COFACTOR

THE LEADERS IN RNA

Cofactor ImmunoPrism Kit

Version 0.7

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Introduction

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 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.

Cofactor has developed the ImmunoPrism Immune Profiling Assay to overcome these challenges. First validated in our centralized laboratory, the Cofactor ImmunoPrism Immune Profiling Kit provides an end-to-end quantitative, high-throughput immuno-profiling solution for Illumina sequencing platforms. Starting with as few as two sections of formalin-fixed paraffin-embedded (FFPE) tissue or 20-40 ng of total RNA (depending on sample quality), Cofactor ImmunoPrism provides comprehensive, quantitative immune profiling of eight immune cell types and ten immune escape genes, resulting in a more complete view of the tumor microenvironment. With the appropriate sample cohorts, the ImmunoPrism Assay may also be used to identify statistically-significant biomarkers within a patient population of interest.

Procedure

Cofactor Genomics ImmunoPrism Kit is used to make high quality libraries ready for sequencing on an Illumina platform. Sequencing data is uploaded to the Prism Portal, analyzed, and a comprehensive, quantitative profile for each individual sample, in the form of the ImmunoPrism Report, is returned to the user. Users may also use the Prism Portal to generate a Biomarker Report highlighting statistically-significant biomarkers which distinguish two patient cohorts.

This protocol requires 20 ng intact or 40 ng highly degraded (FFPE) RNA. If starting with FFPE tissue, we recommend Cofactor Genomics ImmunoPrism FFPE RNA Extraction Kit to maximize RNA quality and quantity. Visit **cofactorgenomics.com/immunoprism-kit/** for more information.

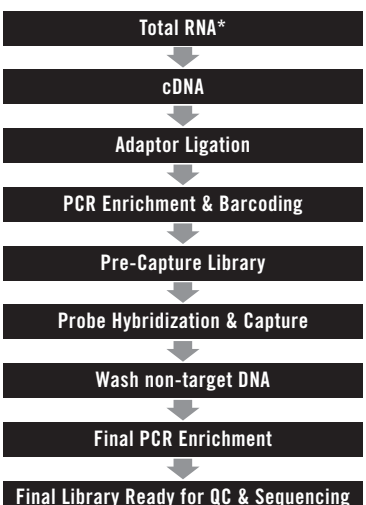


Figure 1:
Overview of Cofactor ImmunoPrism Workflow.

When using Cofactor Genomics ImmunoPrism Kit v0.7, RNA is first converted to cDNA. Adaptors are ligated, and adaptor-ligated cDNA is amplified and barcoded by PCR to create a pre-capture library. Biotinylated ImmunoPrism 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.

*Note: Total RNA must be from human samples; may be intact or degraded (FFPE) RNA as described above.

Protocol Notes

- Use of the included ImmunoPrism Human Control RNA is strongly recommended. This control provides a means to evaluate performance throughout the entire ImmunoPrism process, from library preparation to analysis.

- 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. Return to -20 °C promptly after use.

- Use only pure nuclease-free water. Do not 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.

- 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.

- During *Step 16: Bind Hybridized Target to the Streptavidin Beads*, be sure to vortex every 10-12 minutes to increase the bead capture efficiency. Carefully hold the caps of the warm strip tubes when vortexing to prevent tubes from opening.

- It is critical to perform *Step 17: Wash Streptavidin Beads to Remove Unbound DNA* as it is written in the protocol to avoid high nonspecific contamination. Be sure to completely resuspend the beads at each wash, completely remove the wash buffers, and move the samples to a fresh strip tube (Step 17.16). 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.

- Do not let the streptavidin beads dry out. If needed extend incubation times to avoid drying the beads. If using more than one strip tube work with one strip tube at a time for each wash to avoid drying the beads or rushing, resulting in poor resuspension or other sub-optimal techniques. For first time users, we do not recommend processing more than 8 library reactions at a time.

Thermal Cycler Programs

Program #	Program Name	Conditions
1	Fragmentation and Priming (Intact or partially degraded RNA only)	94 °C for 15 minutes (intact RNA) / 94 °C for 7-8 minutes (partially degraded RNA). Heated lid at 105 °C.
2	Priming (FFPE RNA only)	65 °C for 5 minutes, hold at 4 °C. Heated lid at 105 °C.
3	First Strand Synthesis	25 °C for 10 minutes, 42 °C for 15 minutes, 70 °C for 15 minutes, hold at 4 °C. Heated lid ≥ 80 °C.
4	Second Strand Synthesis	16 °C for 1 hour. Heated lid ≤ 40 °C.
5	End Repair	20 °C for 30 minutes, 65 °C for 30 minutes, hold at 4 °C. Heated lid. ≥ 75 °C.
6	Adaptor Ligation	20 °C for 15 minutes
7	Adaptor Processing	37 °C for 15 minutes. Heated lid ≥ 45 °C.
8	Pre-Capture PCR	98 °C for 30 seconds, 10 (intact RNA) or 15 (FFPE RNA) cycles of: [98 °C for 10 seconds, 65 °C for 75 seconds], 65 °C for 5 minutes, hold at 4 °C. Heated lid at 105 °C.
9	Hybridization	95 °C for 30 seconds, 65 °C for 4 hours, hold at 65 °C. Heated lid at 105 °C.
10	Capture and Wash	Hold at 65 °C. Heated lid at 70 °C.
11	Post-Capture PCR	98 °C for 45 seconds, 14 cycles of: [98 °C for 15 seconds, 60 °C for 30 seconds, 72 °C for 30 seconds], 72 °C for 1 minute, hold at 4 °C. Heated lid at 105 °C.

Optional Protocol Modifications

Throughout protocol: This 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. **Note: it is not recommended for a first-time user to attempt more than 8 library reactions at a time.**

Step 12: Combine Blocking Oligos, Cot-1 DNA, Pre-capture Library DNA, and Dry: To minimize sample loss and avoid switching tubes, Step 12 can be performed in PCR tubes, strip tubes, or a 96-well PCR plate instead of 1.7 mL tubes, if your vacuum concentrator allows. The rotor can be removed on many Speedvac 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.

Step 17: Wash Streptavidin Beads to Remove Unbound DNA: During the room temperature washes, a microplate vortex mixer (such as IKA MS 3 Basic Vortex Mixer) can be used to vortex the samples for the entirety of the 2 minute incubation period for easier resuspension.

Support

For troubleshooting, support, or questions, visit **www.cofactorgenomics.com/support** or email **support@cofactorgenomics.com**.

Protocol Overview

	Protocol Step	Time
1	Fragmentation and Priming	1.5 hours
2	First Strand cDNA Synthesis	
3	First Strand Synthesis Incubation	
4	Second Strand cDNA Synthesis	1 hour
5	Purify double-stranded cDNA	30 minutes
6	Prepare cDNA Ends	1 hour
7	Ligate Adaptors	45 minutes
8	Purify Adaptor Ligated DNA	30 minutes
9	Perform pre-capture PCR enrichment	45 minutes
10	Purify pre-capture PCR fragments	30 minutes
11	Validate and quantify pre-capture library	1 hour
12	Dry blocking oligos, Cot-1, and pre-cap libraries	1 hour
13	Hybridize capture probes with the library	4.25 hours
14 - 15	Prepare buffers and streptavidin beads	1 hour (can be done during hybridization)
16	Bind hybridized targets to streptavidin beads	45 minutes
17	Wash streptavidin beads to remove unbound DNA	30 minutes
18	Perform PCR enrichment	30 minutes
19	Purify postcapture PCR fragments	30 minutes
20	Validate and quantify final library	1 hour
	Ready for Sequencing	**Total: 16 hours**

Library Preparation Kit Reagents

IMPORTANT:
Upon receipt, store ImmunoPrism Human Control at –80 °C. TE Buffer (0.1X) can be stored at room temperature (15-25 °C). Store all other reagents at –20 °C.

The volumes provided are sufficient for preparation of up to 24 reactions.
Colored bullets indicate the color on the cap of the reagent to be used.

Pre-Capture Library Reagents

- ○ (white) First Strand Synthesis Reaction Buffer (#LIB-001)
- ○ (white) Random Primers (#LIB-002)
- ○ (white) Strand Specificity Reagent (#LIB-003)
- ○ (white) First Strand Synthesis Enzyme Mix (#LIB-004)
- (orange) Second Strand Synthesis Reaction Buffer (10X) (#LIB-005)
- (orange) Second Strand Synthesis Enzyme Mix (#LIB-006)
- (green) End Repair Reaction Buffer (#LIB-007)
- (green) End Repair Enzyme Mix (#LIB-008)
- (red) Adaptor (#LIB-009)
- (red) Adaptor Dilution Buffer (#LIB-010)
- (red) Ligation Enhancer (#LIB-011)
- (red) Ligation Master Mix (#LIB-012)
- (red) Adaptor Processing Enzyme (#LIB-013)
- (violet) Pre-Capture PCR Master Mix #(LIB-014)
- (violet) Universal PCR Primer (#LIB-015)
- (violet) Index Primers (1-24) (#LIB-016)

Hybridization and Capture Reagents

- (blue) Blocking Oligos (#CAP-001)
- (blue) 2X Hybridization Buffer (#CAP-002)
- (blue) Hybridization Buffer Enhancer (#CAP-003)
- (blue) ImmunoPrism Probe Panel (#CAP-004)

  2x Bead Wash Buffer (#CAP-005)

- (blue) 10X Wash Buffer 1 (#CAP-006)
- (blue) 10X Wash Buffer 2 (#CAP-007)
- (blue) 10X Wash Buffer 3 (#CAP-008)
- (blue) 10X Stringent Wash Buffer (#CAP-009)
- (black) Post-Capture PCR Master Mix (#CAP-010)
- (black) Post-Capture Primer Mix (#CAP-011)

General ImmunoPrism Reagents

- ○ (white) ImmunoPrism Human Control (**store at -80 °C**) (#PRG-001)
  TE Buffer (0.1X) (#PRG-002)

Additional Materials and Equipment

Materials	Ordering Information
>80% Ethanol	General laboratory supplier
Agencourt® AMPure® XP – PCR Purification beads	Beckman-Coulter, Cat # A63880
Nuclease-free water	General laboratory supplier
Digital electrophoresis chips, such as:	
ExperionTM DNA IK Analysis Kit	Bio-Rad Laboratories, Cat # 700-7107
Agilent High Sensitivity DNA Kit	Agilent Technologies, Cat # 5067-4626
Agilent High Sensitivity D1000 ScreenTape®	Agilent Technologies, Cat # 5067-5584
Dynabeads® M-270 Streptavidin	Life Technologies, Cat # 65305
Invitrogen™ Human Cot-1 DNA®	Life Technologies, Cat # 15279-011
Microcentrifuge tubes, such as: USA Scientific 1.5 mL low-adhesion microcentrifuge tube	USA Scientific, Cat # 1415-2600
0.2 mL PCR 8 tube strip, such as: USA Scientific 0.2 mL PCR 8-tube strip	USA Scientific, Cat # 1402-2700
QIAGEN® Buffer EB (or equivalent: 10 mM Tris-Cl, pH 8.5)	QIAGEN, Cat # 19086 (or general laboratory supplier)
Qubit® Assay Tubes	Life Technologies, Cat # Q32856
Qubit dsDNA HS Assay Kit	Life Technologies, Cat # Q32851

Equipment	Ordering Information
96-well thermal cyclers	General laboratory supplier
Digital electrophoresis system, such as:	
Experion Electrophoresis Station	Bio-Rad Laboratories, Cat # 700-7010
Agilent 2100 Electrophoresis Bioanalyzer	Agilent Technologies, Cat # G2939AA
Agilent 2200 TapeStation	Agilent Technologies, Cat # G2965AA
Magnetic separation rack, such as:	
96-well Magnetic Ring Stand	ThermoFisher Scientific, Cat # AM10050
Microcentrifuge	General laboratory supplier
Qubit 3.0 Fluorometer	Life Technologies, Cat # Q33216
Vacuum concentrator	General laboratory supplier
Vortex mixer	General laboratory supplier
Water bath or heating block	General laboratory supplier

RNA Sample Recommendations

RNA Integrity:
Assess the quality of the input RNA by running the RNA sample on an Agilent Bioanalyzer RNA 6000 Nano/Pico Chip to determine the RNA Integrity Number (RIN) and DV_{200} value.

For intact (RIN > 7) or partially degraded RNA samples (RIN = 2 to 7) follow the library preparation Steps 1A and 2A.

For highly degraded samples (RIN = 1 to 2) (e.g. FFPE), determine the DV_{200} value. We do not recommend proceeding with RNA samples that have a DV_{200} <20%. These samples do not require fragmentation and will follow the instructions for highly degraded RNA (Steps 1B and 2B).

RNA Purity:
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).

Part I: ImmunoPrism Pre-Capture Library Preparation

Starting Material: Quantify RNA using a fluorimetric assay, such as the Qubit Fluorometer. Prepare RNA by diluting in nuclease-free water to a final volume of 5 µl using the guidelines below:

Starting RNA Material	RNA Input (ng)
Intact or partially degraded from fresh frozen tissue (RIN>2)	20
FFPE RNA (DV_{200}>20%)	40

For the ImmunoPrism Human Control sample included with the kit, dilute 1 µl of the provided RNA in 4 µl of nuclease-free water and proceed with steps 1B and 2B.

IMPORTANT: For high quality RNA (such as non-FFPE derived RNA with RIN>2), begin with Step 1A. For FFPE or highly degraded RNA, including the enclosed ImmunoPrism Human control, skip to Step 1B.

1A High Quality RNA Fragmentation and Priming

Note: For highly degraded/FFPE RNA, skip to step 1B

1.1A Assemble the fragmentation and priming reaction on ice in a nuclease-free PCR tube by adding the following components:

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

1.2A Mix thoroughly by pipetting up and down several times.

1.3A Briefly spin down the samples in a microcentrifuge.

1.4A Place the samples in a thermal cycler and incubate the sample at 94 °C following the recommendations:

RNA Type	RIN	Fragmentation Time
Intact RNA	>7	15 minutes at 94 °C
Partially Degraded RNA	2-6	7-8 minutes at 94 °C

Note: If you are preparing both High Quality and FFPE RNA, you can begin prepping the FFPE RNA (Step 1B) during the Fragmentation Incubation.

1.5A Immediately transfer the tubes to ice and proceed to First Strand cDNA Synthesis For High Quality RNA (Step 2A).

2A First Strand cDNA Synthesis for High Quality RNA

2.1A Assemble the first strand synthesis reaction on ice by adding the following components:

First Strand Synthesis	Volume
Fragmented and Primed RNA (Step 1.5A)	10 µl
◯ (white) First Strand Synthesis Specificity Reagent	8 µl
◯ (white) First Strand Synthesis Enzyme Mix	2 µl
Total Volume	20 µl

2.2A Keeping the reactions on ice, thoroughly mix by pipetting up and down several times.
2.3A Briefly spin down the samples in a microcentrifuge.
2.4A Proceed directly to First Strand Synthesis Incubation (Step 3).

1B Priming of Highly Degraded (FFPE) RNA that Does Not Require Fragmentation

Note: For intact or partially degraded RNA, perform Step 1A instead.

1.1B Assemble the priming reaction on ice in a nuclease-free PCR tube by adding the following components:

Priming Reaction	Volume
FFPE RNA (40 ng)	5 µl
◯ (white) Random Primers	1 µl
Total Volume	6 µl

1.2B Mix thoroughly by pipetting up and down several times.

1.3B Briefly spin down the samples in a microcentrifuge.

1.4B Incubate the samples in a preheated thermal cycler as follows:
5 minutes at 65 °C, with heated lid set at 105 °C, Hold at 4 °C

1.5B Transfer the tubes directly to ice and proceed to First Strand cDNA Synthesis for Highly Degraded (FFPE) RNA (Step 2B).

2B First Strand cDNA Synthesis for Highly Degraded (FFPE) RNA

2.1B Assemble the first strand synthesis reaction on ice by adding the following components:

First Strand Synthesis	Volume
Primed RNA (Step 1.5B)	6 µl
◯ (white) First Strand Synthesis Reaction Buffer	4 µl
◯ (white) First Strand Specificity Reagent	8 µl
◯ (white) First Strand Synthesis Enzyme Mix	2 µl
Total Volume	20 µl

2.2B Keeping the reactions on ice, thoroughly mix by pipetting up and down several times.

2.3B Briefly spin down the samples in a microcentrifuge.

2.4B Proceed directly to First Strand Synthesis Incubation.

3 First Strand Synthesis Incubation

3.1 Keeping the tubes on ice, mix thoroughly by pipetting up and down several times.

3.2. Briefly spin down the samples in a microcentrifuge.

3.3 Incubate the samples in a preheated thermal cycler with the heated lid set at ≥ 80 °C as follows:

Step 1: 10 minutes at 25 °C
Step 2: 15 minutes at 42 °C
Step 3: 15 minutes at 70 °C
Step 4: Hold at 4 °C

3.4 Proceed directly to Second Strand cDNA Synthesis.

4 Second Strand cDNA Synthesis

4.1 Prepare the second strand cDNA synthesis reaction on ice by adding the following components to the first strand reaction product from Step 3.3:

Second Strand Synthesis Reaction	Volume
First Strand Synthesis Product (Step 3.3)	20 µl
(orange) Second Strand Synthesis Reaction Buffer	8 µl
(orange) Second Strand Synthesis Enzyme Mix	4 µl
Nuclease-free Water	48 µl
Total Volume	80 µl

4.2 Keeping the tubes on ice, mix thoroughly by pipetting up and down several times.

4.3 Incubate in a thermal cycler for 1 hour at 16 °C with the heated lid set at ≤ 40 °C.

5 cDNA Cleanup Using Ampure XP Beads

5.1 Allow Ampure XP Beads to warm to room temperature for at least 30 minutes before use.

5.2 Vortex Ampure XP Beads for approximately 30 seconds to resuspend.

5.3 Add 144 µl (1.8X) of resuspended beads to the second strand synthesis reaction (~80 µl). Mix well by pipetting up and down at least 10 times.

5.4 Incubate for 5 minutes at room temperature.

5.5 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.

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

5.7 Repeat Step 5.6 once for a total of 2 washing steps.

5.8 Completely remove the residual ethanol. Leave the tubes on the magnetic rack and air dry the beads for approximately 5 minutes with the lid open. Do not over dry the beads, as this may result in lower recovery of DNA.

5.9 Remove the tubes from the magnet and add 53 µl 0.1X TE Buffer to the beads. Pipette up and down at least 10 times to mix thoroughly.

5.10 Incubate for 2 minutes at room temperature.

5.11 Place the tubes on a magnetic rack, allowing beads to fully separate from the supernatant.

5.12 Transfer 50 µl of the supernatant to clean nuclease-free PCR tubes. Be careful not to disturb the beads.

STOP Optional Stopping Point: cDNA samples can be stored at –20 °C.

6 End Repair of cDNA Library

6.1 Assemble the end repair reaction on ice by adding the following components to the second strand synthesis product from Step 5.10:

End Repair Reaction	Volume
Second Strand Synthesis Product (Step 5.10)	50 µl
(green) End Repair Reaction Buffer	7 µl
(green) End Repair Enzyme Mix	3 µl
Total Volume	60 µl

6.2 Set a pipette to 50 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.

Note: It is important to mix well. The presence of a small amount of bubbles will not interfere with performance.

6.3 Incubate the samples in a thermal cycler with the heated lid set at ≥ 75 °C as follows:
Step 1: 30 minutes at 20 °C
Step 2: 30 minutes at 65 °C
Step 3: Hold at 4 °C.

6.4 Proceed immediately to Adaptor Ligation.

7 Adaptor Ligation

7.1 Prior to setting up the ligation reaction, dilute the ● (red) Adaptor in ice-cold ● (red) Adaptor Dilution Buffer as follows, multiplying by the required number of samples, plus 10% extra. Keep the diluted adaptor on ice.

Ligation Dilution	Volume
● (red) Adaptor	0.5 µl
● (red) Adaptor Dilution Buffer	2 µl
Total Volume	2.5 µl

7.2 Assemble the ligation reaction on ice by adding the following components, in the order given, to the end prep reaction product from Step 6.4:

Ligation Reaction	Volume
End Prepped DNA (Step 6.4)	60 µl
Diluted Adaptor (Step 7.1)	2.5 µl
● (red) Ligation Enhancer	1 µl
● (red) Ligation Master Mix	30 µl
Total Volume	93.5 µl

Note: The Ligation Master Mix and Ligation Enhancer can be mixed ahead of time. This mixture is stable for at least 8 hours at 4 °C. Do not premix the Ligation Master Mix, Ligation Enhancer and Adaptor prior to use in the Adaptor Ligation Step.

7.3 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.

Note: The Ligation Master Mix is very viscous. Care should be taken 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.

7.4 Incubate 15 minutes at 20 °C in a thermal cycler.

7.5 Remove the ligation mixture from the thermal cycler and add 3 µl (red) Adaptor Processing Enzyme, resulting in a total volume of 96.5 µl.

7.6 Pippette up and down several times to mix well.

7.7 Incubate for 15 minutes at 37 °C, with the heated lid set to ≥ 45 °C.

7.8 Proceed immediately to Purification of Ligation Reaction.

8 Purification of Ligation Reaction Using Ampure XP Beads

8.1 Allow Ampure XP Beads to warm to room temperature for at least 30 minutes before use.

8.2 Vortex Ampure XP Beads for approximately 30 seconds to resuspend.

8.3 Add 87 µl (0.9X) resuspended Ampure XP Beads and mix well by pipetting up and down at least 10 times.

8.4 Incubate for 10 minutes at room temperature.

8.5 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 minutes), carefully remove and discard the supernatant. Do not discard the beads.

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

8.7 Repeat Step 8.6 once for a total of 2 washing steps.

8.8 Completely remove the residual ethanol. Leave the tubes on the magnetic rack and air dry the beads for approximately 5 minutes with the lid open. Do not over dry the beads, as this may result in lower recovery of DNA.

8.10 Remove the tubes from the magnet and add 17 µl 0.1X TE Buffer to the beads. Pipette up and down at least 10 times to mix thoroughly.

8.11 Incubate for 2 minutes at room temperature.

8.12 Place the tubes on a magnetic rack, allowing beads to fully separate from the supernatant.

8.13 Transfer 15 µl of the supernatant to clean nuclease-free PCR tubes. Be careful not to disturb the beads.

STOP Optional Stopping Point: Adaptor-ligated DNA can be stored at –20 °C.

9 PCR Enrichment of Adaptor Ligated DNA

9.1 Set up the PCR reaction as described below:

PCR Enrichment	Volume
Adaptor ligated DNA (Step 8.9)	15 µl
(violet) Pre-Capture PCR Master Mix	25 µl
(violet) Universal PCR Primer	5 µl
(violet) Index (X) Primer	5 µl
Total Volume	50 µl

Note: 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.

9.2 Mix well by gently pipetting up and down 10 times. Briefly spin the tubes in a microcentrifuge.

9.3 Place the tubes on a thermal cycler with the heated lid set to 105 °C and perform PCR amplification using the following PCR cycling conditions:

IMPORTANT: The number of PCR cycles varies depending on the quality of RNA used. Be sure to use the correct number of cycles for each sample.

Cycle Step	Temp	Time	Number of Cycles
Initial Denaturation	98 °C	30 seconds	1
Denaturation	98 °C	10 seconds	High Quality RNA: 10
Annealing/Extension	65 °C	75 seconds	FFPE RNA: 15
Final Extension	65 °C	5 minutes	1
Hold	4 °C	∞	

Note: PCR cycles are recommended based on internally tested RNA. It may require optimization based on the sample quality to prevent PCR over-amplification. For the ImmunoPrism Human Control RNA, follow recommendations for FFPE RNA (15 cycles).

10 Purification of the PCR Reaction Using Ampure XP Beads

10.1 Allow Ampure XP Beads to warm to room temperature for at least 30 minutes before use.

10.2 Vortex Ampure XP Beads for approximately 30 seconds to resuspend.

10.3 Add 45 µl (0.9X) of resuspended beads to each PCR reaction (~50 µl). Mix well by pipetting up and down at least 10 times.

10.4 Incubate for 5 minutes at room temperature.

10.5 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 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA.

10.6 Add 180 µl of freshly prepared 80% ethanol to the tubes while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

10.7 Repeat Step 10.6 once for a total of 2 washing steps.

10.8 Completely remove the residual ethanol. Leave the tubes on the magnetic rack and air dry the beads for approximately 5 minutes with the lid open. Do not over dry the beads, as this may result in lower recovery of DNA.

10.9 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.

10.10 Incubate for 2 minutes at room temperature.

10.11 Place the tubes on a magnetic rack, allowing beads to fully separate from the supernatant.

10.12 Transfer 20 µl of the supernatant to clean nuclease-free PCR tubes. Be careful not to disturb the beads.

STOP Optional Stopping Point: Pre-Capture Libraries can be stored at –20 °C.

11 Validate and Quantify Pre-Capture Library

11.1 Measure the concentration of the pre-capture library using a Qubit Fluorometer and the Qubit dsDNA HS Assay Kit. A minimum yield of 200 ng is required to proceed to Part II: ImmunoPrism Hybridization and Capture.

11.2 Run 1 µl library on an Agilent Bioanalyzer DNA High Sensitivity Chip or similar electrophoresis. If necessary, dilute the sample to avoid overloading the High Sensitivity Chip, according to the Agilent Bioanalyzer DNA High Sensitivity Chip protocol recommendations.

11.3 Check that the electropherogram shows a narrow distribution with a peak size approximately 250-400 bp (see Appendix Figures A1 and A2).

Note: If a 128 bp (adaptor-dimer) is visible in the Bioanalyzer traces, and the intensity of the signal is ≥ the intensity of 250-400 bp library signal (see Appendix Figure A3), then bring up the sample volume (from Step 10.10) to 50 µl with 0.1X TE Buffer and repeat the Ampure XP Bead purification (Step 10). For FFPE RNA libraries, there may be a second peak around 1000 bp. As long as the intensity of this peak is low relative to the library peak, this should not significantly impact Part II: ImmunoPrism Hybridization and Capture (see Appendix Figures A2 and A3).

STOP Optional stopping point: Pre-capture libraries can be stored at –20 °C before moving on to Part II: ImmunoPrism Hybridization and Capture.

Part II: ImmunoPrism Hybridization and Capture

12 Combine Blocking Oligos, Cot-1 DNA, Pre-capture Library DNA, and Dry

12.1 Mix the following in a nuclease-free 1.5 mL microtube (See Optional Protocol Modifications):

Reagent	Quantity
Barcoded library from Step 10.10	200 ng
Cot-1 DNA	2 µg
(blue) Blocking Oligos	2 µl

12.2 Dry the contents of the tube using a vacuum concentrator (e.g., SpeedVac® System or a similar evaporator device) set to 30-45 °C.

STOP Optional stopping point: After drying, tubes can be stored overnight at room temperature (15–25 °C) or for longer at -20 °C.

13 Hybridize DNA Capture Probes with the Library

13.1 Thaw 2X Bead Wash Buffer and (all blue) 2X 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.

Note: Inspect the tube of 2X Hybridization Buffer for crystallization of salts. If crystals are present, heat the tube at 65 °C, shaking intermittently, until the buffer is completely solubilized; this may require heating for several hours.

13.2 Create the Hybridization Master Mix in a tube. Multiply volumes by the number of samples and add 10% extra:

Hybridization Master Mix	Volume (ul)
(blue) 2X Hybridization Buffer	8.5
(blue) Hybridization Buffer Enhancer	2.7
(blue) ImmunoPrism Probe Panel	5
Nuclease-Free Water	0.8
Total Volume	17

13.3 Vortex or pipette up and down to mix well.
13.4 Add 17 µl of the Hybridization Master Mix to each tube containing dried DNA.
13.5 Seal the tubes and incubate for 5 minutes at room temperature.
13.6 Vortex the samples, ensuring they are completely mixed.
13.7 Spin down the samples briefly in a microcentrifuge.
13.8 Transfer each sample to a nuclease-free PCR tube.
13.9 Place the samples in a thermal cycler and run the following program (with heated lid at 100 °C):

Step 1: 30 seconds at 95 °C
Step 2: 4 hours at 65 °C
Step 3: Hold at 65 °C

Note: During the incubation, prepare the wash buffers (Step 14) and streptavidin beads (Step 15), allowing for sufficient time to preheat buffers and equilibrate the streptavidin beads.

14 Prepare Wash Buffers

14.1 During the Hybridization incubation, dilute the 2X Bead Wash Buffer and the 🔵 (blue) 10X Wash Buffers to create 1X working solutions as follows, multiplying by the required number of samples and adding 10% extra:

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

Note: If 10X Wash Buffer 1 is cloudy, heat the bottle in a 65 °C water bath or heating block to resuspend particulates. The 1X working solutions are stable at room temperature (15-25 °C) for up to 4 weeks or at -20 °C until their expiration date. Frozen 1X Wash Buffers should be mixed after thawing.

14.2 Aliquot the 1X Wash Buffers into nuclease-free PCR tubes and place at the appropriate temperatures as indicated in the table below. 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

14.3 Prepare the Bead Resuspension Mix according to the following table, multiplying by the required number of samples and adding 10% extra:

Bead Resuspension Mix	Volume (µl)
(blue) 2X Hybridization Buffer	8.5
(blue) Hybridization Buffer Enhancer	2.7
Nuclease-Free Water	5.8
Total Volume	17

15 Prepare the Streptavidin Beads

15.1 Equilibrate Dynabeads M-270 Streptavidin beads at room temperature for at least 30 minutes before use.

15.2 Mix the beads thoroughly by vortexing for 15 seconds.

15.3 Aliquot 50 µl of beads per capture into a nuclease-free PCR tube.

15.4 Add 100 µl of 1X Bead Wash Buffer (prepared in Step 14.1) to each tube. Gently pipette up and down 10 times to mix.

15.5 Place the tube on a magnetic rack, allowing beads to fully separate from the supernatant.

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

15.7 Perform the following wash:

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

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

C. Carefully remove and discard the clear supernatant.

15.8 Repeat Step 15.7 once for a total of two washes.

15.9 Remove from magnetic rack. Add 17 µl of Bead Resuspension Mix from Step 14.3 to each tube.

15.10 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.

16 Bind Hybridized Target to the Streptavidin Beads

16.1 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**.

16.2 Using a multichannel pipette, transfer 17 µl fully homogenized beads to the samples.

16.3 Mix thoroughly by pipetting up and down 10 times.

16.4 Bind the DNA to the beads by placing the tubes into the thermal cycler set to 65 °C (**with the heated lid set to 70 °C**) for 45 minutes. During the incubation, briefly remove the strip tubes every 10-12 minutes and gently vortex for 3 seconds to ensure that the beads remain in suspension. Alternatively, mix by pipetting up and down several times.

16.5 Proceed immediately to Wash Streptavidin Beads (Step 17).

17 Wash Streptavidin Beads to Remove Unbound DNA

⚠ **CAUTION: THE FOLLOWING WASHES ARE CRITICAL FOR REMOVING OFF-TARGET DNA. USE CARE AND FOLLOW ALL PROTOCOL STEPS CLOSELY.**

Note: Use the 1X wash buffers from Step 14. Keep heated buffers in the thermal cycler during washes.

17.1 Add 100 µl preheated 1X Wash Buffer 1 to the tubes from Step 16.4.

17.2 Mix thoroughly by pipetting up and down 10 times.

17.3 Place the tubes on a magnetic rack, allowing beads to fully separate from the supernatant.

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

17.5 Perform the following 65 °C wash:

A. Add 150 µl of preheated 1X Stringent Wash Buffer.
B. Mix thoroughly by pipetting up and down at least 10 times. Avoid bubbles during pipetting. **Be sure beads are completely resuspended in all tubes.**
C. Incubate in the thermal cycler at 65 °C for 5 minutes.
D. Place the tubes on a magnetic rack, allowing beads to fully separate from the supernatant.
E. Pipette and discard the supernatant, which contains unbound DNA. Remove from magnetic rack.

17.6 Repeat Step 17.5 for a total of two Stringent Washes.

17.7 Perform the first room temperature wash:

A. Add 150 µl of room temperature 1X Wash Buffer 1.
B. Pipette up and down 10 to 20 times to completely resuspend the beads.
C. Seal the tubes and incubate for 2 minutes, alternating between gently vortexing for 30 seconds and resting for 30 seconds. **Be sure beads in all wells remain completely resuspended in all tubes throughout the entire incubation.**
D. Briefly centrifuge the tubes.
E. Place the tubes on a magnetic rack, allowing beads to fully separate from the supernatant.
F. Pipette and discard the supernatant.
G. Seal the tubes and briefly centrifuge. Return to magnetic rack and use a 10 µl pipette to remove any residual wash buffer.

17.8 Perform the second room temperature wash:

A. Add 150 µl of room temperature 1X Wash Buffer 2.
B. Pipette up and down 10 to 20 times to completely resuspend the beads.
C. Seal the tubes and incubate for 2 minutes, alternating between gently vortexing for 30 seconds and resting for 30 seconds. **Be sure beads in all wells remain completely resuspended in all tubes throughout the entire incubation.**
D. Briefly centrifuge the tubes.
E. 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.**
F. Place the tubes on a magnetic rack, allowing beads to fully separate from the supernatant.
G. Pipette and discard the supernatant.
H. Seal the tubes and briefly centrifuge. Return to magnetic rack and use a 10 µl pipette to remove any residual wash buffer.

17.9 Perform the third room temperature wash:

A. Add 150 µl of room temperature 1X Wash Buffer 3.
B. Pipette up and down 10 to 20 times to completely resuspend the beads.
C. Seal the tubes and incubate for 2 minutes, alternating between gently vortexing for 30 seconds and resting for 30 seconds. **Be sure beads in all wells remain completely**

resuspended in all tubes throughout the entire incubation.

D. Briefly centrifuge the tubes.
E. Place the tubes on a magnetic rack, allowing beads to fully separate from the supernatant.
F. Pipette and discard the supernatant.
G. Seal the tubes and briefly centrifuge. Return to magnetic rack and use a 10 µl pipette to remove any residual wash buffer.

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

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

Important: Do not discard the beads. Use the entire 20 µl of resuspended beads with captured DNA in Step 18.

18 Perform Final, Post-Capture PCR Enrichment

18.1 Prepare the Post-Capture PCR Master Mix according to the following table, multiplying by the required number of samples and adding 10% extra:

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

18.2 Add 30 µl of the Post-Capture PCR Master Mix to each sample for a final reaction volume of 50 µl.

18.3 Mix thoroughly by pipetting up and down 10 times.

18.4 Place the PCR tubes in the thermal cycler, and run the following program **with the heated lid set to 105 °C**:

Cycle Step	Temp	Time	Number of Cycles
Polymerase activation	98 °C	45 seconds	1
Denaturation	98 °C	15 seconds	
Annealing	60 °C	30 seconds	14
Extension	72 °C	30 seconds	
Final extension	72 °C	1 minute	1
Hold	4 °C	Hold	1

STOP Optional stopping point: PCR-enriched captures can be stored at 4 °C overnight.

19 Purify Post-capture PCR Fragments

19.1 Allow Ampure XP Beads to warm to room temperature for at least 30 minutes before use.

19.2 Vortex Ampure XP Beads for approximately 30 seconds to resuspend.

19.3 Add 75 µl (1.5X) of resuspended beads to each PCR-enriched capture (50 µl). Mix well by pipetting up and down at least 10 times.

Note: The streptavidin beads will not interfere with the Ampure XP bead purification.

19.4 Incubate for 5 minutes at room temperature.

19.5 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.

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

19.7 Repeat Step 19.6 once for a total of 2 washing steps.

19.8 Completely remove the residual ethanol. Leave the tube on the magnetic rack and air dry approximately 5 minutes with the lid open. Do not over-dry the beads. This may result in lower recovery of DNA.

19.9 Remove the tube from the magnet. Elute the DNA from the beads by adding 22 µl 10 mM TE Buffer. Mix well by pipetting up and down several times. Incubate for 2 minutes at room temperature. Place the tube on the magnetic rack until the solution is clear.

19.10 Remove 20 µl of the supernatant and transfer to a clean nuclease-free PCR tube, being careful not to disturb the beads.

Optional stopping point: Libraries may be stored at –20 °C.

20 Validate and Quantify Library

20.1 Measure the concentration of the captured library using a Qubit Fluorometer and the Qubit dsDNA HS Assay Kit.

20.2 Measure the average fragment length of the captured library using the Agilent 2100 Bioanalyzer High Sensitivity DNA chip.

20.3 Calculate the average fragment size for each library using the Agilent 2100 Bioanalyzer software. Average fragment size should be approximately 250-400 bp (see Appendix, Figures A4 and A5).

Note: For sequencing, we recommend diluting libraries to 2nM and following Illumina's guidelines for sequencing. Libraries should be sequenced to a minimum depth of 15 million reads. For more information on sequencing and uploading your data for ImmunoPrism analysis, visit www.cofactorgenomics.com/immunoprism-kit.

Stopping point: Libraries may be stored at –20 °C.

Sequencing and Analysis

For sequencing, we recommend diluting libraries to 2nM and following Illumina's guidelines for sequencing. Libraries should be sequenced to a minimum depth of 15 million single end reads of at least 50 bp in length.

The analysis portion of the ImmunoPrism kit is completed via our cloud platform, Prism. In preparation of running analysis on Prism, you are required to upload your sequenced samples onto BaseSpace, Illumina's sequencing hub. If applicable, we recommend you setup your machine before sequencing to automatically upload your sequencing run to Basespace. Please see the Prism instructions at https://cofactorgenomics.com/immunoprism-kit/ for more details.

Appendix: Example Bioanalyzer Traces

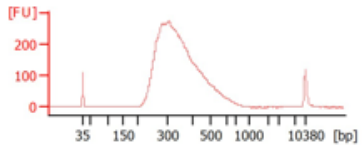


Figure A1: Typical Pre-capture Library Bioanalyzer trace for an intact RNA sample.

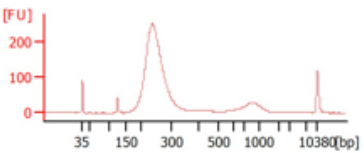


Figure A2: Typical Pre-capture Library Bioanalyzer trace for an FFPE RNA sample. The second peak around 1000 bp is indicative of overamplification. If this peak is small relative to the main peak around 250-400 bp (as shown), it will not interfere with downstream steps or analysis. If the second peak is large relative to the 250-400 bp peak, the pre-capture library can be remade with fewer PCR cycles in order to reduce overamplification.

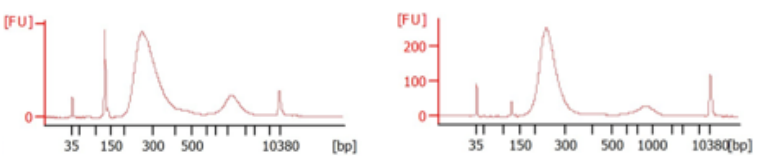


Figure A3: Pre-capture library Bioanalyzer traces showing excessive adaptor dimer (left) and acceptable adaptor dimer (right). The adaptor dimer shows up as a sharp peak around 128 bp. Both traces show evidence of mild over-amplification, but this should not interfere with the ImmunoPrism Assay.

Appendix: Example Bioanalyzer Traces, cont.

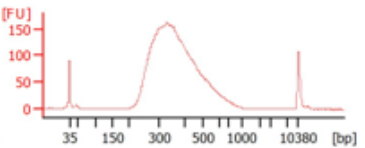


Figure A4: Typical Final Library Bioanalyzer trace for an intact RNA sample.

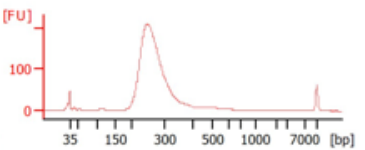
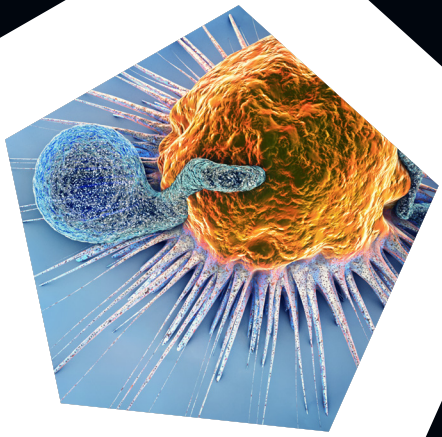


Figure A5: Typical Final Library Bioanalyzer trace for an FFPE RNA sample. Libraries made from FFPE RNA tend to have a smaller average size distribution than libraries made from intact RNA.



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