Video Article

Predictive Immune Modeling of Solid Tumors

Natalie A. LaFranzo¹, Kevin C. Flanagan¹, Danielle Quintanilha¹

¹Cofactor Genomics

Correspondence to: Natalie A. LaFranzo at natalie_lafranzo@cofactorgenomics.com

URL: <https://www.jove.com/video/60645>

DOI: [doi:10.3791/60645](https://doi.org/10.3791/60645)

Keywords: Immunology and Infection, Issue 156, Immune Profiling, RNA, RNA-seq, Machine Learning, Bioinformatics, Solid Tumor, Cancer, Sequencing, Predictive Immune Modeling, Health Expression Models, Immune-Oncology, Gene Expression

Date Published: 2/25/2020

Citation: LaFranzo, N.A., Flanagan, K.C., Quintanilha, D. Predictive Immune Modeling of Solid Tumors. *J. Vis. Exp.* (156), e60645, doi:10.3791/60645 (2020).

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.

Video Link

The video component of this article can be found at <https://www.jove.com/video/60645/>

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^{1,2,3}. Common techniques such as flow cytometry and single-cell ribonucleic acid (RNA) sequencing are useful for fresh tissue and blood⁴, 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 quantification⁵. Platforms such as the nCounter system rely on the expression of single genes to define key immune cells⁶, 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^{7,8,9,10,11,12}. 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 heterogeneous 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.

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. [Please click here to view a larger version of this figure.](#)

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. [Please click here to view a larger version of this figure.](#)

The protocol requires approximately 16 h 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

- 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 (DV₂₀₀) values.
 - 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**).
 - For highly degraded samples (e.g., RIN = 1 to 2 or FFPE), determine the DV₂₀₀ value. These samples do not require fragmentation and will follow the instructions for degraded RNA, starting with Step 2.2.
- 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 DV₂₀₀ > 20%) to 5 µL in nuclease-free water. Processing samples with DV₂₀₀ < 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

- Follow Step 2.1.1 for High-Quality/Intact RNA with RIN > 2.
 - For high-quality RNA, assemble the fragmentation and priming reaction on ice in a nuclease-free PCR tube according to **Table 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.
 - Place the samples in a thermal cycler and use Program #1 (**Supplemental Table 2**).
 - 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.

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

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.

- Follow Step 2.2.1 for Degraded/FFPE RNA with DV₂₀₀ > 20%.
 - 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.
 - Mix thoroughly by pipetting up and down several times. Then, briefly spin down the samples in a microcentrifuge.
 - Place the samples in a thermal cycler and use Program #2 (**Supplemental Table 2**).
 - Transfer the tubes to ice and proceed to **First Strand cDNA Synthesis for Highly Degraded (FFPE) RNA** (Step 3.2).

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

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.

3. First Strand cDNA Synthesis

- Follow Step 3.1.1 for High-Quality/Intact RNA with RIN > 2.
 - For intact RNA (high-quality), assemble the First Strand Synthesis reaction on ice in a nuclease-free PCR tube according to **Table 3**.
 - 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).

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

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.

- Follow Step 3.2.1 for Degraded/FFPE RNA with DV₂₀₀ > 20%.
 - For highly degraded RNA (FFPE), assemble the First Strand Synthesis reaction on ice in a nuclease-free PCR tube according to **Table 4**.
 - 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).

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

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.

4. First Strand Synthesis Incubation

- 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

- 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.

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

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.

- 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

- 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.
- 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.
- 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.
- 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 6.4 once for a total of 2 washing steps.
- 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.
- 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.
- 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

- 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.

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

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.

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

8. Adaptor Ligation

- 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.

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

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

- 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.

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

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.

- 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.
- 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.
- 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 Needs

- 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- 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

- 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.

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

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.

- 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

- 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- 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

- 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.
 - 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.
 - Check that the electropherogram shows a narrow distribution with a peak size approximately 250-400 bp (see Representative Results, **Figure 3** and **Figure 4**).
 - If a 128 bp peak (adaptor-dimer) is visible in the Bioanalyzer traces, and the intensity of the signal is $\geq$ 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

- 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**.

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

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.

- 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

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.
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**.

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

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

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

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.

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

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

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.

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

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.

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.

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

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

16. Prepare the Streptavidin Beads

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.
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.
3. Remove and discard the clear supernatant. Be careful not to disturb the beads.
4. Perform the following wash.
 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.
 2. Place the tube in the magnetic rack, allowing beads to fully separate from the supernatant.
 3. Carefully remove and discard the clear supernatant.
5. Repeat Step 16.4 once for a total of two washes.
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

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

1. Use the 1x Wash Buffers from Step 15.2 and store heated buffers in the thermal cycler during washes.
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.
3. Pipette and discard the supernatant, which contains unbound DNA. Remove from magnetic rack.
4. Perform the following 65 °C wash.
 1. Add 150 μL of preheated 1x Stringent Wash Buffer.
 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.**
 3. Incubate in the thermal cycler at 65 °C for 5 min.
 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.
 5. Repeat Step 18.4 for a total of two Stringent Washes.
5. Perform the first room temperature wash.
 1. Add 150 μL of room temperature 1x Wash Buffer 1.
 2. Pipette up and down 10 to 20 times to completely resuspend the beads.
 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.**
 4. Briefly centrifuge the tubes.
 5. Place the tubes on a magnetic rack, allowing beads to fully separate from the supernatant. Pipette and discard the supernatant.
 6. Seal the tubes and briefly centrifuge. Return to magnetic rack and use a 10 μl pipette to remove any residual wash buffer.
6. Perform the second room temperature wash.
 1. Add 150 μL of room temperature 1x Wash Buffer 2.
 2. Pipette up and down 10 to 20 times to completely resuspend the beads.
 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.**
 4. Briefly centrifuge the tubes.

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.**
6. Place the tubes on a magnetic rack, allowing beads to fully separate from the supernatant. Pipette and discard the supernatant.
7. Seal the tubes and briefly centrifuge. Return to magnetic rack and use a 10 μ L pipette to remove any residual wash buffer.
7. Perform the third room temperature wash.
 1. Add 150 μ L of room temperature 1x Wash Buffer 3.
 2. Pipette up and down 10 to 20 times to completely resuspend the beads.
 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.**
 4. Briefly centrifuge the tubes.
 5. Place the tubes on a magnetic rack, allowing beads to fully separate from the supernatant. Pipette and discard the supernatant.
 6. Seal the tubes and briefly centrifuge. Return to magnetic rack and use a 10 μ L pipette to remove any residual wash buffer.
 7. Remove from the magnetic rack and add 20 μ L of nuclease-free water to the beads.
 8. Pipette up and down 10 times to ensure any beads stuck to the side of the tubes have been resuspended.
8. Important: Do not discard the beads. Use the entire 20 μ L of resuspended beads with captured DNA in Step 19.

19. Perform Final, Post-capture PCR Enrichment

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**.

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

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.

2. Add 30 μ L of the Post-Capture PCR Master Mix to each sample for a final reaction volume of 50 μ L. Mix thoroughly by pipetting up and down 10 times.
3. Place the PCR tubes in the thermal cycler and incubate following Program #11 (**Supplemental Table 2**).

20. Purify Post-capture PCR Fragments

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.
2. Add 75 μ L of resuspended beads to each PCR-enriched capture (50 μ L). 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.
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.
4. Add 180 μ L 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.
5. Repeat Step 20.4 once for a total of 2 washing steps.
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.
7. Remove the tube from the magnet. Elute the DNA from the beads by adding 22 μ L 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.
8. Remove 20 μ L 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 $^{\circ}$ C.

21. Validate and Quantify Library

1. Measure the concentration of the captured library using a fluorometer and High Sensitivity Assay Kit.
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 $^{\circ}$ C.

22. Sequencing on a Sequencing Platform

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

1. Create a Prism account by visiting <https://prism.cofactorgenomics.com/>
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.
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.
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.
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.
 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).

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. [Please click here to view a larger version of this figure.](#)

Care should be taken to avoid overamplification, as indicated by the second peak around 1,000 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.

Figure 4: Typical Pre-capture Library Bioanalyzer trace for an FFPE RNA sample. The second peak around 1,000 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. [Please click here to view a larger version of this figure.](#)

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.

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. [Please click here to view a larger version of this figure.](#)

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** ($DV_{200} = 36$).

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. [Please click here to view a larger version of this figure.](#)

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. [Please click here to view a larger version of this figure.](#)

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

(A) Use Case 1: Individual Sample Immune Profiling

(B) Use Case 2: Multidimensional Biomarker Discovery

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. [Please click here to view a larger version of this figure.](#)

To demonstrate each of these use cases, representative data from a small translational study is included²¹. 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**.

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. [Please click here to view a larger version of this figure.](#)

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.

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. [Please click here to view a larger version of this figure.](#)

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.

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. [Please click here to view a larger version of this figure.](#)

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.

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. [Please click here to view a larger version of this figure.](#)

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. Visit <https://cofactorgenomics.com/product/immunoprism-kit/> for Safety Data Sheets (SDS). [Please click here to view this file \(Right click to download\).](#)

Supplemental Table 2: Thermal Cycler Programs. The recommended cycler programs referenced throughout the protocol are summarized for ease of programming. [Please click here to view this file \(Right click to download\).](#)

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. [Please click here to view this file \(Right click to download\).](#)

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 $-20^{\circ}C$ until ready to use and return to $-20^{\circ}C$ 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.

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. The ImmunoPrism Assay is for Research Use Only, and is not for use in diagnostic procedures.

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.

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