Store at -20°C

InTraSeq™ 3' Assay Kit

#82906

1 Kit
(8 assays)

Support: +1-978-867-2388 (U.S.)
cellsignal.com/support

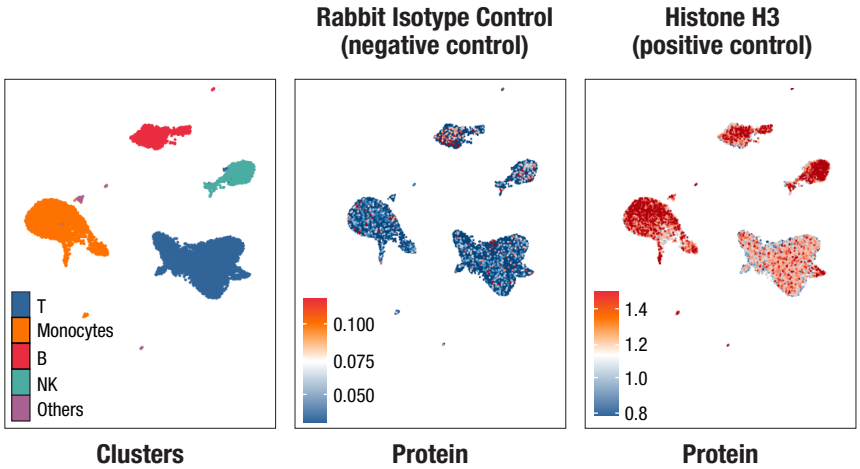
Orders: 877-616-2355 (U.S.)
orders@cellsignal.com

For Research Use Only. Not for Use in Diagnostic Procedures.

Product Includes	Product #	Kit Quantity
InTraSeq™ Wash Buffer	59335	4 x 35 mL
InTraSeq™ Blocking Buffer A	10471	1 x 7 mL
InTraSeq™ RNase Inhibitor	79298	1 x 250 µL
InTraSeq™ Blocking Buffer B	31701	1 x 1.1 mL
InTraSeq™ Staining Buffer	46005	1 x 500 µL
InTraSeq™ Reducing Agent	63760	1 x 400 µL
Rabbit (DA1E) mAb IgG XP® Isotype Control (InTraSeq™ 3' Conjugate 3000)	81472	1 x 25 µL
Histone H3 (D1H2) XP® Rabbit mAb (InTraSeq™ 3' Conjugate 3002)	68984	1 x 25 µL

Description: The InTraSeq™ method enables researchers to measure proteins (cytoplasmic, surface, and nuclear), including post-translational modifications (PTMs), along with RNA in their single-cell assays. While single-cell RNA sequencing (scRNAseq) has transformed the field of cellular and molecular biology, it only measures RNA transcripts within single cells. This presents a significant challenge, as RNA levels don't necessarily translate to protein abundance or activity. The InTraSeq technology addresses this limitation and can unravel missing information in scRNAseq experiments, uncovering new cell states and crucial biological data in single-cell analyses, especially since RNA does not reflect PTMs.

The InTraSeq™ 3' Assay Kit provides important controls to ensure a successful experiment. The kit contains a negative control Rabbit (DA1E) mAb IgG XP® Isotype Control (InTraSeq™ 3' Conjugate 3000) #81472 conjugated to a 10x Genomics Feature Barcode, a positive control Histone H3 (D1H2) XP® Rabbit mAb (InTraSeq™ 3' Conjugate 3002) #68984 conjugated to a 10x Genomics Feature Barcode, and a protocol for performing 8 assays. The InTraSeq™ 3' Assay Kit is specifically designed to be used with the InTraSeq™ 3' Antibody Cocktails and InTraSeq™ 3' Conjugates and is validated by CST using the 10x Genomics Chromium Single Cell 3' Kits with Feature Barcoding technology.



Human peripheral blood mononuclear cells (PBMCs) were processed in accordance with the IntraSeq™ 3' protocol and analyzed using the Seurat computational package. The left figure shows the Uniform Manifold Approximation and Projection (UMAP) of the annotated PBMC clusters. The FeaturePlot in the middle figure displays the Rabbit (DA1E) mAb IgG XP® Isotype Control (InTraSeq™ 3' Conjugate 3000) #81472 expression and the FeaturePlot in the right figure displays the Histone H3 (D1H2) XP® Rabbit mAb (InTraSeq™ 3' Conjugate 3002) #68984 expression. To generate similar plots, use the FeaturePlot command template: FeaturePlot(your_object, features = your_protein, max.cutoff = "q95", min.cutoff = "q5", order = TRUE) + scale_color_gradientn(colours = c("#22578B", "#73A9D2", "grey90", "#FDB393", "#b22222"))

Storage: All components in this kit are stable for at least 12 months when stored at -20ºC.

Please visit cellsignal.com for validation data and a complete listing of recommended companion products.

Applications: W—Western **IP**—Immunoprecipitation **IHC**—Immunohistochemistry **ChIP**—Chromatin Immunoprecipitation **IF**—Immunofluorescence **F**—Flow cytometry **FC-FP**— Flow cytometry-Fixed/Permeabilized **FC-L**— Flow cytometry-Live **E-P**—ELISA-Peptide
Species Cross-Reactivity: H—human **M**—mouse **R**—rat **Hm**—hamster **Mk**—monkey **Mi**—mink **C**—chicken **Dm**—D. melanogaster **X**—Xenopus **Z**—zebrafish **B**—bovine **Dg**—dog **Pg**—pig **Sc**—S. cerevisiae **Ce**—C. elegans **Hr**—Horse
All—all species expected Species enclosed in parentheses are predicted to react based on 100% homology.

InTraSeq™ 3' Protocol

For best results:

- Maintain an RNase-free and sterile workspace.
- It is highly recommended to use filtered pipette tips.
- When starting section **B. Blocking (Day 2)** until the end of the protocol, it is crucial to perform the centrifugations at 850 x g (not RPM).
- When starting section **C. Immunostaining (Day 2)** in the protocol, it is crucial to maintain the cell pellet submerged (in ~40 µL) when removing and discarding the supernatant.
- Do not vacuum aspirate when discarding the supernatant in any step; always use a pipette.
- Make sure the reagents are completely thawed before use.

NOTES:

- All InTraSeq™ 3' Assay Kit components are validated by CST in the 10x Genomics Chromium Single Cell 3' Kits with Feature Barcoding technology.
- **SAFE STOP –** This indicates a safe stopping point in the protocol if stopping is necessary.
- This protocol is designed for a single InTraSeq™ assay (one sample). If processing multiple samples, all quantities should be adjusted proportionally.

Solutions and Reagents

Materials Included in Kit (Store at -20°C)

- InTraSeq™ Blocking Buffer A #10471
- InTraSeq™ Blocking Buffer B #31701
- InTraSeq™ Staining Buffer #46005
- InTraSeq™ Wash Buffer #59335
- InTraSeq™ Reducing Agent #63760
- InTraSeq™ RNase Inhibitor #79298
- Rabbit (DA1E) mAb IgG XP® Isotype Control (InTraSeq™ 3' Conjugate 3000) #81472
- Histone H3 (D1H2) XP® Rabbit mAb (InTraSeq™ 3' Conjugate 3002) #68984

Required Reagents Not Included

- InTraSeq™ 3' Conjugates/Antibody Cocktails
- Two sterile 40-micron Falcon cell strainers
- Vortex mixer
- 1.5 mL Sterile Polypropylene Microcentrifuge Tubes - LoBind
- 15 mL Conical Sterile Polypropylene Centrifuge Tubes (preferably LoBind)
- 50 mL Conical Sterile Polypropylene Centrifuge Tubes (preferably LoBind)
- Phosphate Buffered Saline (PBS-1X) pH7.2 (Sterile) #9872
- Methanol #13604

Before starting:

(Optional) When you receive the kit, thaw and pipette mix the InTraSeq™ Blocking Buffer A, then aliquot 870 µL into 8 separate 1.5 mL sterile microcentrifuge tubes.

A. Cell Fixation (Day 1)

NOTE: If performing an immunostaining step prior to this protocol, we highly recommend beginning immunostaining with at least 2 million cells to guarantee 1-5 million cells in step 1 below.

1. Count and transfer 1-5 million cells to a 15 mL tube.

   NOTE: Do not proceed with the protocol with less than 1 million cells.
2. Centrifuge the cells at 300 x g for 5 min at 4°C.
3. Remove and discard the supernatant, then wash the cells with 10 mL of ice-cold 1X PBS.
4. Centrifuge the cells at 300 x g for 5 min at 4°C.
5. Remove and discard supernatant, without disturbing the cell pellet, then resuspend the cells in 0.5 mL of ice-cold 1X PBS.
6. Turn on the vortex mixer and keep it on the "**On**" switch (**not the "Auto/Touch" mode**) at low speeds (~speed 1-4 depending on your mixer). **The sample should NOT be vortexed, but it should rather be constantly mixed**. If the mixer is fast enough to vortex the sample, decrease the speed accordingly.

   NOTE: It is very important to **drop the ice-cold methanol slowly (>30 sec)** in the step below.
7. Place the 15 mL tube on the vortex mixer while it is on the "On" switch, then slowly add 4.5 mL of ice-cold methanol in a **dropwise manner (>30 sec)**.
8. Incubate the cells overnight in the -20°C freezer. **(SAFE STOP - sample can be stored in the -20°C freezer for up to 7 days)**

B. Blocking (Day 2)

From this point forward, at every centrifugation step, it is crucial to spin down the cells at 850 x g. Decreasing the spin down speed will result in cell loss.

1. Thaw the following reagents at room temperature:
   - Thaw 1 bottle of InTraSeq™ Wash Buffer.
   - Thaw the InTraSeq™ Blocking Buffer B.
   - Thaw and pipette mix the InTraSeq™ Blocking Buffer A.
   - Thaw and vortex mix the InTraSeq™ Reducing Agent until the precipitate disappears.
2. Once thawed, place the reagents on ice.
3. Prepare the heat/cooled InTraSeq™ Blocking Buffer B:

   a. Aliquot 130 µL InTraSeq™ Blocking Buffer B into a sterile 1.5 mL microcentrifuge tube. Return the stock InTraSeq™ Blocking Buffer B to the freezer.

   b. Heat the aliquoted tube at 95°C for 5 min.

InTraSeq™ 3' Protocol

c. Spin the tube down briefly then place it on ice. Use this heat/cooled InTraSeq™ Blocking Buffer B in the steps below.

4. Prepare the **scBlock** (see table below), pipette mix. Store on ice.

scBlock	Volume
InTraSeq™ Blocking Buffer A	860 µL
Heat/cooled InTraSeq™ Blocking Buffer B	98 µL
InTraSeq™ Reducing Agent	40 µL
InTraSeq™ RNase Inhibitor	2 µL
TOTAL Volume	**1,000 µL**

5. Centrifuge the cells at 850 x g for 5 min at 4°C.

6. Remove and discard the supernatant, then add the **scBlock** to the cells. Pipette mix to resuspend the cells, then keep the cells on ice for at least 30 min.

7. During the **scBlock** incubation, prepare the **Antibody Master Mix** as follows:

a. Determine the volume of Antibodies **(Vol. of Abs.)** based on the number of InTraSeq™ 3' Antibody Cocktails and Individual InTraSeq™ 3' Conjugates (Ind. InTraSeq™ 3' Conj. or Ind. Conj.) being used (see table below). Limit the total volume to 30 µL.

	Volume (Cocktails only)	Volume (Ind. Conj. only)	Volume (Cocktails + Ind. Conj.)
InTraSeq™ 3' Cocktail	5 µL (per cocktail)	N/A	___ µL (5 µL per cocktail)
Ind. InTraSeq™ 3' Conj.	N/A	3 µL (per Ind. Conj.)	___ µL (3 µL per Ind. Conj.)
Vol. of Abs.	___ µL	___ µL	___ µL

b. Prepare the **Antibody Master Mix** (see table below) using the **Vol. of Abs.** (max 30 µL) calculated above. Gently pipette mix to minimize bubble formation, then store on ice.

Antibody Master Mix	Volume (Cocktails only)	Volume (Ind. Conj. only)	Volume (Cocktails + Ind. Conj.)
Vol. of Abs.	___ µL	___ µL	___ µL
InTraSeq™ Wash Buffer	30 µL - **Vol. of Abs.** (see row above)		
InTraSeq™ Staining Buffer	55 µL	55 µL	55 µL
Heat/cooled InTraSeq™ Blocking Buffer B	10 µL	10 µL	10 µL
Rabbit (DA1E) mAb IgG XP® Isotype Control (InTraSeq™ 3' Conjugate 3000)	3 µL	3 µL	3 µL
Histone H3 (D1H2) XP® Rabbit mAb (InTraSeq™ 3' Conjugate 3002)	3 µL	3 µL	3 µL
InTraSeq™ RNase Inhibitor	2 µL	2 µL	2 µL
TOTAL Volume	**103 µL**	**103 µL**	**103 µL**

8. After the 30 min **scBlock** incubation, add 2 mL InTraSeq™ Wash Buffer to the cells and pipette mix.

9. Filter the cells through a sterile 40-micron Falcon cell strainer into a 50 mL tube.

NOTE: To maximize cell recovery, apply gentle pressure to the pipette tip against the strainer while filtering. (See image)

10. Transfer the flow through/filtered cells into a new 15 mL tube and immediately start section **C. Immunostaining (Day 2)**.

NOTE: Store the remaining InTraSeq™ Wash Buffer at 4°C for use on Day 3.

InTraSeq™ 3' Protocol

C. Immunostaining (Day 2)

From this point forward, whenever removing and discarding the supernatant, it is crucial to maintain the cell pellet submerged (in ~40 µL). Drying the cells will negatively impact the RNA signal and result in cell loss.

1. Centrifuge the cells at 850 x g for 5 min at 4°C.
2. Remove and discard supernatant while retaining ~40 µL in the tube.
3. Add 100 µL **Antibody Master Mix** to the cells. Pipette mix to resuspend the cells.
4. Transfer to a sterile 1.5 mL LoBind microcentrifuge tube and incubate at 4°C overnight (for ~16 hr).

   NOTES:

   - Do not incubate the cells for longer than 20 hr.
   - It is crucial to maintain the overnight incubation at 4°C.

D. Washes and Count (Day 3)

Before starting:

Prepare the **Modified InTraSeq™ Wash Buffer**. Gently mix to minimize bubble formation, then keep on ice.

NOTE: If there is remaining InTraSeq™ Wash Buffer in the stock bottle, store it at -20°C.

Modified InTraSeq™ Wash Buffer	Volume
InTraSeq™ Wash Buffer	14 mL
InTraSeq™ RNase Inhibitor	26 µL

1. Add 1 mL **Modified InTraSeq™ Wash Buffer** to the 1.5 mL microcentrifuge tube containing the cells. Pipette mix then transfer the mixture to a 15 mL tube.
2. Add 2 mL **Modified InTraSeq™ Wash Buffer** so the total volume is 3 mL.
3. Centrifuge at 850 x g for 5 min at 4°C. Remove and discard supernatant while retaining ~40 µL in the tube.
4. Add 1 mL **Modified InTraSeq™ Wash Buffer**. Pipette mix to resuspend the cells, then add another 3 mL **Modified InTraSeq™ Wash Buffer** so the total volume is 4 mL.
5. Repeat steps "3" and "4", then filter the 4 mL cell suspension through a sterile 40-micron Falcon cell strainer into a 50 mL tube. Transfer the flow through/filtered cells into a new 15 mL tube.

   NOTE: To maximize cell recovery, apply gentle pressure to the pipette tip against the strainer while filtering. (See image)

6. Centrifuge the cells at 850 x g for 5 min at 4°C. Remove and discard supernatant while retaining ~40 µL in the tube.
7. Add 100 µL **Modified InTraSeq™ Wash Buffer** to the cells. Pipette mix to resuspend the cells, then keep the cells on ice.

   NOTE: From this point forward, keep the cells in the **Modified InTraSeq™ Wash Buffer** including any necessary dilutions. **Using any other buffer or solution will result in RNA degradation.**
8. Count the cells. Refer to the Cell Suspension Volume Calculator Table in the 10x Genomics Chromium Single Cell 3' Kits with Feature Barcoding technology protocol to determine the desired cell concentration.

   IMPORTANT: Dilute the cells using **ONLY** the **Modified InTraSeq™ Wash Buffer** to reach the desired cell concentration.

   NOTE: If cell clumps are observed after counting, additional pipette mixing is recommended before proceeding to the single-cell experiment.
9. Process the cells using the 10x Genomics Chromium Single Cell 3' Kits with Feature Barcoding technology.