript Draft--

Article Type:	Corporate Submission
Manuscript Number:	JoVE60645R2
Full Title:	Predictive Immune Modeling of Solid Tumors using the ImmunoPrism Assay
Keywords:	RNA; Computational Biology; Tumor Microenvironment; Immunotherapy; Informatics; biomarkers
Corresponding Author:	Natalie A LaFranzo, Ph.D. Cofactor Genomics, Inc. San Francisco, California UNITED STATES
Corresponding Author's Institution:	Cofactor Genomics, Inc.
Corresponding Author E-Mail:	natalie_lafranzo@cofactorgenomics.com
Order of Authors:	Natalie A LaFranzo, Ph.D.
	Kevin C. Flanagan
	Danielle Quintanilha
Additional Information:	
Question	**Response**
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Saint Louis, Missouri, United States

TITLE:

Predictive Immune Modeling of Solid Tumors

AUTHORS AND AFFILIATIONS:

Natalie A. LaFranzo[1], Kevin C. Flanagan[1], Danielle Quintanilha[1]

[1]Cofactor Genomics, San Francisco, CA USA

Email addresses of co-authors:

Kevin C. Flanagan (kevin_flanagan@cofactorgenomics.com)

Danielle Quintanilha (danielle_quintanilha@cofactorgenomics.com)

Corresponding author:

Natalie A. LaFranzo (natalie_lafranzo@cofactorgenomics.com)

KEYWORDS:

Immune Profiling, RNA, RNA-seq, Machine Learning, Bioinformatics, Solid Tumor, Cancer, Sequencing, Predictive Immune Modeling, Health Expression Models, Immune-Oncology, Gene Expression

SUMMARY:

The use of an RNA-based approach to determine quantitative immune profiles of solid tumor tissues and leverage clinical cohorts for immune-oncology biomarker discovery is described through a molecular and informatics protocols.

ABSTRACT:

Immunotherapies show promise in the treatment of oncology patients, but complex heterogeneity of the tumor microenvironment makes predicting treatment response challenging. The ability to resolve the relative populations of immune cells present in and around the tumor tissue has been shown to be clinically-relevant to understanding response, but is limited by traditional techniques such as flow cytometry and immunohistochemistry (IHC), due the large amount of tissue required, lack of accurate cell type markers, and many technical and logistical hurdles. One assay (e.g., the ImmunoPrism Immune Profiling Assay) overcomes these challenges by accommodating both small amounts of RNA and highly degraded RNA, common features of RNA extracted from clinically archived solid tumor tissue. The assay is accessed via a reagent kit and cloud-based informatics that provides an end-to-end quantitative, high-throughput immuno-profiling solution for Illumina sequencing platforms. Researchers start with as few as two sections of formalin-fixed paraffin-embedded (FFPE) tissue or 20-40 ng of total RNA (depending on sample quality), and the protocol generates an immune profile report quantifying eight immune cell types and ten immune escape genes, capturing a complete view of the tumor microenvironment. No additional bioinformatic analysis is required to make use of the resulting data. With the appropriate sample cohorts, the protocol may also be used to identify statistically significant biomarkers within a patient population of interest.

INTRODUCTION:

Quantification of tumor-infiltrating lymphocytes (TILs) and other immune-related molecules in formalin-fixed and paraffin embedded (FFPE) solid tumor human tissue samples has demonstrated value in clinical research[1-3]. Common techniques such as flow cytometry and single-cell ribonucleic acid (RNA) sequencing are useful for fresh tissue and blood[4], but are unsuitable for analysis of FFPE materials due to the inability to create viable cell suspensions. Current methods that have been used to quantify these cells in FFPE tissue suffer from major challenges. Immunohistochemistry (IHC) and other similar imaging workflows require specific antibodies to detect cell-surface proteins, which can be difficult to standardize across laboratories to enable reproducible quantification[5]. Platforms such as the nCounter system rely on the expression of single genes to define key immune cells[6], limiting sensitivity and specificity of detection. More generic RNA sequencing methods, coupled with standalone software tools, are available but require significant optimization and validation prior to use[7-12]. Recent advances in combining laser capture microdissection (LCM) with RNA sequencing for FFPE tissue has shown promise; however, a more high-throughput, turnkey solution is required for translational studies aimed at identifying robust biomarkers[13,14]. Methods to generate multidimensional biomarkers, such as Predictive Immune Modeling, that define patient cohorts including therapy responders, cancer subtypes, or survival outcomes with high predictive accuracy and statistical significance are becoming increasingly important in the age of precision medicine and immunotherapy[15,16].

To address this need, an immune profiling assay was developed to enable sensitive and specific quantification of immune cells in solid tumor FFPE tissue using standardized RNA-sequencing reagents and cloud-based informatics. In addition to accommodating degraded RNA from FFPE tissue, the protocol is able to accommodate RNA derived from limiting tissue samples such as core needle biopsies, needle aspirates, and micro- or macro-dissected tissue. RNA data from each sample is compared to a database of gene expression models of immune cells, called immune Health Expression Models, to quantify immune cells as a percentage of total cells present in the sample. Briefly, these models were built using machine-learning methods to identify unique multigenic expression patterns from whole-transcriptome data generated from purified immune cell populations (isolated using canonical cell-surface markers)[17,18]. The multidimensional Health Expression Models underlying the technology enables the assay to quantify each immune cell as a percent of the total cells present in the heterogenous mixture. This enables the researcher to generate inter- and intra-sample immune cell comparisons, which have been shown to have clinical value[19,20]. Other applications include quantification of immune response pre- and post-treatment, as described in the representative results. The assay reports on multiple features of immune contexture of the tumor and tumor microenvironment including the absolute percentages of eight immune cell types (derived from gene expression models): CD4+ T cells, CD8+ T cells, CD56+ Natural Killer cells, CD19+ B cells, CD14+ monocytes, Tregs, M1 macrophages, and M2 macrophages. In addition, the assay reports the expression (in transcripts per million, or TPM) of ten immune escape genes: PD-1, PD-L1, CTLA4, OX40, TIM-3, BTLA, ICOS, CD47, IDO1, and ARG1.

The reagent kit is used to make high quality libraries ready for sequencing on an Illumina platform following a hybrid capture-based library preparation method, as shown in **Figure 1**. If a

researcher does not have an Illumina sequencing platform in their laboratory, they may submit their samples to a core laboratory for sequencing. Once generated, sequencing data is uploaded to the Prism Portal for automated analysis, and a comprehensive, quantitative profile for each individual sample, in the form of the Immune Report (**Figure 2A**), is returned to the user. Users may also define sample groupings in the Prism Portal to generate a Biomarker Report (**Figure 2B**), highlighting statistically significant biomarkers that distinguish two patient cohorts. Importantly, the data generated by the reagent kit is for research use only and may not be used for diagnostic purposes.

[Place **Figures 1 and 2** here]

The protocol requires approximately 16 hours of preparation time (from total RNA to libraries ready for sequencing); however, there are a number of optional stopping points, as noted in the protocol. The assay makes use of the rich, dynamic nature of transcriptomics to move beyond legacy single-analyte biomarkers to multidimensional gene expression models, thereby enabling comprehensive biological characterization of tissue samples with standardized reagents and easy-to-use software tools. It empowers researchers to utilize a contemporary technology in their own laboratory, by leveraging machine-learning and a database of Health Expression Models to derive more accurate, quantitative immune profiles of precious clinical samples, and discover multidimensional RNA biomarkers with full statistical analysis.

PROTOCOL:

The human tissue samples utilized in the Representative Results shown here were purchased from a reputable entity (TriStar Technology Group) and have informed donor consent permitting academic and commercial research, as well as approval from a competent ethical committee.

Part I: Pre-Capture Library Preparation

1. RNA quantification and qualification

1.1 Quantify RNA using a fluorometric assay to determine the appropriate input to the assay. Assess the quality of the input RNA using electrophoresis to determine the RNA Integrity Number (RIN) and the percentage of fragments >200 nucleotides (DV_{200}) values.

1.1.1 For intact (RIN > 7) or partially degraded RNA samples (RIN = 2 to 7) follow the library preparation steps for high quality/intact RNA, starting with Step 2.1. The quality of the RNA is important for selecting the correct fragmentation time in Thermal Cycler Program #1 (**Supplemental Table 2**).

1.1.2 For highly degraded samples (e.g., RIN = 1 to 2 or FFPE), determine the DV_{200} value. These samples do not require fragmentation and will follow the instructions for degraded RNA, starting with Step 2.2.

1.2 Prepare the appropriate amount of total RNA for each sample for by diluting 20 ng of RNA (High-Quality/Intact RNA with RIN > 2) or 40 ng of RNA (Degraded/FFPE RNA with DV_{200} > 20%) to 5 µL in nuclease-free water. Processing samples with DV_{200} < 20% is not recommended. For the control RNA samples provided with the kit, dilute 1 µL of the appropriate RNA in 4 µL of nuclease-free water. Control samples will follow the same processing as described for either High-Quality (Intact) or Degraded (FFPE) RNA materials, as labeled. See **Supplemental Table 1** for all reagents included in the kit.

2. RNA fragmentation and priming

2.1 Follow Step 2.1.1 for High-Quality/Intact RNA with RIN > 2.

2.1.1 For high-quality RNA, assemble the fragmentation and priming reaction on ice in a nuclease-free PCR tube according to **Table 1**.

[Place **Table 1** here]

2.1.1.1 Mix thoroughly by pipetting up and down several times. Then, briefly spin down the samples in a microcentrifuge

NOTE: For all centrifuge spins in the protocol, a speed of ≥ 1,000 x *g* for at least 3 s is recommended.

2.1.1.2 Place the samples in a thermal cycler and use Program #1 (**Supplemental Table 2**).

2.1.1.3 Immediately transfer the tubes to ice and proceed to **First Strand cDNA Synthesis for High Quality RNA** (Step 3.1). For concurrent preparation of both High Quality and FFPE RNA, begin preparation of the FFPE RNA (Step 2.2) during the Fragmentation Incubation.

2.2 Follow Step 2.2.1 for Degraded/FFPE RNA with DV_{200} > 20%.

2.2.1 For highly degraded (FFPE) RNA that does not require fragmentation, assemble the priming reaction as described in **Table 2**. For intact RNA, remember to follow Step 2.1.

[Place **Table 2** here]

2.2.1.1 Mix thoroughly by pipetting up and down several times. Then, briefly spin down the samples in a microcentrifuge.

2.2.1.2 Place the samples in a thermal cycler and use Program #2 (**Supplemental Table 2**).

2.2.1.3 Transfer the tubes to ice and proceed to **First Strand cDNA Synthesis for Highly Degraded** (FFPE) RNA (Step 3.2).

3. First strand cDNA synthesis

3.1 Follow Step 3.1.1 for High-Quality/Intact RNA with RIN > 2.

3.1.1 For intact RNA (high-quality), assemble the fragmentation and priming reaction on ice in a nuclease-free PCR tube according to **Table 3**.

[Place **Table 3** here]

3.1.1.1 Keeping the reactions on ice, thoroughly mix by pipetting up and down several times. Briefly spin down the samples in a microcentrifuge, and proceed directly to **First strand synthesis incubation** (Step 4).

3.2 Follow Step 3.2.1 for Degraded/FFPE RNA with DV_{200} > 20%.

3.2.1 For highly degraded RNA (FFPE), assemble the fragmentation and priming reaction on ice in a nuclease-free PCR tube according to **Table 4**.

[Place **Table 4** here]

3.2.1.1 Keeping the reactions on ice, thoroughly mix by pipetting up and down several times. Briefly spin down the samples in a microcentrifuge, and proceed directly to **First strand synthesis incubation** (Step 4).

4. First strand synthesis incubation

4.1 Keeping the tubes on ice, mix thoroughly by pipetting up and down several times. Briefly spin down the samples in a microcentrifuge. Incubate the samples in a preheated thermal cycler following Program #3 (**Supplemental Table 2**).

5. Second strand cDNA synthesis

5.1 Prepare the second strand cDNA synthesis reaction on ice by assembling the components listed in **Table 5**, including the first strand reaction product from Step 4.1.

[Place **Table 5** here]

5.2 Keeping the tubes on ice, mix thoroughly by pipetting up and down several times. Incubate in a thermal cycler following Program #4 (**Supplemental Table 2**).

6. cDNA cleanup using SPRI (Solid Phase Reversible Immobilization) beads

6.1 Allow the SPRI Beads to warm to room temperature for at least 30 min before use, and then vortex SPRI Beads for approximately 30 s to resuspend.

6.2 Add 144 µL of resuspended beads to the second strand synthesis reaction (~80 µL). Mix well by pipetting up and down at least 10 times and incubate for 5 min at room temperature.

6.3 Briefly spin the tubes in a microcentrifuge and place the tubes on a magnetic rack to separate beads from the supernatant. After the solution is clear, carefully remove and discard the supernatant. Be careful not to disturb the beads, which contain DNA.

6.4 Add 180 µL of freshly prepared 80% ethanol to the tubes while on the magnetic rack. Incubate at room temperature for 30 s, and then carefully remove and discard the supernatant.

6.5 Repeat Step 6.4 once for a total of 2 washing steps.

6.6 Completely remove the residual ethanol. Leave the tubes on the magnetic rack and air dry the beads for approximately 3 min with the lid open, or until visibly dry. Do not over dry the beads, as this may result in lower recovery of DNA.

6.7 Remove the tubes from the magnet and add 53 µL 0.1x TE Buffer (included in reagent kit, see **Supplemental Table 1**) to the beads. Pipette up and down at least 10 times to mix thoroughly. Incubate for 2 min at room temperature.

6.8 Place the tubes on a magnetic rack, allowing beads to fully separate from the supernatant. Transfer 50 µL of the supernatant to clean nuclease-free PCR tubes. Be careful not to disturb the beads. This is an optional stopping point in the protocol, cDNA samples may be stored at –20 °C.

7. End repair of cDNA library

7.1 Assemble the end repair reaction on ice by assembling the components listed in **Table 6** to the second strand synthesis product from Step 6.8.

[Place **Table 6** here]

7.2 Set a pipette to 50 µL and then pipette the entire volume up and down at least 10 times to mix thoroughly. Briefly centrifuge to collect all liquid from the sides of the tubes. **It is important to mix well. The presence of a small amount of bubbles will not interfere with performance.**

7.3 Incubate the samples in a thermal cycler following Program #5 (Supplemental Table 2).

8. Adaptor ligation

8.1 Prior to setting up the ligation reaction, dilute the Adaptor in ice-cold Adaptor Dilution Buffer as shown in **Table 7**, multiplying by the required number of samples, plus 10% extra. Keep the diluted adaptor on ice.

[Place **Table 7** here]

8.2 Assemble the ligation reaction on ice by adding the components as described in **Table 8**, in the order listed, to the end prep reaction product from Step 7.3. Note that the Ligation Master Mix and Ligation Enhancer can be mixed ahead of time. This mixture is stable for at least 8 h at 4 °C. Do not premix the Ligation Master Mix, Ligation Enhancer and Adaptor prior to use in the Adaptor Ligation Step.

[Place **Table 8** here]

8.3 Set a pipette to 80 µL and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tubes. The Ligation Master Mix is very viscous. Take care to ensure adequate mixing of the ligation reaction, as incomplete mixing will result in reduced ligation efficiency. The presence of a small amount of bubbles will not interfere with performance.

8.4 Incubate following Program #6 (**Supplemental Table 2**), and then remove the ligation mixture from the thermal cycler and add 3 µL of Adaptor Processing Enzyme, resulting in a total volume of 96.5 µL.

8.5 Pipette up and down several times to mix well, and then incubate following Program #7 (**Supplemental Table 2**) before proceeding immediately to Purification of Ligation Reaction.

9. Purification of ligation reaction using SPRI neads

9.1 Allow SPRI Beads to warm to room temperature for at least 30 min before use, and then vortex SPRI Beads for approximately 30 s to resuspend.

9.2 Add 87 µL of resuspended SPRI Beads and mix well by pipetting up and down at least 10 times. Incubate for 10 min at room temperature.

9.3 Briefly spin the tubes in a microcentrifuge and place the tubes on a magnetic rack to separate beads from the supernatant. After the solution is clear (~5 min), carefully remove and discard the supernatant. Do not discard the beads.

9.4 Add 180 µL of freshly prepared 80% ethanol to the tubes while on the magnetic rack. Incubate at room temperature for 30 s, and then carefully remove and discard the supernatant. Repeat Step 9.4 once for a total of 2 washing steps.

9.5 Completely remove the residual ethanol. Leave the tubes on the magnetic rack and air dry the beads for approximately 3 min with the lid open, or until visibly dry. Do not over dry the beads, as this may result in lower recovery of DNA.

9.6 Remove the tubes from the magnet and add 17 µL of 0.1x TE buffer to the beads. Pipette up and down at least 10 times to mix thoroughly. Incubate for 2 min at room temperature, and then place the tubes on a magnetic rack, allowing beads to fully separate from the supernatant.

9.7 Transfer 15 µL of the supernatant to clean nuclease-free PCR tubes. Be careful not to disturb the beads. This is an optional stopping point in the protocol, the Adaptor-ligated DNA may be stored at –20 °C.

10. PCR enrichment of adaptor ligated DNA

10.1 Set up the PCR reaction as described in **Table 9**. A Master Mix containing the Pre-Capture PCR Master Mix and the Universal Primer can be made and added to the Adaptor ligated DNA. For multiplexed sequencing, use unique index primers for each reaction and add to each sample individually.

[Place **Table 9** here]

10.2 Mix well by gently pipetting up and down 10 times. Briefly spin the tubes in a microcentrifuge and place in a thermal cycler and perform PCR amplification using Program #8 (**Supplemental Table 2**).

11. Purification of the PCR reaction using SPRI beads

11.1 Allow SPRI Beads to warm to room temperature for at least 30 min before use, and then vortex SPRI Beads for approximately 30 s to resuspend.

11.2 Add 45 µL of resuspended beads to each PCR reaction (~50 µL). Mix well by pipetting up and down at least 10 times, before incubating for 5 min at room temperature.

11.3 Briefly spin the tubes in a microcentrifuge and place the tubes on a magnetic rack to separate beads from the supernatant. After the solution is clear (~5 min), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA.

11.4 Add 180 µL of freshly prepared 80% ethanol to the tubes while in the magnetic rack. Incubate at room temperature for 30 s, and then carefully remove and discard the supernatant. Repeat Step 11.4 once for a total of 2 washing steps.

11.5 Completely remove the residual ethanol. Leave the tubes on the magnetic rack and air dry the beads for approximately 3 min with the lid open, or until visibly dry. Do not over dry the beads, as this may result in lower recovery of DNA.

11.6 Remove the tubes from the magnet and add 23 µL 0.1x TE Buffer to the beads. Pipette up and down at least 10 times to mix thoroughly. Incubate for 2 min at room temperature.

11.7 Place the tubes on a magnetic rack, allowing beads to fully separate from the supernatant. Transfer 20 µL of the supernatant to clean nuclease-free PCR tubes. Be careful not to disturb the beads. This is an optional stopping point in the protocol, Pre-Capture Libraries may be stored at –20 °C.

12. Validate and quantify pre-capture library

12.1 Measure the concentration of the pre-capture library using a fluorometer and high sensitivity assay kit. A minimum yield of 200 ng is required to proceed to Part II: Hybridization and Capture.

12.1.1 Run 1 µL of library on a digital electrophoresis system. If necessary, dilute the sample to avoid overloading the High Sensitivity Chip, according to the manufacturer's protocol recommendations.

12.1.2 Check that the electropherogram shows a narrow distribution with a peak size approximately 250-400 bp (see Representative Results, **Figure 3** and **Figure 4**).

12.1.3 If a 128 bp peak (adaptor-dimer) is visible in the Bioanalyzer traces, and the intensity of the signal is ≥ the intensity of 250-400 bp library signal (see Representative Results, **Figure 5**), and then bring up the sample volume (from Step 11.7) to 50 µL with 0.1x TE Buffer and repeat the SPRI Bead purification (Step 11). This is an optional stopping point in the protocol, Pre-capture libraries may be stored at –20 °C before moving on to Part II: ImmunoPrism Hybridization and Capture.

Part II: Hybridization and Capture

13. Combine blocking oligos, Cot-1 DNA, pre-capture library DNA, and dry

13.1 Mix the barcoded library prepared in Step 11 and Quantified in Step 12, with Cot-1 DNA and Blocking Oligos in a nuclease-free PCR tube or 1.5 mL microtube, as shown in **Table 10**.

[Place **Table 10** here]

13.2 Dry the contents of the tube using a vacuum concentrator set to 30-45 °C. This is an optional stopping point in the protocol. After drying, tubes may be stored overnight at room temperature (15–25 °C) or for longer at -20 °C.

14. Hybridize DNA capture probes with the library

14.1 Thaw 2x Bead Wash Buffer and Hybridization Buffer, Hybridization Buffer Enhancer, ImmunoPrism Probe Panel, 10x Wash Buffer 1, 10x Wash Buffer 2, 10x Wash Buffer 3, and 10x Stringent Wash Buffer at room temperature. Before use, inspect the Hybridization Buffer for crystallization of salts. If crystals are present, heat the tube at 65 °C, shaking intermittently, until the buffer is completely solubilized.

14.2 At room temperature, create the Hybridization Master Mix in a tube. Multiply volumes by the number of samples and add 10% extra, following **Table 11**.

[Place **Table 11** here]

14.3 Vortex or pipette up and down to mix well. Then, add 17 µL of the Hybridization Master Mix to each tube containing dried DNA. Seal the tubes and incubate for 5 min at room temperature.

14.4 Vortex the samples, ensuring they are completely mixed, and spin down the samples briefly in a microcentrifuge. If applicable, transfer each sample from a 1.5 mL microtube to a nuclease-free PCR tube.

14.5 Place the samples in a thermal cycler and run Program #9 (**Supplemental Table 2**).

14.5.1 During the incubation, prepare the wash buffers (Step 15) and streptavidin beads (Step 16), allowing for sufficient time to preheat buffers and equilibrate the streptavidin beads.

15. Prepare wash buffers

NOTE: Wash buffers are supplied as 2x (Bead Wash Buffer) or 10x (all other wash buffers) concentrated solutions.

15.1 During the Hybridization incubation, dilute the 2x Bead Wash Buffer and the 10x Wash Buffers to create 1x working solutions, multiplying by the required number of samples and adding 10% extra, following **Table 12**. If 10x Wash Buffer 1 is cloudy, heat the bottle in a 65 °C water bath or heating block to resuspend particulates. Frozen 1x Wash Buffers should be mixed after thawing.

[Place **Table 12** here]

15.2 Aliquot the 1x Wash Buffers into nuclease-free PCR tubes and place at the appropriate temperatures as indicated in **Table 13**. Be sure to include sufficient overage for pipetting. For heated buffers, use a thermal cycler set to 65 °C with the lid set to 70 °C.

[Place **Table 13** here]

15.3 Prepare the Bead Resuspension Mix at room temperature as shown in **Table 14**, multiplying by the required number of samples and adding 10% extra.

[Place **Table 14** here]

16. Prepare the streptavidin beads

16.1 Equilibrate streptavidin beads at room temperature for at least 30 min before use. Mix the beads thoroughly by vortexing for 15 s and aliquot 50 µL of beads per capture into a nuclease-free PCR tube.

16.2 Add 100 µL of 1x Bead Wash Buffer (prepared in Step 15.1) to each tube. Gently pipette up and down 10 times to mix. Place the tube on a magnetic rack, allowing beads to fully separate from the supernatant.

16.3 Remove and discard the clear supernatant. Be careful not to disturb the beads.

16.4 Perform the following wash.

16.4.1 Remove from magnetic rack. Add 100 µL of 1x Bead Wash Buffer to each tube containing beads, and then pipette up and down 10 times to mix.

16.4.2 Place the tube in the magnetic rack, allowing beads to fully separate from the supernatant.

16.4.3 Carefully remove and discard the clear supernatant.

16.5 Repeat Step 16.4 once for a total of two washes.

16.6 Remove from magnetic rack. Add 17 µL of Bead Resuspension Mix from Step 15.3 to each tube. Pipette up and down several times to thoroughly mix. Ensure that beads are not stuck to the sides of the tubes. If needed, briefly spin the tubes to collect the beads at the bottom.

17. Bind hybridized target to the streptavidin beads

17.1 After the 4 hour Hybridization incubation is complete, remove the samples from the thermal cycler and set the thermal cycler to incubate at 65 °C with the heated lid set to 70 °C.

17.2 Using a multichannel pipette, transfer 17 µL of fully homogenized beads to the samples. Mix thoroughly by pipetting up and down 10 times.

17.3 Bind the DNA to the beads by placing the tubes into the thermal cycler following Program #10 (**Supplemental Table 2**). During the incubation, briefly remove the strip tubes every 10-12 min and gently vortex for 3 s to ensure that the beads remain in suspension. Alternatively, mix by pipetting up and down several times. Proceed immediately to Wash Streptavidin Beads (Step 18).

18. Wash streptavidin beads to remove unbound DNA

18.1 Use the 1x Wash Buffers from Step 15.2 and store heated buffers in the thermal cycler during washes.

18.2 Add 100 µL preheated 1x Wash Buffer 1 to the tubes from Step 17.3. Mix thoroughly by pipetting up and down 10 times. Place the tubes on a magnetic rack, allowing beads to fully separate from the supernatant.

18.3 Pipette and discard the supernatant, which contains unbound DNA. Remove from magnetic rack.

18.4 Perform the following 65 °C wash.

18.4.1 Add 150 µL of preheated 1x Stringent Wash Buffer.

18.4.2 Mix thoroughly by pipetting up and down at least 10 times. Avoid bubbles during pipetting. **Be sure beads are completely resuspended in all tubes.**

18.4.3 Incubate in the thermal cycler at 65 °C for 5 min.

18.4.4 Place the tubes on a magnetic rack, allowing beads to fully separate from the supernatant. Pipette and discard the supernatant, which contains unbound DNA. Remove from magnetic rack.

18.4.5 Repeat Step 18.4 for a total of two Stringent Washes.

18.5 Perform the first room temperature wash.

18.5.1 Add 150 µL of room temperature 1x Wash Buffer 1.

18.5.2 Pipette up and down 10 to 20 times to completely resuspend the beads.

18.5.3 Seal the tubes and incubate for 2 min, alternating between gently vortexing for 30 s and resting for 30 seconds. **Be sure beads in all wells remain completely resuspended in all tubes throughout the entire incubation.**

18.5.4 Briefly centrifuge the tubes.

18.5.5 Place the tubes on a magnetic rack, allowing beads to fully separate from the supernatant. Pipette and discard the supernatant.

18.5.6 Seal the tubes and briefly centrifuge. Return to magnetic rack and use a 10 µl pipette to remove any residual wash buffer.

18.6 Perform the second room temperature wash.

18.6.1 Add 150 µL of room temperature 1x Wash Buffer 2.

18.6.2 Pipette up and down 10 to 20 times to completely resuspend the beads.

18.6.3 Seal the tubes and incubate for 2 min, alternating between gently vortexing for 30 s and resting for 30 seconds. **Be sure beads in all wells remain completely resuspended in all tubes throughout the entire incubation.**

18.6.4 Briefly centrifuge the tubes.

18.6.5 Transfer the entire volume of beads resuspended in Wash Buffer 2 to clean nuclease-free PCR tubes. **Important: Transferring the beads to fresh tubes is important to avoid off-target contamination.**

18.6.6 Place the tubes on a magnetic rack, allowing beads to fully separate from the supernatant. Pipette and discard the supernatant.

18.6.7 Seal the tubes and briefly centrifuge. Return to magnetic rack and use a 10 µl pipette to remove any residual wash buffer.

18.7 Perform the third room temperature wash.

18.7.1 Add 150 µL of room temperature 1x Wash Buffer 3.

18.7.2 Pipette up and down 10 to 20 times to completely resuspend the beads.

18.7.3 Seal the tubes and incubate for 2 min, alternating between gently vortexing for 30 s and resting for 30 seconds. **Be sure beads in all wells remain completely resuspended in all tubes throughout the entire incubation.**

18.7.4 Briefly centrifuge the tubes.

18.7.5 Place the tubes on a magnetic rack, allowing beads to fully separate from the supernatant. Pipette and discard the supernatant.

18.7.6 Seal the tubes and briefly centrifuge. Return to magnetic rack and use a 10 µL pipette to remove any residual wash buffer.

18.7.7 Remove from the magnetic rack and add 20 µL of nuclease-free water to the beads.

18.7.8 Pipette up and down 10 times to ensure any beads stuck to the side of the tubes have been resuspended.

18.8 Important: Do not discard the beads. Use the entire 20 µL of resuspended beads with captured DNA in Step 19.

19. Perform final, post-capture PCR enrichment

19.1 Prepare the Post-Capture PCR Master Mix according to the following table, multiplying by the required number of samples and adding 10% extra, according to **Table 15**.

19.2 Add 30 µL of the Post-Capture PCR Master Mix to each sample for a final reaction volume of 50 µL. Mix thoroughly by pipetting up and down 10 times.

19.3 Place the PCR tubes in the thermal cycler and incubate following Program #11 (**Supplemental Table 2**).

20. Purify post-capture PCR fragments

20.1 Allow SPRI Beads to warm to room temperature for at least 30 min before use, and then vortex SPRI Beads for approximately 30 s to resuspend.

20.2 Add 75 µL of resuspended beads to each PCR-enriched capture (50 µL). Mix well by pipetting up and down at least 10 times. The streptavidin beads will not interfere with the SPRI bead purification. Incubate for 5 min at room temperature.

20.3 Briefly spin the tubes in a microcentrifuge and place the tubes on a magnetic rack to separate beads from the supernatant. After the solution is clear, carefully remove and discard the supernatant. Be careful not to disturb the beads, which contain DNA.

20.4 Add 180 µL of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 s, and then carefully remove and discard the supernatant.

20.5 Repeat Step 20.4 once for a total of 2 washing steps.

20.6 Completely remove the residual ethanol. Leave the tube on the magnetic rack and air dry 3 min with the lid open, or until visibly dry. Do not over-dry the beads. This may result in lower recovery of DNA.

20.7 Remove the tube from the magnet. Elute the DNA from the beads by adding 22 µL of 0.1x TE Buffer. Mix well by pipetting up and down several times. Incubate for 2 min at room temperature. Place the tube on the magnetic rack until the solution is clear.

20.8 Remove 20 µL of the supernatant and transfer to a clean nuclease-free PCR tube, being careful not to disturb the beads. This is an optional stopping point in the protocol, libraries may be stored at –20 °C.

21. Validate and quantify library

21.1 Measure the concentration of the captured library using a fluorometer and High Sensitivity Assay Kit.

21.2 Measure the average fragment length of the captured library using a digital electrophoresis High Sensitivity DNA chip and calculate the average fragment size for each library using the system software. Average fragment size should be approximately 250-400 bp (see Representative Results, **Figure 6** and **Figure 7**). This is an optional stopping point in the protocol, completed libraries may be stored at –20 °C.

22. Sequencing on a sequencing platform

22.1 For sequencing, dilute libraries to 2 nM and follow the manufacturer's guidelines for loading and operating the sequencer. Sequence libraries to a minimum depth of 15 million single end reads of at least 50 bp in length.

23. Analysis of sequencing data to generate immune profiles and discover biomarkers with the Prism Portal, a cloud-based informatics tool

23.1 Create a Prism account by visiting https://prism.cofactorgenomics.com/

23.2 Once logged in, click **Submit New Project** in the top toolbar from any page in Prism to upload the demultiplexed FASTQ sequencing files, or upload files stored on BaseSpace with the Prism account.

23.3 Complete the New Project form including the project name, and samples by group or cohort. The grouping of samples, and the corresponding grouping names, are necessary to generate the Biomarker Discovery Report. Note that a minimum of 3 samples per group are required to generate the Biomarker Discovery Report. Click the **Launch Application** button to submit the form; a confirmation page will appear if successful.

23.4 While logged in, click **See Results** in the top toolbar or any page of Prism. Prism enables a user to see the status of submitted projects and to view sample and biomarker reports per project. There will be a table of projects the user has created on Prism. The table has three columns for the status, name, and the date of submission.

NOTE: The status of each project can be:

- "Running", where the project analysis is currently running, or,
- "Success", where the project analysis is complete and reports are available.

23.5 If a project has finished analysis (indicated by a "Success" status), view the Individual Sample Reports and a Biomarker Discovery Report. Note that the Biomarker Discovery Report will only be available if the project includes the required minimum of three samples per group.

23.5.1 To access these reports, return to the table of projects and click on the name of the project. On this project page, there will be a table with a row for each sample in the project. Click the link in each row, under the **Report** column, to access the Individual Report of each sample. Immediately below the table, click the link for the Biomarker Discovery Report. If no links are in this page, your project has not completed analysis.

REPRESENTATIVE RESULTS:

There are a number of checkpoints throughout the protocol that enable a user to evaluate the quality and quantity of generated materials. Following Step 12 described in the protocol, an electropherogram is generated as shown in **Figure 3**, representative of a typical pre-capture library for an intact RNA sample (RIN = 7.8).

[Place **Figure 3** here]

Care should be taken to avoid overamplification, as indicated by the second peak around 1000 bp shown in **Figure 4**, a representative electropherogram of a pre-capture library generated from an FFPE RNA sample (DV_{200} = 46). If this peak is small relative to the main peak (around 250-400 base pairs (bp), as shown), it will not interfere with downstream steps or analysis. If the second peak is large relative to the 250-400 bp peak, the pre-capture library can be remade with fewer PCR cycles in order to reduce overamplification.

[Place **Figure 4** here]

As described in Step 12.1.3, the presence of adaptor dimers should be evaluated to determine if additional cleanup is necessary. The electropherograms shown in **Figure 5** are representative of unacceptable (**Figure 5A**, DV_{200} = 33) and acceptable (**Figure 5B**, DV_{200} = 46) levels of adaptor dimer, appearing as the sharp peak around 128 bp.

[Place **Figure 5** here]

At the completion of the protocol, prior to sequencing, the final libraries are again evaluated using digital electrophoresis. Libraries made from FFPE RNA tend to have a smaller average size distribution than libraries made from intact RNA. For intact RNA samples, the resulting trace should look similar to **Figure 6** (RIN = 9.5). For degraded or FFPE RNA, the resulting trace should look similar to **Figure 7** (DV_{200} = 36).

[Place **Figures 6 & 7** here]

As described, the results generated with this protocol may be applied in two key ways, as shown in **Figure 8**.

[Place **Figure 8** here]

To demonstrate each of these use cases, representative data from a small translational study is included[21]. The samples used in this study are a set of specimens from 7 patients diagnosed and treated for non-small cell lung cancer (NSCLC). The samples are patient-matched solid tumor tissue from pre and post treatment biopsies. First, individual samples were analyzed to generate an immune profile, such as the example report shown in **Figure 9**.

[Place **Figure 9** here]

The immune profiles pre- and post-treatment may be used to understand how a therapy (chemotherapy or radiation, in this study) has modified the tumor microenvironment. An example is shown in **Figure 10**, where the changes in percentage for each immune cell and total immune content are shown pre- and post chemotherapy, for a single patient.

[Place **Figure 10** here]

Patients may be grouped by criteria such as clinical outcomes or phenotypes for comparison. For example, in **Figure 11**, the samples in the NSCLC study were compared according to time to disease progression following treatment. A subset of the patients showed disease recurrence in >18 months, and another subset progressed faster, in ≤18 months. The median delta value (difference between pre- and post-treatment values) are compared for each sample to identify putative biomarkers of disease progression.

[Place **Figure 11** here]

Finally, similar sample groupings may be used to look specifically at pre-treatment samples to identify predictive biomarkers by using the Prism Portal to generate a Biomarker Report. Shown in **Figure 12**, the same clinical phenotype (disease progression) as described above defines the sample groupings. In this example, two immune escape genes were identified as statistically significant differentiators of the sample groupings (CD47 and OX40, shown in the lower panel of **Figure 12A**). In this example, because the individual gene biomarkers are robust with clear statistical significance, the multidimensional biomarker does not add significant predictive value (ImmunoPrism, as labeled in the top right bar chart of **Figure 12B**). The full table of data, including results for all 18 analytes for the assay, is summarized on the reverse side of the report, including statistical analysis and a brief methods summary.

[Place **Figure 12** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Overview of Workflow. In this protocol, RNA is first converted to cDNA. Sequencing adaptors are ligated, and adaptor-ligated cDNA is amplified and barcoded by PCR to create a pre-capture library. Biotinylated probes are then hybridized to specific cDNA targets which are then captured using streptavidin beads. Unbound, non-targeted cDNA is removed by washing. A final PCR enrichment yields a post-capture library ready for sequencing. *Total RNA must be from human samples; may be intact or degraded (FFPE) RNA.

Figure 2: Representative Immune Reports. The workflow generates two reports, an individual immune report (A) for each sample processed, and a biomarker report (B) for defined patient cohorts.

Figure 3: Typical Pre-capture Library Bioanalyzer trace for an intact RNA sample. Pre-capture libraries appear as a broad peak around 250-400 base pairs (bp) in size.

Figure 4: Typical Pre-capture Library Bioanalyzer trace for an FFPE RNA sample. The second peak around 1000 bp is indicative of over-amplification. If this peak is small relative to the main peak around 250-400 bp (as shown), it will not interfere with downstream steps or analysis. If the second peak is large relative to the 250-400 bp peak, the pre-capture library can be remade with fewer PCR cycles in order to reduce over-amplification.

Figure 5: Pre-capture library Bioanalyzer traces. The adaptor dimer shows up as a sharp peak around 128 bp. (**A**) Excessive adaptor dimers are present in this electropherogram. (**B**) Acceptable adaptor dimer levels are depicted in this trace. Both traces show evidence of mild over-amplification, but this should not interfere with the ImmunoPrism Assay.

Figure 6: Typical Final Library Bioanalyzer trace for an intact RNA sample.
Final libraries appear as a broad peak around 250-400 base pairs (bp) in size.

Figure 7: Typical Final Library Bioanalyzer trace for an FFPE RNA sample.
Libraries made from FFPE RNA tend to have a smaller average size distribution than libraries made from intact RNA.

Figure 8: Two use cases of the protocol. The results generated by this immune profiling assay are applied in two key translational applications. (**A**) The first use case starts from human solid tumor tissue (including FFPE archives) and generates an individual immune profile for the sample. (**B**) Once generated for a cohort of human samples, the data is combined using the Prism Portal to generate a multidimensional biomarker and corresponding Biomarker Report.

Figure 9: Example individual immune report for a NSCLC sample. The Prism Portal pipeline generates a graphical report for each sample processed, with a representative report generated for a NSCLC solid tumor sample shown here. (**A**) The front side of the report graphically depicts

the breakdown of immune cells present in the RNA sample extracted from the FFPE tissue. (**B**) The reverse side of the report includes a table of immune cells (in absolute percentages) and escape gene expression (in transcripts per million, or TPM), as well as a statement of performance for the assay.

Figure 10: Example Pre and Post Treatment Results. Individual immune cell and total immune content data generated from pre- and post-treatment samples from a single NSCLC patient are shown. In this example, the patient received a chemotherapy regimen as treatment.

Figure 11: Example Clinical Outcome Comparison. Quantitative changes between the immune cell percentages in matched pre and post-treatment NSCLC samples were calculated and reported as the "delta" value. Those highlighted in yellow show clear signal changes between the survival status. Blue bars represent median delta values for >18 months until disease progression, orange bars represent median delta values for ≤18 months until disease progression.

Figure 12: Example Biomarker Report for NSCLC samples. The Biomarker Discovery pipeline delivers a visual report of individual biomarkers, and a machine-learning multidimensional biomarker, with detailed statistics. (**A**) For this study, the pipeline identified two individual biomarkers (CD47 and OX40) as statistically-significant for defining disease progression with a threshold of 18 months. (**B**) Details on the method and full results are included on the reverse side of the report.

Table 1: Fragmentation and priming reaction for high-quality RNA. Components of the fragmentation and priming reaction for high-quality RNA should be assembled and mixed on ice according to the volumes shown. A master mix of First Strand Synthesis Reaction Buffer and Random Primers can be made and added to the RNA samples.

Table 2: Random priming reaction for highly degraded RNA. Components of the priming reaction for highly degraded RNA should be assembled on ice in a nuclease-free PCR tube.

Table 3: First Strand Synthesis reaction for high-quality RNA. Components of the fragmentation and priming reaction for high quality RNA should be assembled and mixed on ice according to the volumes given. A master mix of First Strand Synthesis Specificity Reagent and First Strand Synthesis Enzyme Mix can be made and added to the fragmented and primed RNA samples.

Table 4: First Strand Synthesis reaction for highly degraded RNA. Components of the fragmentation and priming reaction for highly degraded RNA should be assembled and mixed on ice according to the volumes shown. A master mix of First Strand Synthesis Reaction Buffer, First Strand Synthesis Specificity Reagent, and First Strand Synthesis Enzyme Mix can be made and added to the primed RNA samples.

Table 5. Second Strand Synthesis reaction. Components of the second strand cDNA synthesis reaction should be assembled and mixed on ice according to the volumes shown. A master mix of the Second Strand Synthesis Reaction Buffer, Second Strand Synthesis Enzyme Mix, and

Nuclease-free Water can be made and added to the First Strand Synthesis Product.

Table 6. End Repair reaction. Components of the end repair reaction should be assembled and mixed on ice according to the volumes shown. A master mix of the End Repair Reaction Buffer and the End Repair Enzyme Mix can be made and added to the Second Strand Synthesis Product.

Table 7. Adaptor Dilution. The adaptor should be diluted on ice with adaptor dilution buffer according to the volumes shown.

Table 8. Ligation reaction. Components of the adaptor ligation reaction should be assembled on ice according to the volumes shown in the order shown. A master mix of Ligation Enhancer and Ligation Master Mix can be made and added to the End Prepped DNA with Diluted Adaptor. Do not mix the diluted Adaptor and the Ligation Master Mix or Ligation Enhancer prior to mixing the with the End Prepped DNA.

Table 9. PCR enrichment of adaptor ligated DNA. Components of the PCR enrichment of adaptor ligated DNA reaction should be assembled and mixed on ice according to the volumes shown. A master mix of the Pre-Capture PCR Master Mix and the Universal PCR Primer can be made and added to the adaptor ligated DNA. For multiplexed sequencing, each sample should be given a unique Index Primer.

Table 10. Hybridization Preparation and drying down. Components to be combined for drying down of libraries in preparation of hybridization should be assembled according to the quantities shown.

Table 11. Hybridization Master Mix. Components of Hybridization Master Mix should be assembled and mixed at room temperature according to the volumes shown.

Table 12. Wash Buffer Dilution. The concentration wash buffers should be diluted with nuclease-free water at room temperature according to the volumes shown.

Table 13. Diluted Wash Buffers. The diluted wash buffers should be aliquoted into separate tubes according to the volumes and number of tubes per sample shown. Wash buffers must be held at the indicated temperature before use.

Table 14. Bead Resuspension Mix. Components of Bead Resuspension Mix should be assembled and mixed at room temperature according to the volumes shown.

Table 15. Post-Capture PCR Master Mix. Components of Post-Capture PCR Master Mix should be assembled and mixed on ice according to the volumes shown.

Supplemental Table 1. Reagent Kit Materials. A list of materials provided in the ImmunoPrism Kit are listed, along with the part numbers that referenced in the manufacturer's protocol. All other equipment and materials required are listed in the Table of Materials.

Supplemental Table 2. Thermal Cycler Programs. The recommended cycler programs referenced throughout the protocol are summarized for ease of programming.

Supplemental Table 3. Sequencing Index Guide. The index primers provided in the reagent kit are listed; a unique primer is added to each reaction for post-sequencing demultiplexing. Recommended low-level multiplexing combinations are also provided.

DISCUSSION:

The protocol requires 20 ng intact or 40 ng highly degraded (FFPE) RNA. The RNA sample should be free of DNA, salts (e.g., Mg^{2+}, or guanidinium salts), divalent cation chelating agents (e.g., EDTA, EGTA, citrate), or organics (e.g., phenol and ethanol). It is not recommended to proceed with RNA samples that have a DV_{200} <20%. Use of the in-kit control RNA is strongly recommended as these controls provide a means to evaluate performance throughout the entire protocol, from library preparation to analysis.

The protocol is designed to be performed using 0.2 mL PCR strip tubes. If preferred, the protocol can also be performed using the wells in a 96-well PCR plate. Simply use the wells of a 96-well PCR plate in place of all references to PCR tubes or strip tubes. Use PCR plates with clear wells only, as it is critical to visually confirm complete resuspension of beads during bead purifications and wash steps.

Throughout the protocol, keep reagents frozen or on ice unless otherwise specified. Do not use reagents until they are completely thawed. Be sure to thoroughly mix all reagents before use. Keep enzymes at -20 °C until ready to use and return to -20 °C promptly after use. Use only molecular-grade nuclease-free water; it is not recommended to use DEPC-treated water. When pipetting to mix, gently aspirate and dispense at least 50% of the total volume until the solutions are well mixed. Pipette mix all master mixes containing enzymes. Using vortex to mix the enzymes could lead to denaturation and compromise their performance. During bead purifications, use freshly made 80% ethanol solutions from molecular grade ethanol. Using ethanol solutions that are not fresh may result in lower yields. Avoid over drying the beads, as this can reduce elution efficiency (beads look cracked if over dried).

As described in Step 10, unique index primers are added to each reaction. Based on the sequences of these indices, for low-level multiplexing, certain index combinations are optimal. The sequences of these indices are required for demultiplexing the data post-sequencing. The sequences and recommended multiplexing combinations are provided in **Supplemental Table 3**. In this same step, it is important to note that the number of recommended PCR cycles varies depending on the quality of RNA used, and, some optimization may be required to prevent PCR over-amplification. For the ImmunoPrism Intact Control RNA and other high-quality RNA, start optimization with 10 PCR cycles. For the ImmunoPrism FFPE Control RNA and other highly degraded/FFPE RNA, start optimization with 15 PCR cycles. Producing a test library using RNA representative of the material to be analyzed in order to optimize PCR cycles is recommended. The minimum number of PCR cycles that consistently yield sufficient pre-capture library yields

(>200 ng) should be used. A secondary peak around 1000 bp on the Bioanalyzer trace is indicative of over-amplification (**Figure 4**). Over-amplification should be minimized, but the presence of a small secondary peak will not interfere with assay results.

To minimize sample loss and avoid switching tubes, Step 13 may be performed in PCR tubes, strip tubes, or a 96-well PCR plate instead of 1.5 mL microtubes, if your vacuum concentrator allows. The rotor can be removed on many concentrators. This enables the strip tubes or plates to fit in the vacuum. The vacuum concentration can then be run using the aqueous desiccation setting with no centrifugation. Consult the manual for your vacuum concentrator for instructions. If the samples are dried down in strip tubes or a 96-well plate, the hybridization step can be performed in the same vessel.

During Step 17, be sure to vortex every 10-12 min to increase the bead capture efficiency. Carefully hold the caps of the warm strip tubes when mixing to prevent tubes from opening.

The washes described in Step 18 are critical to avoid high nonspecific contamination and must be followed closely. Be sure to completely resuspend the beads at each wash, completely remove the wash buffers, and during the Wash Buffer 2 wash, transfer the samples to a fresh strip tube (Step 18.6.5). Ensure that the streptavidin beads are completely resuspended and remain in suspension during the entire incubation. Splashing on the tube caps will not negatively impact the capture. During the room temperature washes, a microplate vortex mixer may be used to vortex the samples for the entirety of the two-minute incubation period for easier resuspension. Do not let the streptavidin beads dry out. If needed, extend incubations in the buffers to avoid drying the beads. If using more than one strip tube, work with one strip tube at a time for each wash while the other strip tubes sit in the thermocycler. This can help avoid over drying the beads or rushing, resulting in poor resuspension or other sub-optimal techniques. For first time users, it is not recommended to process more than 8 library reactions at a time.

Current immune profiling techniques deliver a continuum of information – from thousands of data points that require significant interpretation (RNA sequencing) to an individual, discrete data point (single-plex IHC). The protocol described here represents an approach that is somewhere in the middle, with a focused scope enabling high sensitivity, but capturing only a subset of clinically relevant transcriptomic data. Due to the nature of bulk RNA extraction, this protocol does not provide information about the spatial relationships between immune cells and the tumor microenvironment, however, results may be complemented with imaging technologies to add this information. There are a myriad of applications for the data generated by this protocol, as there is much to be learned about biology of cancer as a disease, and the therapies being developed to treat it. As shown in the representative results, the individual immune report is useful for understanding how a patient's immune profile may change in response to events such as disease progression or treatment. While the results presented here provide some example use cases, other applications including investigating the mechanism of action of a therapy and identifying putative biomarkers of clinical outcomes such as progression free and overall survival are also practical. When using this protocol for biomarker discovery applications, it is important to practice good study design to ensure homogenous populations are

analyzed, sufficient samples are included for statistical power, and sources of bias are considered. Due to the focused, streamlined nature of the assay, it is feasible to imagine a path towards clinical validation and downstream application of these biomarkers once discovered.

ACKNOWLEDGMENTS:

The authors wish to acknowledge TriStar Technology Group for providing the biological specimens for the representative results, as well as the entire molecular, analysis, product, and commercial teams at Cofactor Genomics for their technical expertise and support.

DISCLOSURES:

All authors are employed by Cofactor Genomics, Inc. the company that developed and produces the ImmunoPrism reagent kit and informatics tools used in this article.

REFERENCES:

1 Brambilla, E. et al. Prognostic Effect of Tumor Lymphocytic Infiltration in Resectable Non-Small-Cell Lung Cancer. *Journal of Clinical Oncology.* **34** (11), 1223-1230 (2016).

2 Iacono, D. et al. Tumour-infiltrating lymphocytes, programmed death ligand 1 and cyclooxygenase-2 expression in skin melanoma of elderly patients: clinicopathological correlations. *Melanoma Research.* **28** (6), 547-554 (2018).

3 Fridman, W. H., Zitvogel, L., Sautes-Fridman, C., Kroemer, G. The immune contexture in cancer prognosis and treatment. *Nature Reviews Clinical Oncology.* **14** (12), 717-734 (2017).

4 Sierant, M. C., Choi, J. in *Genomics Inform* Vol. 16 (2018).

5 Klauschen, F. et al. Scoring of tumor-infiltrating lymphocytes: From visual estimation to machine learning. *Seminars in Cancer Biology.* **52** (Pt 2), 151-157 (2018).

6 Danaher, P. et al. Gene expression markers of Tumor Infiltrating Leukocytes. *Journal for ImmunoTherapy of Cancer.* **5**, 18 (2017).

7 Aran, D., Hu, Z., Butte, A. J. xCell: digitally portraying the tissue cellular heterogeneity landscape. *Genome Biology.* **18** (1), 220 (2017).

8 Newman, A. M. et al. Robust enumeration of cell subsets from tissue expression profiles. *Nature Methods.* **12** (5), 453-457 (2015).

9 Becht, E. et al. Estimating the population abundance of tissue-infiltrating immune and stromal cell populations using gene expression. *Genome Biology.* **17** (1), 218 (2016).

10 Newman, A. M., Gentles, A. J., Liu, C. L., Diehn, M., Alizadeh, A. A. Data normalization considerations for digital tumor dissection. *Genome Biology.* **18** (1), 128 (2017).

11 Chen, S. H. et al. A gene profiling deconvolution approach to estimating immune cell composition from complex tissues. *BMC Bioinformatics.* **19** (Suppl 4), 154 (2018).

12 Yoshihara, K. et al. Inferring tumour purity and stromal and immune cell admixture from expression data. *Nature Communications.* **4**, 2612 (2013).

13 Foley, J. W. et al. Gene-expression profiling of single cells from archival tissue with laser-capture microdissection and Smart-3SEQ. *Genome Research.* (2019).

14 Civita, P. et al. Laser Capture Microdissection and RNA-Seq Analysis: High Sensitivity Approaches to Explain Histopathological Heterogeneity in Human Glioblastoma FFPE Archived Tissues. *Front Oncol.* **9**, 482 (2019).

15 OncologyPRO. *PD-L1 in cancer: ESMO Biomarker Factsheet | OncologyPRO*,

1006 <https://oncologypro.esmo.org/Education-Library/Factsheets-on-Biomarkers/PD-L1-in-Cancer>
1007 (2019).
1008 16 Haslam, A., Prasad, V. Estimation of the Percentage of US Patients With Cancer Who Are
1009 Eligible for and Respond to Checkpoint Inhibitor Immunotherapy Drugs. *JAMA Network Open.* **2**
1010 (5), e192535 (2019).
1011 17 Maecker, H. T., McCoy, J. P., Nussenblatt, R. Standardizing immunophenotyping for the
1012 Human Immunology Project. *Nature Reviews Immunology.* **12** (3), 191-200 (2012).
1013 18 Schillebeeckx, I. et al. Analytical Performance of an Immunoprofiling Assay Based on RNA
1014 Models. Association for Molecular Pathology 2019 Annual Meeting, November 7-9, Baltimore,
1015 MD. *Journal of Molecular Diagnostics*. **21** (2019).
1016 19 Uryvaev, A., Passhak, M., Hershkovits, D., Sabo, E., Bar-Sela, G. The role of tumor-
1017 infiltrating lymphocytes (TILs) as a predictive biomarker of response to anti-PD1 therapy in
1018 patients with metastatic non-small cell lung cancer or metastatic melanoma. *Medical Oncology.*
1019 **35** (3), 25 (2018).
1020 20 Wang, K., Shen, T., Siegal, G. P., Wei, S. The CD4/CD8 ratio of tumor-infiltrating
1021 lymphocytes at the tumor-host interface has prognostic value in triple-negative breast cancer.
1022 *Human Pathology.* **69**, 110-117 (2017).
1023 21 Carney, W.P., Bhagat, M., LaFranzo, N. Multidimensional gene expression models for
1024 characterizing response and metastasis in solid tumor samples [abstract]. In: Proceedings of the
1025 American Association for Cancer Research Annual Meeting 2019; Atlanta, GA. Philadelphia (PA):
1026 AACR; *Cancer Research*. **79** (13 Suppl) (2019).
1027

Figure1

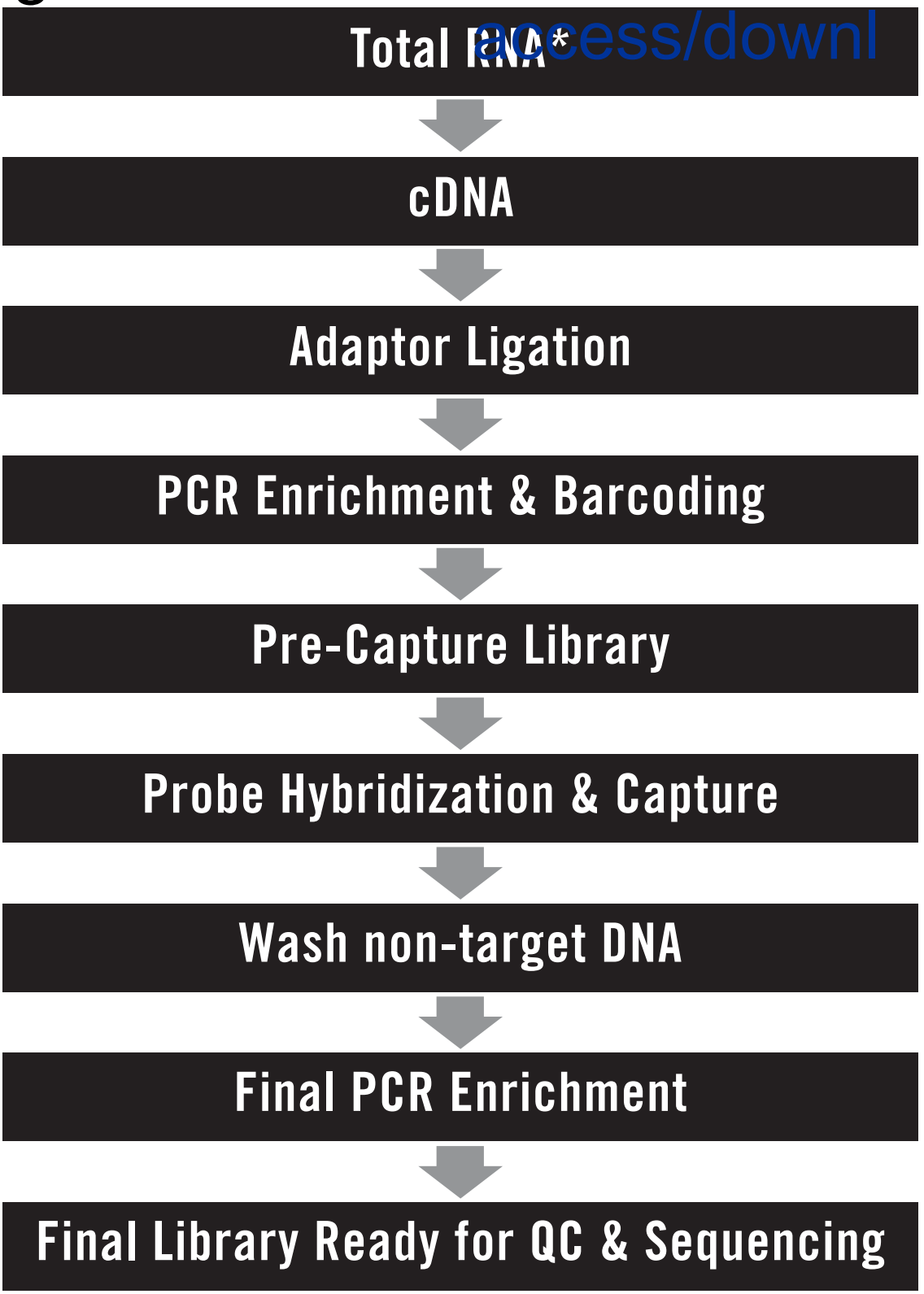
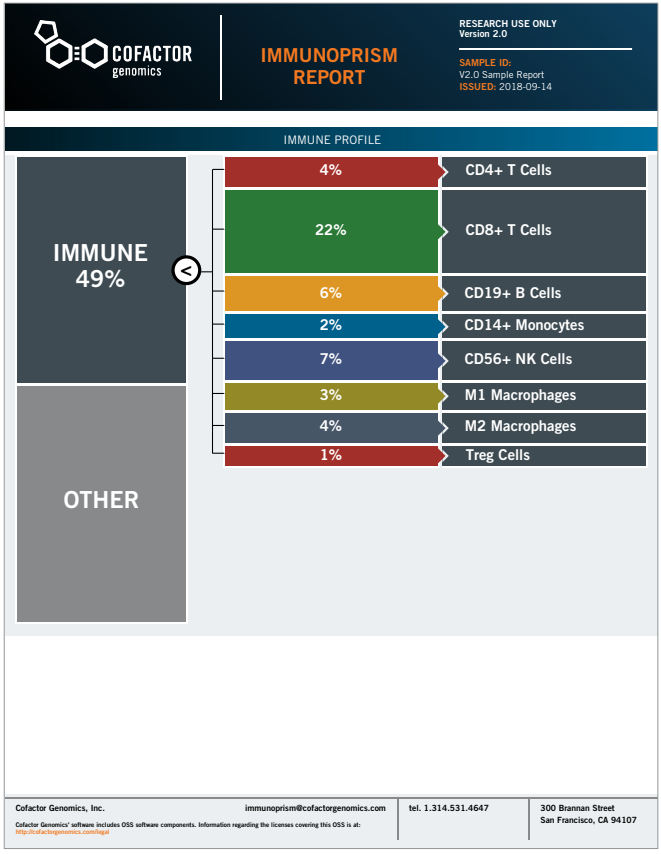


Figure2 (A)



(B)

Click here to access/download;Figure;Figure2.ai

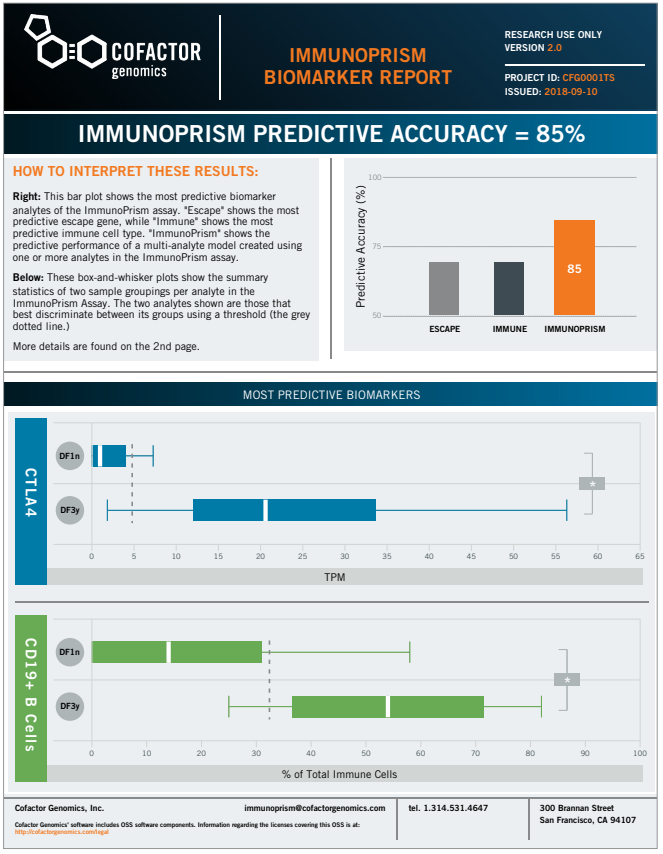


Figure3

Click here to access/download;Figure;Figure3.tif

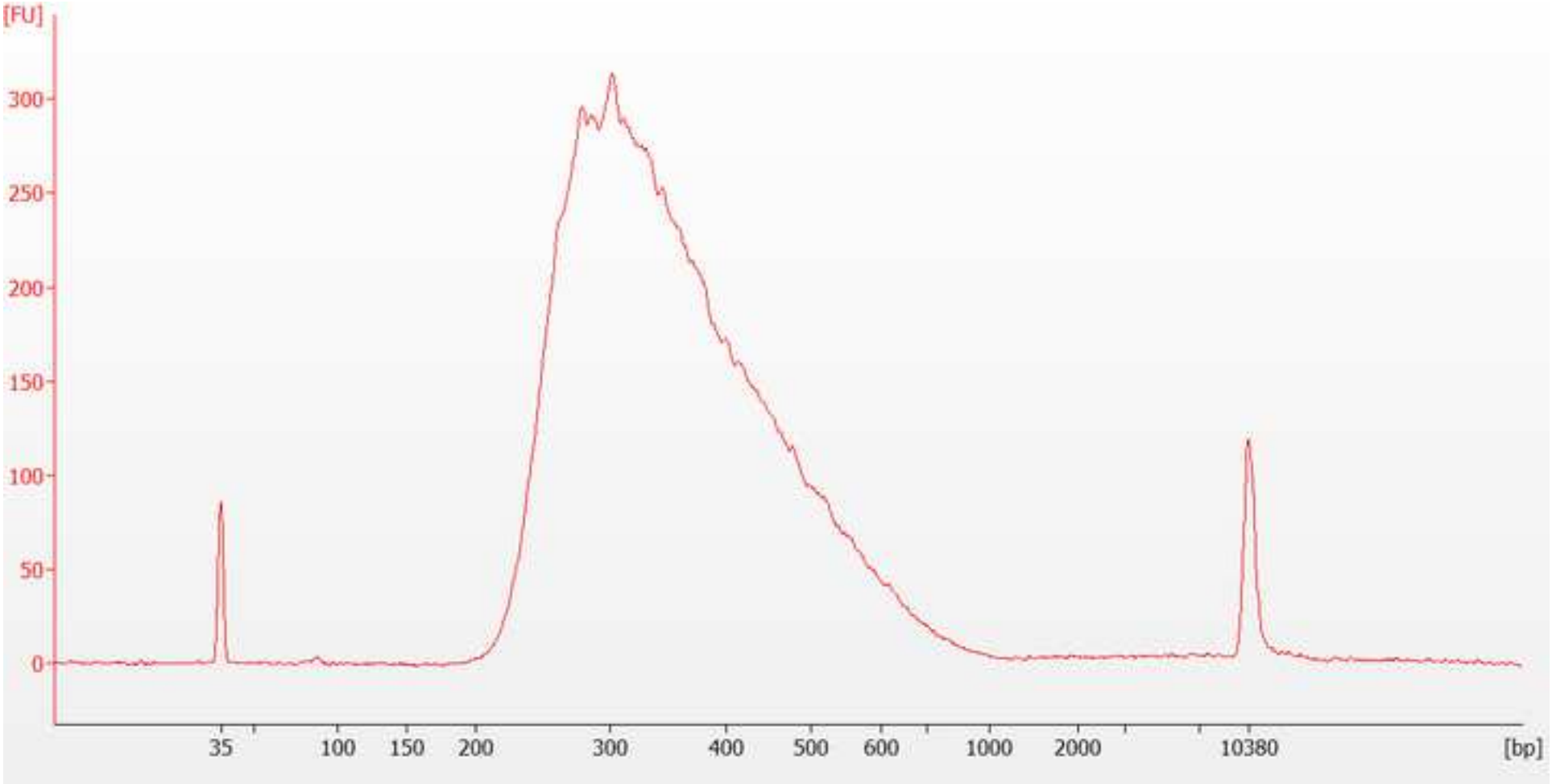
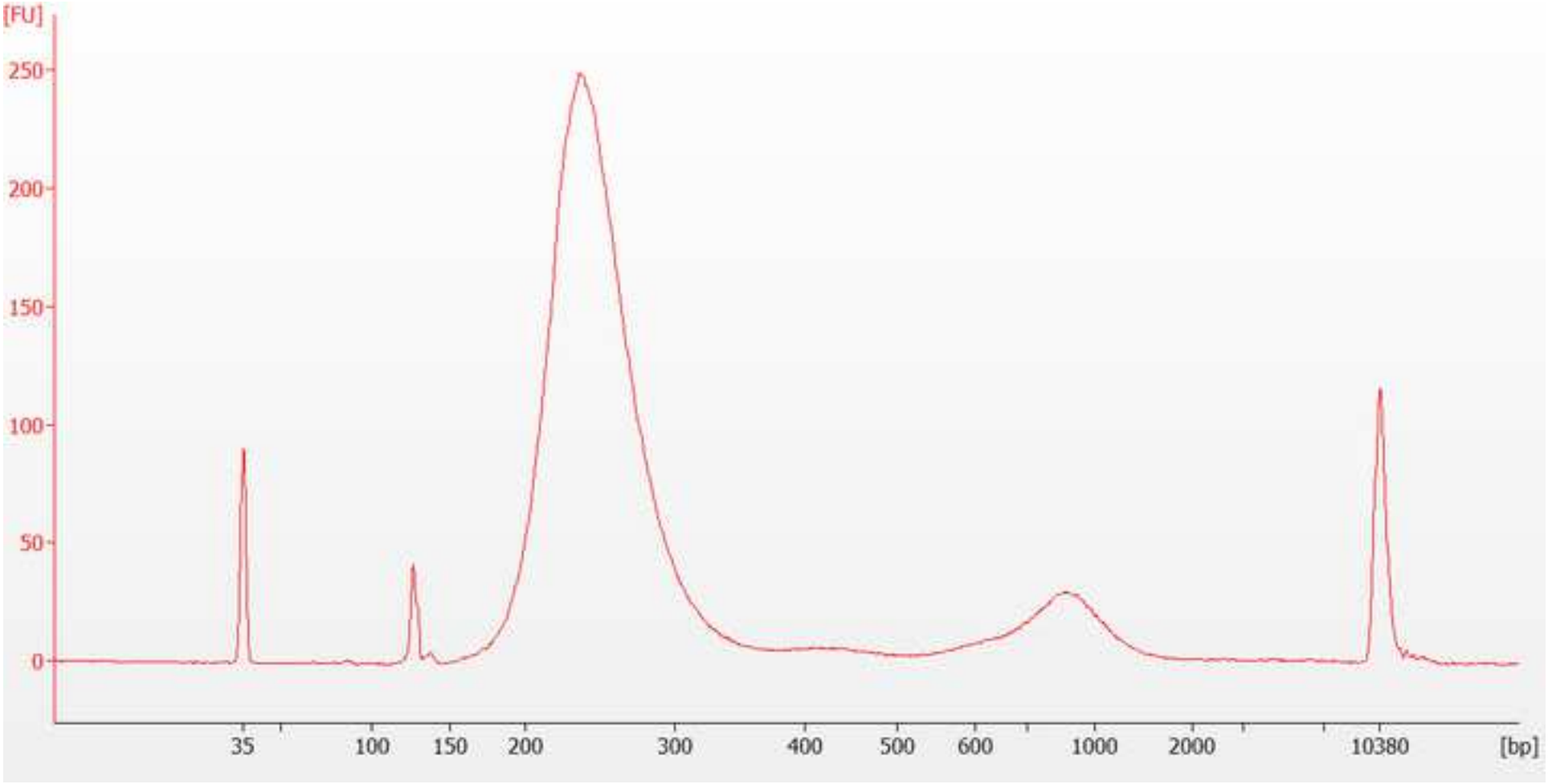
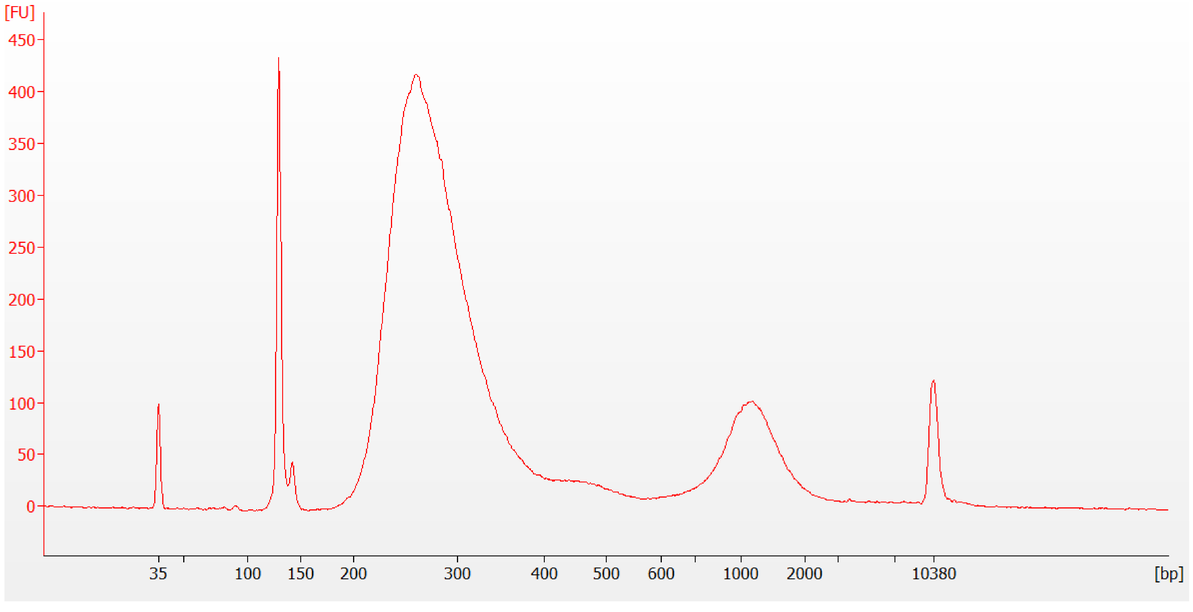


Figure4

Click here to access/download;Figure;Figure4.tiff



(A)



(B)

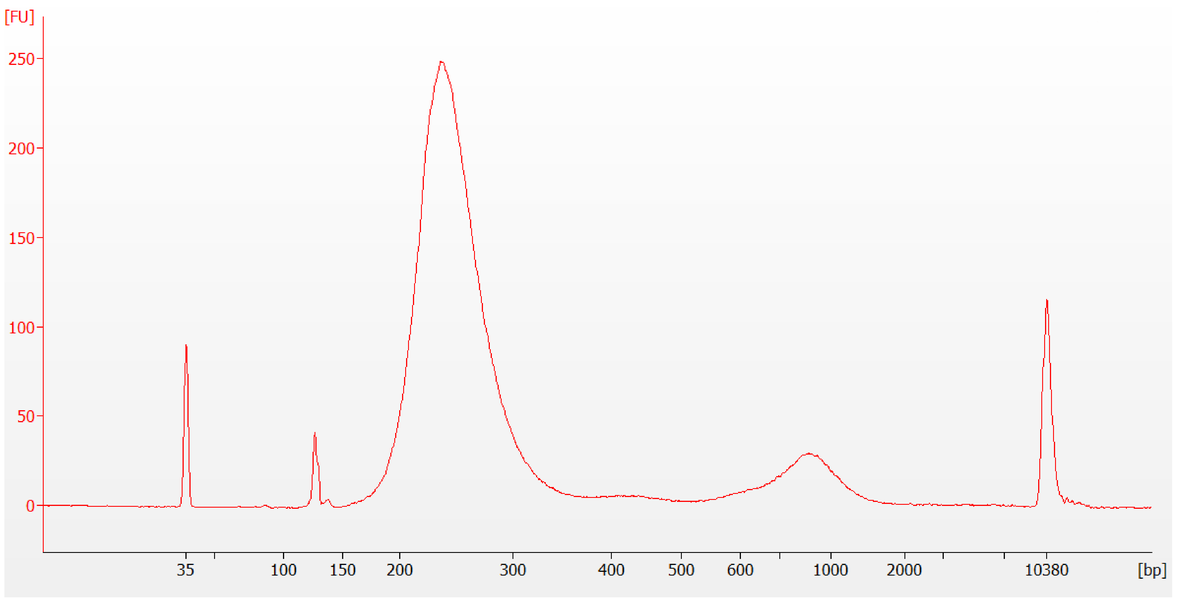


Figure6

Click here to access/download;Figure;Figure6.tif

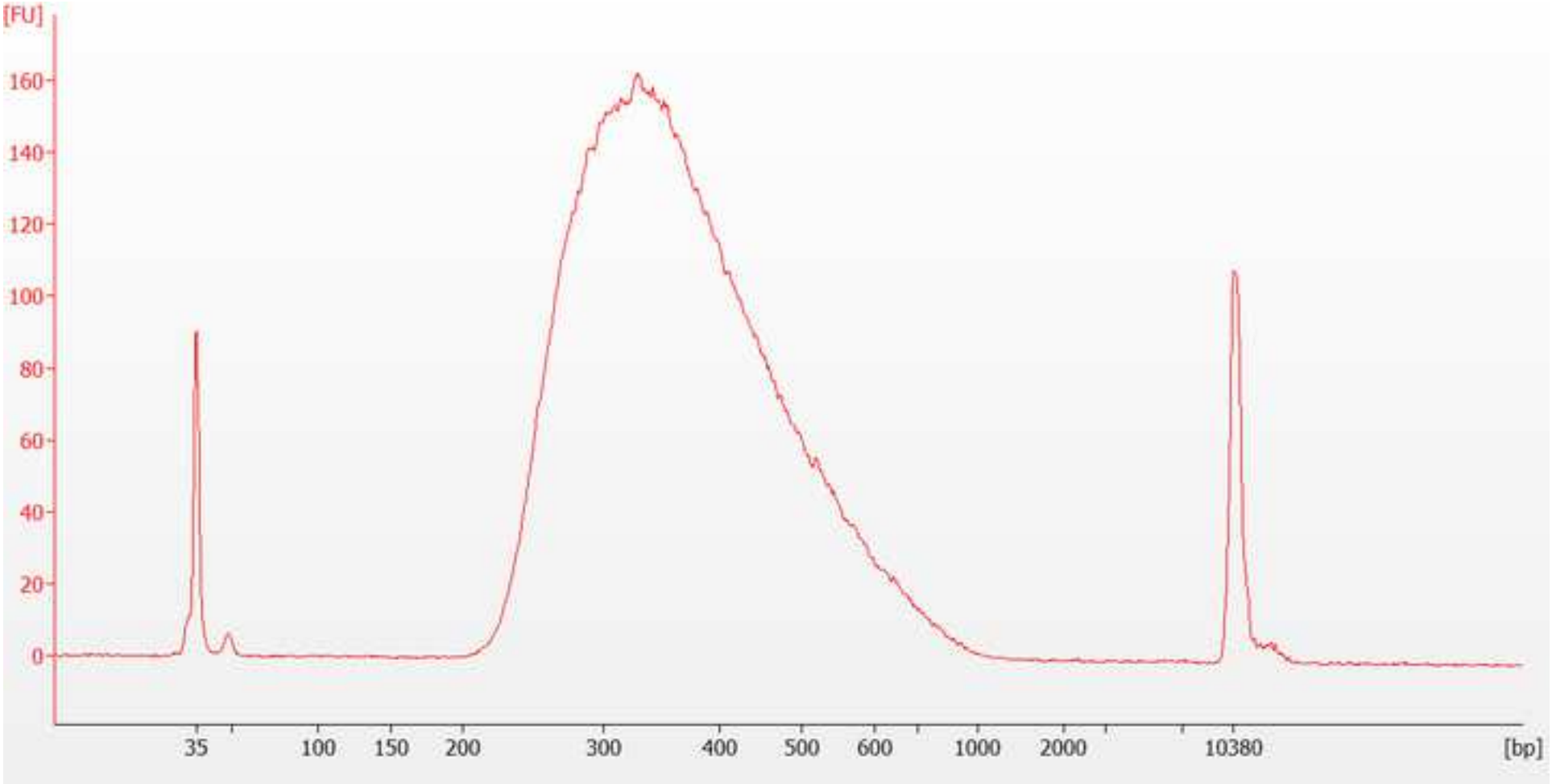
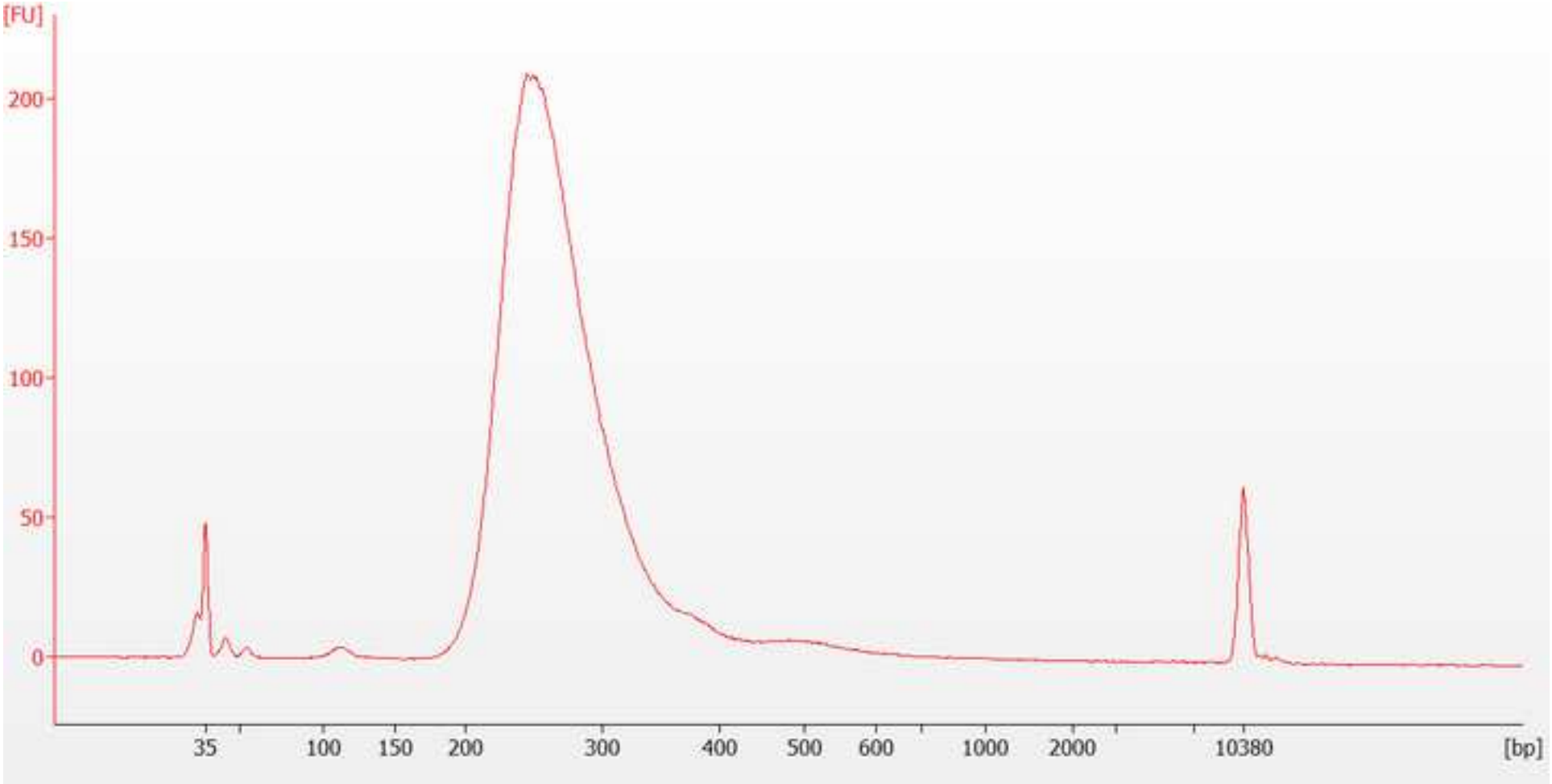
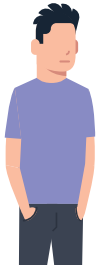


Figure7

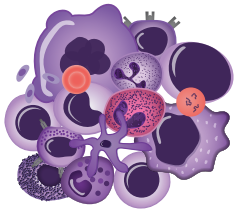
Click here to access/download;Figure;Figure7.tif



(A) Use Case 1: Individual Sample Immune Profiling

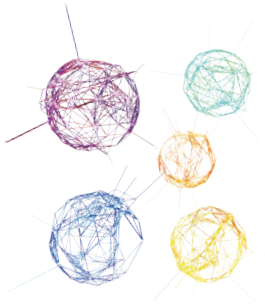


Biopsy is collected from solid tumor of cancer patient

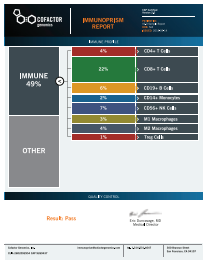


Tissue is archived as heterogenous mixture of cells in FFPE

RNA is extracted and sequenced using ImmunoPrism Assay

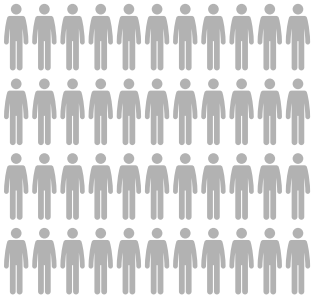


Database of Health Expression Models enable quantification of immune cells



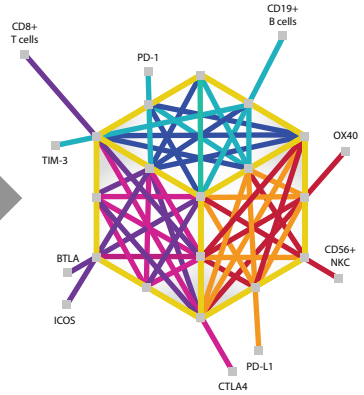
Immune profiling report is generated for each sample

(B) Use Case 2: Multidimensional Biomarker Discovery

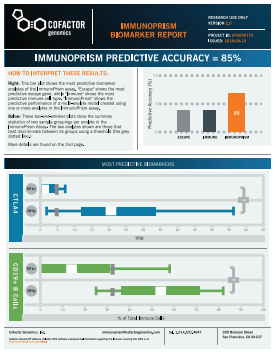


ImmunoPrism data for multiple patients is collected, along with clinical outcomes/meta data

Individual immune profiles are combined using machine-learning to identify optimal features



The output is a multidimensional biomarker that defines patient cohorts with high predictive accuracy



The multidimensional biomarker report provides statistical anlaysis, and can be used to predict outcomes of future samples

Figure9 (A)

(B) Click here to access/download;Figure;Figure9.ai

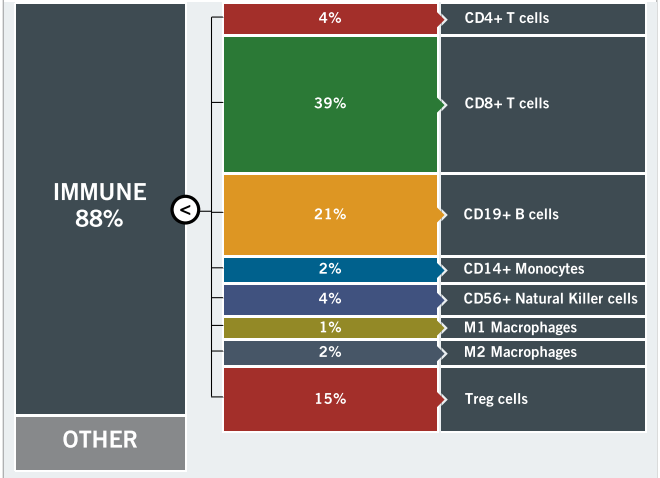
COFACTOR genomics

IMMUNOPRISM REPORT

RESEARCH USE ONLY
Version 2.0

SAMPLE ID: TS-18-1758-A_sequence
ISSUED: 2019-04-09

IMMUNE PROFILE



ESCAPE GENES

Gene Name	TPM for Sample
PD-1	6262.270
PD-L1	3145.368
CTLA4	6989.549
OX40	7326.921
TIM-3	8500.031
BTLA	2482.715
ICOS	2356.334
CD47	16032.422
IDO1	15265.082
ARG1	37.336

IMMUNE CONTENT

Cell Type	Percentage
CD4+ T cells	4
CD8+ T cells	39
CD19+ B cells	21
CD14+ Monocytes	2
CD56+ Natural Killer cells	4
M1 Macrophages	1
M2 Macrophages	2
Treg cells	15

STATEMENT OF PERFORMANCE

The ImmunoPrism assay was developed and characterized by Cofactor Genomics in San Francisco, CA. ImmunoPrism is for Research Use Only and not for use in diagnostics procedures. The results of the included report are subject to change in future releases. Expression levels of escape genes are calculated for the sample in comparison to mean expression of a specific cancer. Immune profiling in ImmunoPrism has been shown to significantly correlate with flow cytometry measurements for PBMC samples across 8 different immune cell types. In addition, ImmunoPrism immune profiling performs consistently across fresh-frozen and formalin-fixed samples. Furthermore, ImmunoPrism immune profiling has been validated in dissociated tumor cells. For technical support or additional information on assay performance or validation, please visit: https://cofactorgenomics.com/immunoprism-assay/.

Figure10 - revised

Click here to access/download;Figure;Figure10_revised.ai

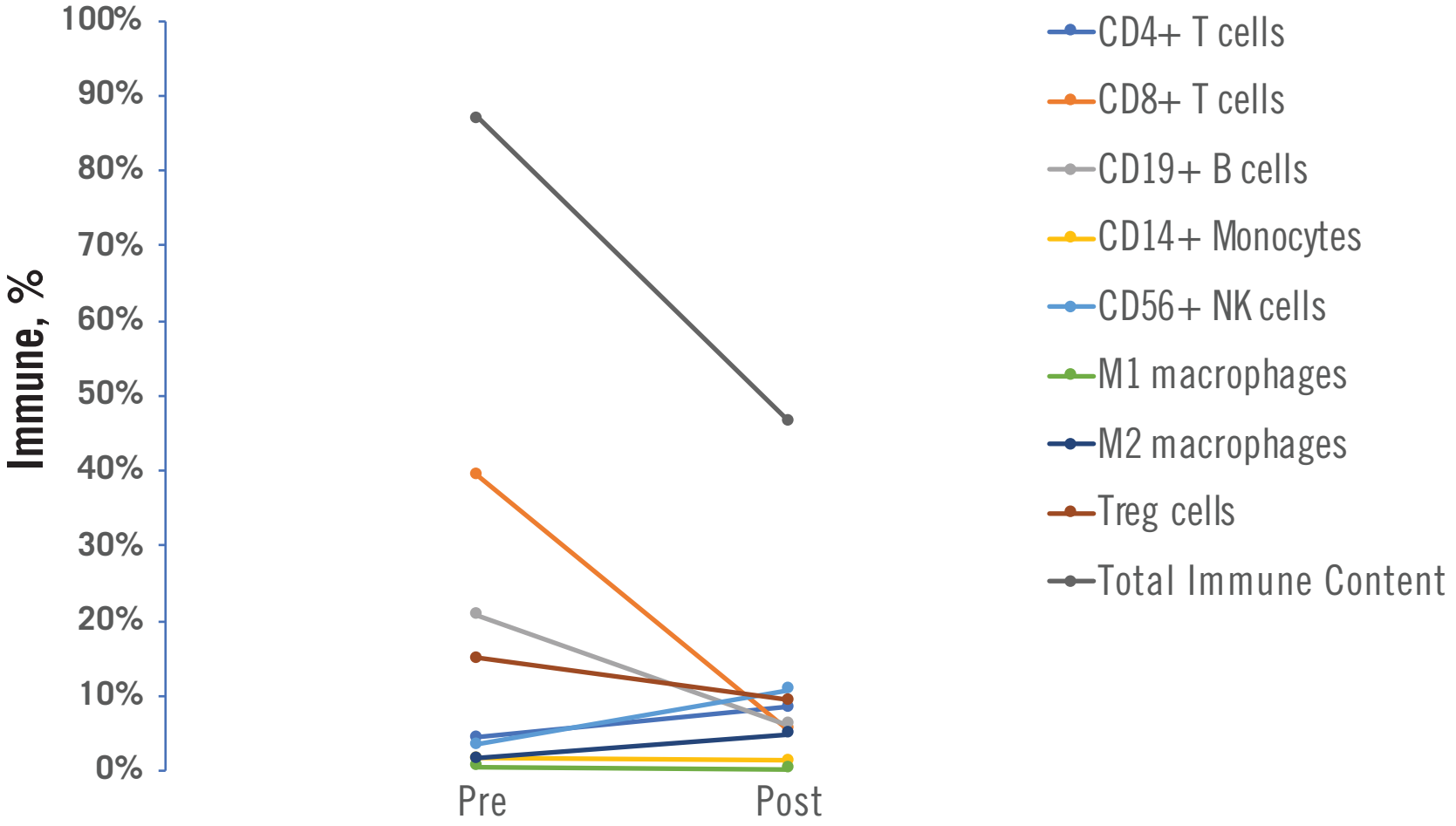


Figure11 - revised

Click here to access/download;Figure;Figure11_revised.ai

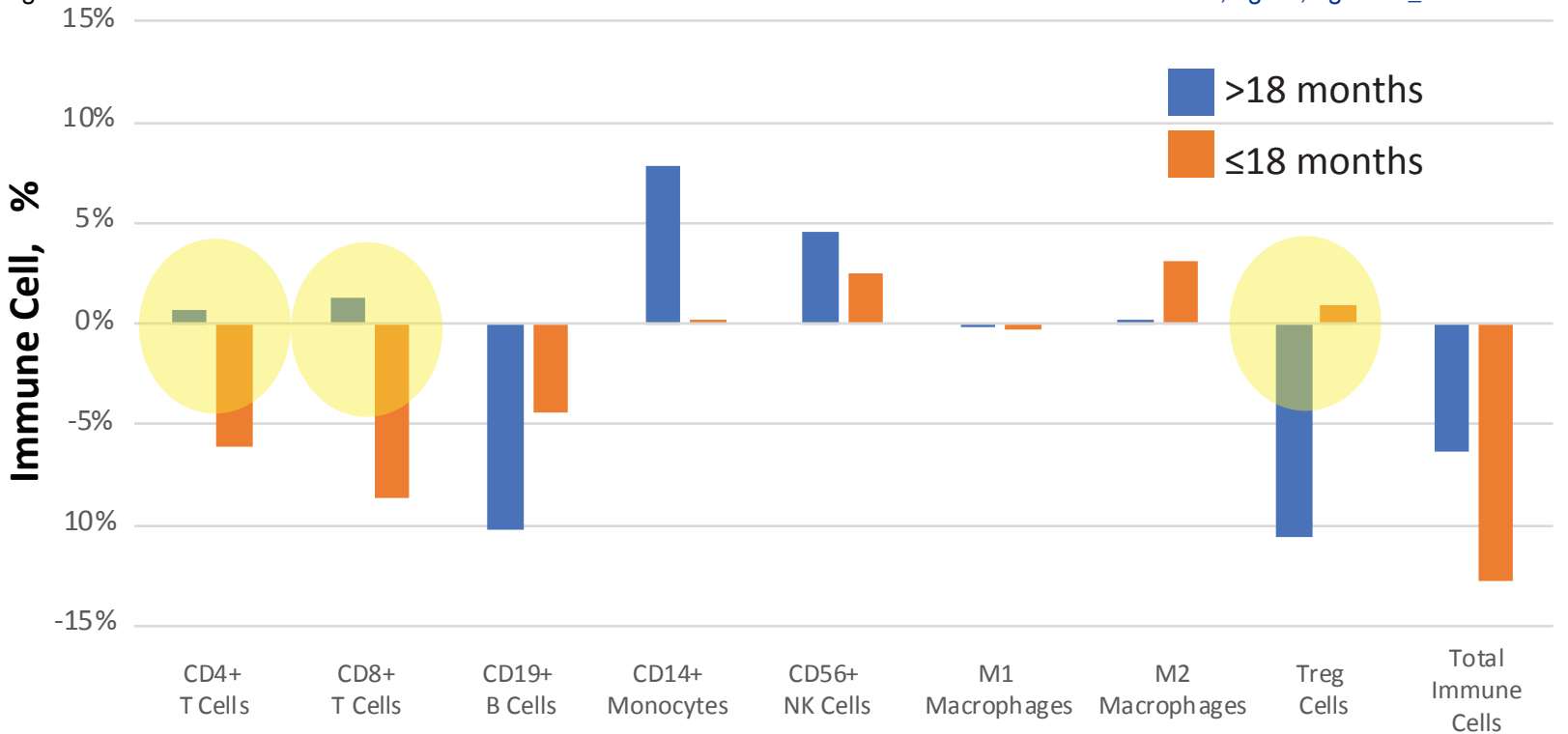


Figure12_revised - AI

(A)

(B) Click here to access/download;Figure;Figure12_revised.ai

COFACTOR genomics

IMMUNOPRISM BIOMARKER REPORT

RESEARCH USE ONLY
VERSION 2.2

PROJECT ID: TRISTAR19
ISSUED: 2019-04-09

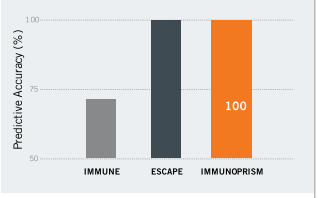
IMMUNOPRISM PREDICTIVE ACCURACY = 100%

HOW TO INTERPRET THESE RESULTS:

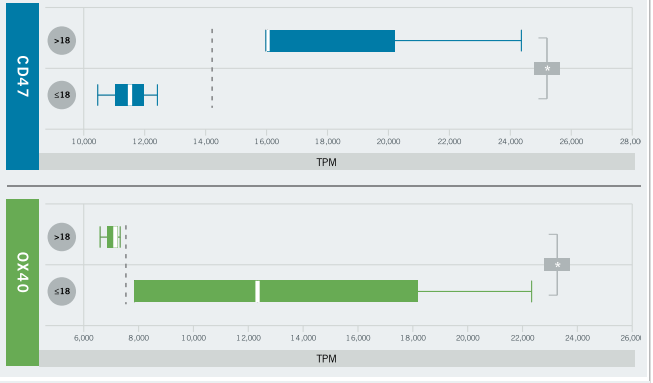
Right: This bar plot shows the most predictive biomarker analytes of the ImmunoPrism assay. "Escape" shows the most predictive escape gene, while "Immune" shows the most predictive immune cell type. "ImmunoPrism" shows the predictive performance of a multi-analyte model created using one or more analytes in the ImmunoPrism assay.

Below: These box-and-whisker plots show the summary statistics of two sample groupings per analyte in the ImmunoPrism Assay. The two analytes shown are those that best discriminate between its groups using a threshold (the grey dotted line.)

More details are found on the 2nd page.



MOST PREDICTIVE BIOMARKERS



Cofactor Genomics, Inc. support@cofactorgenomics.com tel. 1.888.602.0448 300 Brannan Street San Francisco, CA 94107

COFACTOR genomics

IMMUNOPRISM BIOMARKER REPORT

RESEARCH USE ONLY
VERSION 2.2

PROJECT ID: TRISTAR19
ISSUED: 2019-04-09

ANALYTE PERFORMANCE

Analyte	>18 Median	≤18 Median	p value	threshold	accuracy (%)	ppv (%)	npv (%)
CD47	16032.42 TPM	11518.23 TPM	0.0339	14213.99 TPM	100	100	100
OX40	7154.26 TPM	12338.12 TPM	0.0339	7537.95 TPM	100	100	100
IDO1	9609.78 TPM	3381.65 TPM	0.0339	5740.94 TPM	85.71	100	80
ARG1	83.05 TPM	250.75 TPM	0.4795	210.87 TPM	71.43	75	66.67
CTLA4	6840.07 TPM	5517.87 TPM	0.7237	5638.75 TPM	71.43	66.67	75
PD-1	4441.55 TPM	2462.25 TPM	0.7237	2613.87 TPM	71.43	66.67	75
TIM-3	8500.03 TPM	6059.33 TPM	1	6169.95 TPM	71.43	66.67	75
PD-L1	2066.41 TPM	1874.61 TPM	0.2888	1913.42 TPM	57.14	50	66.67
ICOS	1956.02 TPM	1733.86 TPM	0.7237	1955.02 TPM	57.14	50	66.67
BTLA	1169.16 TPM	1807.41 TPM	0.7237	1184.99 TPM	57.14	66.67	50
.							
CD56+ NK Cells	5.98 %	1.92 %	0.0339	3.43 %	71.43	66.67	75
CD8+ T Cells	17.2 %	9.41 %	0.0771	15.83 %	71.43	66.67	75
CD14+ Monocytes	8.72 %	6.57 %	0.7237	6.94 %	71.43	66.67	75
CD4+ T Cells	14.5 %	10.09 %	1	11.4 %	71.43	66.67	75
CD19+ B Cells	12.1 %	17.91 %	0.2888	13.6 %	57.14	66.67	50
M1 Macrophages	0.39 %	0.27 %	0.4795	0.32 %	57.14	50	66.67
Treg Cells	6.14 %	11.88 %	0.5959	8.78 %	57.14	66.67	50
M2 Macrophages	1.8 %	1.56 %	0.4795	1.79 %	42.86	33.33	50
.							
ImmunoPrism	N/A	N/A	N/A	N/A	100	100	100

ADDITIONAL DETAILS

Predictive Accuracy Bar Plot: These bars show the predictive accuracy for the most predictive escape gene ("ESCAPE") and immune cell type ("IMMUNE"). Predictive accuracy refers to the leave-one-out cross validation accuracy (See "Leave-one-out Cross Validation", below). Also shown is the predictive accuracy of using one or more analytes in the ImmunoPrism Assay ("IMMUNOPRISM"). This "marker" is created by learning a machine learning model that incorporates information from one or more analytes. **Most Predictive Biomarkers Box Plot:** These box-and-whisker plots visualize the statistics for groups of samples for the 2 most predictive analytes. The left and right sides of the box indicate the 1st and 3rd quartiles of the respective data set. The median is indicated by the white line inside the box. The minimum and maximum inlier datapoints are denoted by the ends of the whiskers, while outliers are shown as empty circles. The optimal threshold for a given analyte is shown as a vertical dotted line. Wilcoxon rank-sum testing is used to test the null hypothesis that the two groups are sampled from the same distribution. Significance of rejecting this hypothesis is denoted for p values of <0.05, <0.01, and <0.001 by 1, 2, and 3 stars, respectively. **Analyte Performance Table:** Leave-one-out cross validation is used to calculate the accuracy, positive predictive value (ppv), and negative predictive value (npv) for each analyte. **Leave-one-out Cross Validation:** For a dataset limited in size, leave-one-out cross validation gives the best approximation to how an estimator will generalize to future, independent samples. The process works by iterating n times (where there are n datapoints), each time learning a threshold considering n-1 points and testing the prediction of the nth, left out, point. Then, all n predictions are considered to calculate prediction statistics. **Thresholds:** Thresholds are determined by optimizing equally for sensitivity and specificity using all samples. For datapoints that are normally distributed, this threshold would be the same threshold optimized for accuracy.

STATEMENT OF PERFORMANCE

The ImmunoPrism assay was developed and characterized by Cofactor Genomics in San Francisco, CA. ImmunoPrism is for Research Use Only and not for use in diagnostics procedures. The results of the included report are subject to change in future releases. For technical support or additional information on assay analytical performance, please contact: support@cofactorgenomics.com

Cofactor Genomics, Inc. support@cofactorgenomics.com tel. 1.888.602.0448 300 Brannan Street San Francisco, CA 94107

Cofactor Genomics' software includes OSS software components. Information regarding the licenses covering this OSS is at: http://cofactorgenomics.com/legal

Fragmentation and Priming Mix	**Volume (µL)**
Intact or partially degraded RNA (20 ng)	5
First Strand Synthesis Reaction Buffer	4
Random Primers	1
Total Volume	10

Priming Reaction	Volume (µL)
FFPE RNA (40 ng)	5
Random Primers	1
Total Volume	6

First Strand Synthesis	Volume (µL)
Fragmented and Primed RNA (Step 2.1.3)	10
First Strand Synthesis Specificity Reagent	8
First Strand Synthesis Enzyme Mix	2
Total Volume	20

First Strand Synthesis	Volume (µL)
Primed RNA (Step 2.2.3)	6
First Strand Synthesis Reaction Buffer	4
First Strand Specificity Reagent	8
First Strand Synthesis Enzyme Mix	2
Total Volume	20

Second Strand Synthesis Reaction	**Volume (µL)**
First Strand Synthesis Product (Step 4.1)	20
Second Strand Synthesis Reaction Buffer	8
Second Strand Synthesis Enzyme Mix	4
Nuclease-free Water	48
Total Volume	80

End Repair Reaction	Volume (µL)
Second Strand Synthesis Product (Step 6.8)	50
End Repair Reaction Buffer	7
End Repair Enzyme Mix	3
Total Volume	60

Ligation Dilution	Volume (µL)
Adaptor	0.5
Adaptor Dilution Buffer	2
Total Volume	2.5

Ligation Reaction	**Volume (µL)**
End Prepped DNA (Step 7.3)	60
Diluted Adaptor (Step 8.1)	2.5
Ligation Enhancer	1
Ligation Master Mix	30
Total Volume	93.5

PCR Enrichment	**Volume (µL)**
Adaptor ligated DNA (Step 10.1)	15
Pre-Capture PCR Master Mix	25
Universal PCR Primer	5
Index (X) Primer	5
Total Volume	50

Reagent	**Quantity/Volume**
Barcoded library from Step 10.10	200 ng
Cot-1 DNA	2 µg
Blocking Oligos	2 µL

Hybridization Master Mix	**Volume (µL)**
Hybridization Buffer	8.5
Hybridization Buffer Enhancer	2.7
ImmunoPrism Probe Panel	5
Nuclease-Free Water	0.8
Total Volume	17

Wash Buffers	Concentrated Buffer (µL)	Nuclease-free water (µL)	Total (µL)
Bead Wash Buffer	150	150	300
Wash Buffer 1	25	225	250
Wash Buffer 2	15	135	150
Wash Buffer 3	15	135	150
Stringent Wash Buffer	30	270	300

Wash Buffers	Holding Temperature	Volume/Tube (µL)	Number of Tubes/Sample
Bead Wash Buffer	RT (15-25 °C)	100	3
Wash Buffer 1	65 °C	100	1
Wash Buffer 1	RT (15-25 °C)	150	1
Wash Buffer 2	RT (15-25 °C)	150	1
Wash Buffer 3	RT (15-25 °C)	150	1
Stringent Wash Buffer	65 °C	150	2

Bead Resuspension Mix	**Volume (µL)**
Hybridization Buffer	8.5
Hybridization Buffer Enhancer	2.7
Nuclease-Free Water	5.8
Total Volume	17

Post-Capture PCR Master Mix Component	**Volume (µL)**
Post-Capture PCR MasterMix	25
Post-Capture PCR Primer Mix	1.25
Nuclease-Free Water	3.75
Total Volume	30

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials_revised.xlsx

Name of Material/Equipment	Company	Catalog Number
0.2 mL PCR 8 tube strip	USA Scientific	1402-2700
200 Proof Ethanol	MilliporeSigma	EX0276-1
96-well thermal cyclers	BioRad	1861096
Solid-phase Reversible Immobilization (SPRI)	Beckman-Coulter	A63882
Digital electrophoresis chips and kit	Agilent Technologies	5067-4626
Digital electrophoresis system	Agilent Technologies	G2939AA
Streptavidin Beads	ThermoFisher Scientific	65306
ImmunoPrism Kit – 24 reaction	Cofactor Genomics	CFGK-302
Human Cot-1 DNA	ThermoFisher Scientific	15279011
Magnetic separation rack	Alpaqua/Invitrogen	A001322/12331D
Microcentrifuge	Eppendorf	22620701
Microcentrifuge tubes	USA Scientific	1415-2600
NextSeq550	Illumina	SY-415-1002
Nuclease-free water	ThermoFisher Scientific	AM9937
Prism Extraction Kit	Cofactor Genomics	CFGK-401
Purified RNA	-	-
Fluorometer	ThermoFisher Scientific	Q33226
Fluorometric Assay Tubes	Axygen	PCR-05-C
High Sensitivity Fluorometric Reagent Kit	Life Technologies	Q32854
Vacuum concentrator	Eppendorf	22820001
Vortex mixer	VWR	10153-838
Water bath or heating block	VWR/USA Scientific	NA/2510-1102

Comments/Description

USA Scientific 0.2 mL PCR 8-tube strip
Prepare 80% by mixing with nuclease-free water on the day of the experiment

Agencourt AMPure XP – PCR Purification beads
Agilent High Sensitivity DNA chips and kit
Agilent 2100 Electrophoresis Bioanalyzer
Dynabeads M-270 Streptavidin
Cofactor ImmunoPrism Immune Profiling Kit – 24 reactions
Invitrogen brand

96-well Magnetic Ring Stand

USA Scientific 1.5 mL low-adhesion microcentrifuge tube
Any Illumina sequencer may be used for this protocol

Cofactor Prism FFPE Extraction Kit – 24 samples
Purified from human tissue samples
Qubit 4 System
0.5mL Thin Wall PCR Tubes with Flat Caps
Qubit dsDNA HS Assay Kit
VacufugePlus

VWR water bath/USA Scientific heating block

This document contains the authors' responses to the Editorial and Reviewers' Comments. The authors' comments are in bolded font in line, below.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version. **Noted and confirmed.**
2. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please reduce the number of instances of "ImmunoPrism" within your text. The term may be introduced but please use it infrequently and when directly relevant. Otherwise, please refer to the term using generic language. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: AMPure XP, Dynabeads, **This has been addressed in the revised document. The authors have removed many of the ImmunoPrism references, and all of the trade names aside from Illumina because it is a requirement that this sequencing platform be used. The protocol will not work with another platform.**
3. Please define all abbreviations before use: DV200, etc. **This has been addressed in the revised document.**
4. Please insert a one line spacer between all steps and substeps in the protocol. **This has been addressed in the revised document.**
5. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader. **The authors have selected what they believe are the most essential parts of the protocol to ensure a reader/viewer obtains useful information that will enable them to perform the protocol successfully. The protocol is very long so, select steps from Part I and Part II of the protocol were highlighted but it still may be too much. If this is the case, the authors wish to remove Steps 3 and 10, and focus on those highlighted in Part II of the protocol.**
6. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. **Please see notes above.**
7. Please discuss some limitations of the technique in the discussion. **This has been addressed in the revised document.**

8. Please do not abbreviate journal titles in the references. **The EndNote plugin does not allow this. The authors request editorial support in formatting as desired.**
9. Figure 9/11: Please remove the company branding: Cofactor, etc. **The reports shown in Figures 9 and 11 are generated by the analysis software, and as generated, have our logo on them. To remove this would make the report look very different than a user would see in real life. We'd prefer to keep these as is for the sake of realistic representation. We have consciously removed as many trade names (ImmunoPrism) as possible throughout the manuscript in an effort to minimize our brand/logo.**
10. Tables: Please ensure that SI abbreviations are used: µL instead of µl, etc. **This has been addressed in the revised document and tables.**

Reviewers' comments

Reviewer #1:
Manuscript Summary:
This manuscript presents an interesting method for determining immune cell composition of tumour tissue, and immune cell/immune escape associated biomarkers. The key application appears to be for use with FFPE tissue where RNA may be significantly degraded, and single cell isolation is not possible.

Major Concerns:
Whilst the protocol very clearly demonstrates that libraries can be prepared and sequenced from FFPE tissue, it is unclear the relative quality of these samples. Inclusion of the initial RIN obtained for the samples used to generate libraries analysed in figures 3 and 4 would help support the quality of the method. Otherwise it is difficult to assert that this method can be used on the types of degraded sample as suggested in the introduction and protocol section 1. **The authors have added RIN and DV_{200} values into the manuscript text for Figures 3, 4, 5, 6, and 7. Values range from RIN of 9.5 (very high quality) to DV_{200} = 33 (low-quality, FFPE). The protocol is not recommended for samples with DV_{200} < 20, so this is not shown.**

The protocol shows how to prepare samples for sequencing, which for FFPE will be a mixed population of cells. This data is then used to generate an immune profile showing percentages of different immune cell types within this mixed tumour tissue. Whilst the cell markers and bioinformatics used by prism.cofactorgenomics.com may be proprietary, an explanation of the deconvolution method to take sequencing data of mixed cells to single cell populations would be useful for the reader. It is difficult to assess the quality of the results in figures 9-11 without more information about the markers used to identify these populations. **The authors have added a brief description of how the models are generated (and the markers used to identify the immune cell populations during database preparation) in the Introduction and referenced an analytical validation study. An in depth description of the underlying technology is outside of the scope of this manuscript.**

One of the applications suggested by the authors is to compare pre- and post-treatment patient samples. They have obtained and tested samples using their method, however they only show an example of one profile and do not compare pre- and post-treatment samples graphically. If they have this data, I would recommend they include it as this is listed as an example application. **While this comparison is shown for all samples in the cohort in Figure 11, it is certainly also interesting to look at individual patient's profiles. The authors have added a new figure, now Figure 10, to address this. The new figure shows an example of a pre- and post-treatment comparison for an individual patient. This addition results in re-numbering for the subsequent figures, which is also addressed in the manuscript.**

Minor Concerns:
As one of the key applications is for use with FFPE tissue where single cell isolation is not possible. The authors should address in the introduction advances in laser-capture microdissection that are making grounds in isolation of single cells from FFPE tissue. **This has been addressed in the revised document.**

Addition of a table of reagents included in the ImmunoPrism Kit would be useful to include as they are referenced throughout the protocol alongside reagents that the user is providing. **Supplemental Table 1 - ImmunoPrism Reagent List - has been added as a separate file from the JoVE Materials table, however, the authors are unsure of whether to reference this in the text seeing as the JoVE Materials table does not get referenced. It has been referenced as Supplemental Table 1 in the document to address the points below, however, we are open to feedback if this is not appropriate. This revision has resulted in the renumbering of the other two Supplemental Tables, reflected in the text.**

For some buffers e.g. wash buffers (line 343 onwards), the authors describe how to generate 1X solutions. At other points in the protocol they refer to 1.8X (line 184) 1.5X (line 466), 0.9X (line 241) and 0.1X (lines 197 and 479). It is unclear whether these are provided at these concentrations, require dilution to these concentrations before use, or if these are the final concentrations obtained after adding to the tubes. **The authors have addressed this in the following way:**

- **1.8X (line 184): removed for clarity, not required**
- **1.5X (line 466): removed for clarity, not required**
- **0.9X (line 241): removed for clarity, not required**
- **0.1X (lines 197 and 479): added Supplemental Table 1 and referenced in first instance of 0.1X**

The protocol mentions brief spins of centrifuge tubes a several points, do the authors recommend a speed/force for this? If so, it should be included. **The authors have added a note at Step 2.1.1.1. to address this.**

The two distinct steps for high and low quality in section 2 (2.1 and 2.2 respectively)

could be more clearly separated. **The authors have added header instructions of 2.1 and 2.2 and additional spacing to delineate the different paths here. The same has been done for 3.1 and 3.2 for consistency and additional clarity.**

Reviewer #2:

Manuscript Summary:

Predictive Immune Modeling of Solid Tumors using the ImmunoPrism Assay in formalin-fixed and paraffin embedded samples.

Major Concerns:

- Related Figures must be merged. **When possible, related figures have been merged in an Adobe file, however, some source files may also be attached which are showing up as individual files. If there are specific figures that the editor would like us to merge, please confirm and we are happy to address this.**
- How you know the accurate cycles for your PCR amplification? **This has been addressed in the revised document by adding details about the required amount of material needed to proceed to the next step in the protocol.**

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	Predictive Immune Modeling of Solid Tumors using the ImmunoPrism Assay
Author(s):	Natalie A. LaFranzo, PhD, Kevin C. Flanagan, PhD, Danielle Quintanilha, PhD

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☐ Standard Access ☑ Open Access

Item 2: Please select one of the following items:

☑ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Natalie LaFranzo
Department:	Marketing
Institution:	Cofactor Genomics
Title:	VP Market Development
Signature:	Natalie LaFranzo
Date:	08/02/2019

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

612542.6 For questions, please contact us at submissions@jove.com or +1.617.945.9051.

Signature Certificate

Document Ref.: T53VM-Q3MBL-DFOMW-ETX2R

Document signed by:

Natalie LaFranzo

Verified E-mail:
natalie_lafranzo@cofactorgenomics.com

IP: 162.238.119.37 | Date: 03 Aug 2019 04:25:47 UTC

Natalie LaFranzo

Document completed by all parties on:
03 Aug 2019 04:25:47 UTC

Name of Material	Reagent Number
First Strand Synthesis Reaction Buffer	#LIB-001
Random Primers	#LIB-002
Strand Specificity Reagent	#LIB-003
First Strand Synthesis Enzyme Mix	#LIB-004
Second Strand Synthesis Reaction Buffer	#LIB-005
Second Strand Synthesis Enzyme Mix	#LIB-006
End Repair Reaction Buffer	#LIB-007
nd Repair Enzyme Mix	#LIB-008
Adaptor	#LIB-009
Adaptor Dilution Buffer	#LIB-010
Ligation Enhancer	#LIB-011
Ligation Master Mix	#LIB-012
Adaptor Processing Enzyme	#LIB-013
Pre-Capture PCR Master Mix	#LIB-014
Universal PCR Primer	#LIB-015
Index Primers for CFGK-301	#LIB101 - #LIB108
Index Primers for CFGK-302	#LIB101 - #LIB124
Blocking Oligos	#CAP-001
Hybridization Buffer	#CAP-002
Hybridization Buffer Enhancer	#CAP-003
ImmunoPrism Probe Panel	#CAP-004
2X Bead Wash Buffer	#CAP-005
10X Wash Buffer 1	#CAP-006
10X Wash Buffer 2	#CAP-007
10X Wash Buffer 3	#CAP-008
10X Stringent Wash Buffer	#CAP-009
Post-Capture PCR Master Mix	#CAP-010
Post-Capture Primer Mix	#CAP-011
ImmunoPrism Intact Control	#CFG-001
ImmunoPrism FFPE Control	#CFG-002
TE Buffer 0.1X	#CFG-003

Visit https://cofactorgenomics.com/product/immunoprism-kit/ for Safety Da

Comments/Description
Pre-Capture Library Reagents
Pre-Capture Library Reagents
Pre-Capture Library Reagents
Pre-Capture Library Reagents
Pre-Capture Library Reagents
Pre-Capture Library Reagents
Pre-Capture Library Reagents
Pre-Capture Library Reagents
Pre-Capture Library Reagents
Pre-Capture Library Reagents
Pre-Capture Library Reagents
Pre-Capture Library Reagents
Pre-Capture Library Reagents
Pre-Capture Library Reagents
Pre-Capture Library Reagents
Pre-Capture Library Reagents
Pre-Capture Library Reagents
Hybridization and Capture Reagents
Hybridization and Capture Reagents
Hybridization and Capture Reagents
Hybridization and Capture Reagents
Hybridization and Capture Reagents
Hybridization and Capture Reagents
Hybridization and Capture Reagents
Hybridization and Capture Reagents
Hybridization and Capture Reagents
Hybridization and Capture Reagents
Hybridization and Capture Reagents
General ImmunoPrism Reagents, IMPORTANT: store at -80 °C
General ImmunoPrism Reagents, IMPORTANT: store at -80 °C
General ImmunoPrism Reagents

ıta Sheets (SDS).

Thermal Cycler Programs

Program #	Program Name
1	Fragmentation and Priming (Intact or partially degraded RNA only)
2	Priming (FFPE RNA only)
3	First Strand Synthesis
4	Second Strand Synthesis
5	End Repair
6	Adaptor Ligation
7	Adaptor Processing
8	Pre-Capture PCR
9	Hybridization
10	Capture and Wash
11	Post-Capture PCR

Conditions
94 °C for 15 minutes (intact RNA) / 94 °C for 7-8 minutes (partially degraded RNA). Heated lid at 105 °C.
65 °C for 5 minutes, hold at 4 °C. Heated lid at 105 °C.
25 °C for 10 minutes, 42 °C for 15 minutes, 70 °C for 15 minutes, hold at 4 °C. Heated lid ≥ 80 °C.
16 °C for 1 hour. Heated lid ≤ 40 °C.
20 °C for 30 minutes, 65 °C for 30 minutes, hold at 4 °C. Heated lid. ≥ 75 °C.
20 °C for 15 minutes
37 °C for 15 minutes. Heated lid ≥ 45 °C.
98 °C for 30 seconds, 10 (intact RNA) or 15 (FFPE RNA) cycles of: [98 °C for 10 seconds, 65 °C for 75 secor
95 °C for 30 seconds, 65 °C for 4 hours, hold at 65 °C. Heated lid at 100 °C.
Hold at 65 °C. Heated lid at 70 °C.
98 °C for 45 seconds, 14 cycles of: [98 °C for 15 seconds, 60 °C for 30 seconds, 72 °C for 30 seconds], 72 °

ıds], 65 °C for 5 minutes, hold at 4 °C. Heated lid at 105 °C.

'C for 1 minute, hold at 4 °C. Heated lid at 105 °C.

Sequencing Index Guide

Index #	Index Primer Sequence Read
1	AGTCAA
2	CCGTCC
3	GTCCGC
4	GTGGCC
5	GAGTGG
6	ACTGAT
7	CGTACG
8	ATTCCT
9	ATCACG
10	CGATGT
11	TTAGGC
12	TGACCA
13	ACAGTG
14	GCCAAT
15	CAGATC
16	ACTTGA
17	GATCAG
18	TAGCTT
19	GGCTAC
20	CTTGTA
21	AGTTCC
22	ATGTCA
23	GTGAAA
24	GTTTCG

Note: For low-level multiplexing for sequencing, it is recommended that the following index primer Pool of 2 samples: Index 3 and 6 | Pool of 3 samples: Index 1, 3 and 5 | Pool of 4 samples: Index 2, 3

s be used:
3, 4 and 5