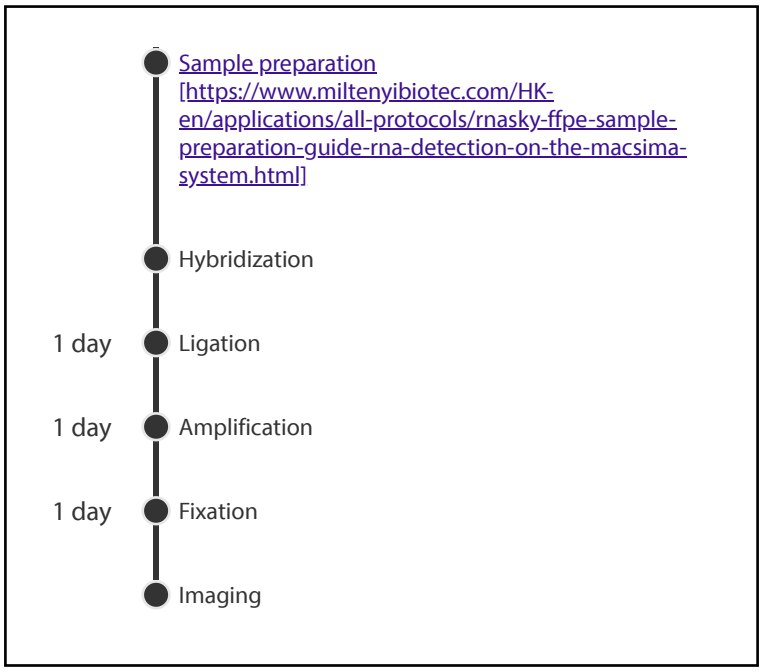
RNAsky™ User Guide: RNA detection on the MACSima™ System

This protocol describes step-by-step the molecular preparation of mounted samples before an RNAsky Assay. The RNAsky assay enables sensitive and specific detection of RNA targets in their spatial context using the MACSima System. The MACSima System is a fully automated instrument capable of same-section high-plex protein and RNA detection using the MACSima Imaging Cyclic Staining (MICS) technology.

Protocol



1. Detailed timelines

Table 1: Detailed timeline of day 1. Tissue sectioning, deparaffinization, antigen retrieval, and MACSwell Assembly steps are part of the RNAsky FFPE Sample Preparation Guide.

RNAsky FFPE Sample Preparation Guide [https://www.miltenyibiotec.com/HK-en/applications/all-protocols/rnasky-ffpe-sample-preparation-guide-rna-detection-on-the-macsima-system.html]

Day 1 (200 min)	
90 min	Tissue sectioning
60 min	Deparaffinization
20 min	Antigen retrieval
10 min	MACSwell™ Assembly
20 min	Preparation of hybridization mixture
Overnight	Hybridization

Table 2: Detailed timeline of day 2.

Day 2 (80 min)	
60 min	Post-hybridization wash
20 min	Preparation of ligation mixture
Overnight	Ligation

Table 3: Detailed timeline of day 3.

Day 3 (60 min)	
10 min	Ligation wash
30 min	Amplification priming
20 min	Preparation of amplification mixture
Overnight	Amplification

Table 4: Detailed timeline of day 4.

Day 4 (90 min)	
10 min	Amplification wash
10 min	Preparation of fixation mixture
60 min	Fixation
10 min	Fixation wash

2. General information

Capacity of panel and kits

- One RNAsky Experiment in the MACSima System supports the staining of:
  - One well in MACSwell One Small Imaging Frames or
  - Two wells in MACSwell Four Imaging Frames or
  - Two wells across two MACSwell Four Imaging Frames

- Each RNAsky Design Panel (custom RNAsky panel) provides enough reagents for:
  - Five experiments in the MACSima System
  - Each RNAsky Design Probe Mix provides enough probes for the preparation of a maximum of:
    - Ten reactions in MACSwell Four Imaging Frames (one reaction per well) or
    - Five reactions in MACSwell One Small Imaging Frames (one reaction per well)

- Each RNAsky IO Explore Panel, human provides enough reagents for:
  - Two experiments in the MACSima System
  - Each RNAsky IO Explore Probe Mix, human provides enough probes for the preparation of a maximum of:
    - Four reactions in MACSwell Four Imaging Frames (one reaction per well) or
    - Two reactions in MACSwell One Small Imaging Frames (one reaction per well)

- Each RNAsky Detection Plate is used on the MACSima System to automatically stain the prepared samples and detect the RNA targets in their spatial context using the MICS technology. The RNAsky Assay uses one RNAsky Detection Plate per experiment.

- Each RNAsky Sample Preparation Kit provides enough reagents for preparing a maximum of:

› Eight reactions in MACSwell Four Imaging Frames (one reaction per well) or
› Four reactions in MACSwell One Small Imaging Frames (one reaction per well)

- Each RNAsky Support Kit provides enough reagents for:
› Two experiments in the MACSima System

Handling of MACSwell Imaging Frames and MACSwell HighRes Slides

- MACSwell HighRes Slides are made of thin glass. Always handle with care.
- Once a MACSwell HighRes Slide has been mounted into a MACSwell Imaging Frame, take care to pipet reagents along the side of the well without touching the tissue or glass. Pipet gently to avoid generating bubbles.
- Carefully remove all liquid from the wells in each step where washing or solution removal is required.
- Cover the sample with the lid of the MACSwell Imaging Frame on each incubation step.

Temperature-specific incubations and steps

- To maintain the correct temperature, minimize the opening and closing of the incubators between steps.
- Always allow solutions to equilibrate to the correct temperature as described in this guide.

3. Protocol

Day 1: Probe hybridization for RNAsky Design (custom RNAsky panels)

Preparation of buffers and reagents

1. Prepare 1× PBST (0.05% v/v TWEEN® 20), e.g., by adding 90 mL nuclease-free water and 50 µL TWEEN® 20 to 10 mL of 10× PBS, pH 7.4. Store at room temperature during the duration of the protocol. Prepare fresh for every new preparation. Avoid adding air bubbles to the sample.
2. Thaw Buffer A. Make sure that there is no precipitate. If a precipitate is present, vortex tube until dissolved.
3. Incubate Buffer B at +60 °C for 10 minutes before use. Vortex a few times during incubation. Cool down to room temperature.
   ▲ **Note:** Buffer B can re-crystalize with temperature changes. If precipitates are still present, reheat the tube at +60 °C for an additional 10 minutes and mix until completely dissolved.
4. Once thawed, Buffer A and Buffer B can remain at room temperature during the remainder of the protocol. If samples are not prepared within one week, store Buffer A and Buffer B at –20 °C long term.

RNAsky Design Probe Mix resuspension

- Wear fresh gloves while handling the tube to avoid nucleases.
- Lyophilized contents may appear as either film or a white powder. This variance does not affect the quality of the probes.

1. Before opening the vial, briefly spin it down to ensure any dried DNA is at the bottom of the tube. Some of the product may have been dislodged during shipping.
2. Add 500 µL of Resuspension buffer into the tube (5× concentration)
3. After suspension, shake content for 1–2 hours or allow content to resuspend overnight.
4. Store at –20 °C after use.

Preparation of the probe hybridization mix

1. Immediately before making the hybridization mixture, take the Blocking Reagent and Stability Enzyme from –20 °C and place them on ice.
   ▲ **Note:** Blocking Reagent and Stability Enzyme should be kept on ice during use.
2. Prepare the appropriate volume of probe hybridization mix at room temperature in a 1.5 mL DNA low-binding tube according to table 5. Mix by gently pipetting up and down at least ten times.
3. Take the sample(s) assembled with the MACSwell Imaging Frame and prepared according to RNAsky™ FFPE Sample Preparation Guide: RNA detection on the MACSima™ System.
4. Completely remove 1× PBST from the sample(s).
5. Add 250 µL (MACSwell Four Imaging Frames) or 500 µL (MACSwell One Small Imaging Frame) of probe hybridization mix to the sample well(s).

6. Place the sample (with the MACSwell lid on) on top of the humidified chamber's grid. Close the chamber and seal it with parafilm.
   ▲ **Note:** To set up the humidified chamber, briefly remove the grid from a T100 BIOTUBE™ Storage Box (or equivalent), add 50 mL of RNase-free water, and place the grid back. Ensure sufficient water is present throughout all incubation steps.
7. Incubate at +45 °C overnight (between 16–22 hours).
8. End of day 1. Immediately proceed to day 2.

- The required control probes are already included in the RNAsky Design Probe Mix.

Table 5: Volumes for preparation of 1× probe hybridization mix for one well of MACSwell Four or one well of MACSwell One Small Imaging Frames. Use the 1× volumes of the table to calculate the volumes required for multiple preparations.

Probe hybridization mix	Volume required for one well of a MACSwell Four Imaging Frame	Volume required for one well of a MACSwell One Small Imaging Frame
RNAse-free water	41 µL	82 µL
Buffer A	75 µL	150 µL
Buffer B	75 µL	150 µL
RNAsky Design Probe Mix	50 µL	100 µL
Blocking Reagent	2.5 µL	5 µL
Stability Enzyme	6.5 µL	13 µL
Total volume	**250 µL**	**500 µL**

Day 1: Probe hybridization for IO Explore Panel, human

Preparation of buffers and reagents

1. Prepare 1× PBST (0.05% v/v TWEEN® 20), e.g., by adding 90 mL nuclease-free water and 50 µL TWEEN® 20 to 10 mL of 10× PBS, pH 7.4. Store at room temperature during the duration of the protocol. Prepare fresh for every new preparation. Avoid adding air bubbles to the sample.
2. Thaw IO Explore Probe Mix, human, and Control Probe Mix, human at room temperature. After thawing, vortex, centrifuge briefly, and place on ice.
3. Thaw Buffer A. Make sure that there is no precipitate. If a precipitate is present, vortex tube until dissolved.
4. Incubate Buffer B at +60 °C for 10 minutes before use. Vortex a few times during incubation. Cool down to room temperature.
   ▲ **Note:** Buffer B can re-crystalize with temperature changes. If precipitates are still present, reheat the tube at +60 °C for an additional 10 minutes and mix until completely dissolved.
5. Once thawed, Buffer A and Buffer B can remain at room temperature during the remainder of the protocol. If samples are not prepared within one week, store Buffer A and Buffer B at –20 °C long term.

Preparation of the probe hybridization mix

1. Immediately before making the hybridization mixture, take the Blocking Reagent and Stability Enzyme from –20 °C and place them on ice.
   ▲ **Note:** Blocking Reagent and Stability Enzyme should be kept on ice during use.
2. Prepare the appropriate volume of probe hybridization mix at room temperature in a 1.5 mL DNA low binding tube according to table 6. Mix by gently pipetting up and down at least ten times.
   ▲ **Note:** Wear fresh gloves while handling the tube to avoid nucleases.
3. Take the sample(s) assembled with the MACSwell Imaging Frame and prepared according to RNAsky™ FFPE Sample Preparation Guide: RNA detection on the MACSima™ System.
4. Completely remove 1× PBST from the sample(s).
5. Add 250 µL (MACSwell Four Imaging Frames) or 500 µL (MACSwell One Small Imaging Frame) of probe hybridization mix to the sample well(s).
6. Place the sample (with the MACSwell lid on) on top of the humidified chamber's grid. Close the chamber and seal it with parafilm.
   ▲ **Note:** To set up the humidified chamber, briefly remove the grid from a T100 BIOTUBE™ Storage Box (or equivalent), add 50 mL of RNase-free water, and place the grid back. Ensure sufficient water is present throughout all incubation steps.

7. Incubate at +45 °C overnight (between 16–22 hours).

8. End of day 1. Immediately proceed to day 2.

Table 6: Volumes for preparation of 1× probe hybridization mix for one well of MACSwell Four or one well of MACSwell One Small Imaging Frames. Use the 1× volumes of the table to calculate the volumes required for multiple preparations.

Probe hybridization mix	Volume required for one well of a MACSwell Four Imaging Frame	Volume required for one well of a MACSwell One Small Imaging Frame
RNAse-free water	41 µL	82 µL
Buffer A	75 µL	150 µL
Buffer B	75 µL	150 µL
IO Explore Probe Mix, human	25 µL	50 µL
Control Probe Mix, human	25 µL	50 µL
Blocking Reagent	2.5 µL	5 µL
Stability Enzyme	6.5 µL	13 µL
Total volume	250 µL	500 µL

Day 2: Ligation

Post-hybridization wash

- The temperature of the post-hybridization washes is critical for optimal results. It is recommended to use a thermometer to check the correct temperature of the heat block or incubator before proceeding with this step.

1. Check Buffer B: If a precipitate is present, heat at +37 °C using a heat block for at least of 10 minutes, then vortex.
2. Prepare post-hybridization wash buffer in a 15 mL conical tube according to table 7.
3. Pre-heat post-hybridization wash buffer to +37 °C by incubating on a heat block or incubator for at least 20 minutes. While post-hybridization wash buffer is preheating, leave the sample(s) in +45 °C incubator.
4. Remove sample(s) from +45 °C incubator, immediately prior to washing.
5. Set the incubator to +37 ºC in preparation for the next section.
6. Remove the whole volume of probe hybridization mix from the sample(s). Add post-hybridization wash buffer to the sample(s). For MACSwell Four Imaging Frames, add 500 µL per well, for MACSwell One Small Imaging Frames, add 1 mL per well.
7. Wash three times with post-hybridization wash buffer for 10 minutes each by placing the sample(s) directly onto a heat block set to +37 °C. To maintain temperature, gently cover the MACSwell with aluminum foil during incubations. During incubations, place the empty humidified chamber into the +37 °C incubator to maintain temperature.
8. Wash an additional three times in 1× PBST at room temperature for 5 min each. For MACSwell Four Imaging Frames, add 500 µL per well, for MACSwell One Small Imaging Frames, add 1 mL per well.
9. Immediately proceed to the next step.

Table 7: Volumes for preparation of 1× post-hybridization wash buffer for one well of MACSwell Four or one well of MACSwell One Small Imaging Frames.

Post-hybridization wash buffer	Volume required for one well of a MACSwell Four Imaging Frame	Volume required for one well of a MACSwell One Small Imaging Frame Frame
RNAse-free water	450 µL	900 µL
Buffer A	150 µL	300 µL
Buffer B	900 µL	1800 µL
Total volume	1500 µL	3000 µL

Preparation of Ligation Buffer

1. Thaw Ligation Buffer at room temperature.
2. After thawing, vortex, centrifuge briefly, and place on ice.

Preparation of ligation mix

- Prepare ligation mix on ice in a 1.5 mL DNA low binding tube according to table 8.
  ▲ **Note:** The Ligation Enzyme is temperature-sensitive. Always keep the enzyme at –20 °C. Do not vortex.

Table 8: Volumes for preparation of ligation mix for one well of MACSwell Four and MACSwell One Small Imaging Frames.

Ligation mixture	Volume required for one well of a MACSwell Four Imaging Frame	Volume required for one well of a MACSwell One Small Imaging Frame
RNase-free water	211 µL	422 µL
Ligation Buffer	25 µL	50 µL
Blocking Reagent	2.5 µL	5 µL
Stability Enzyme	6.5 µL	13 µL
Ligation Enzyme	5 µL	10 µL
Total volume	250 µL	500 µL

Ligation

1. Gently remove 1× PBST at once with a p1000 pipette.
2. Add the ligation mix to the well.
3. Place sample(s) into pre-warmed humidified chamber and seal with parafilm.
4. Incubate at +37 °C overnight (16–22 hours).
5. End of day 2. Immediately proceed to day 3.

Day 3: Amplification

Post-ligation wash

1. Remove sample(s) from +37 °C incubator, immediately before washing.
2. Set the incubator to +30 ºC in preparation for the next section.
3. Remove the whole volume of ligation mix from the sample(s).
4. Wash the sample(s) three times for 2 min each in 1× PBST. For MACSwell Four Imaging Frames, add 500 µL per well, for MACSwell One Small Imaging Frames, add 1 mL per well. During incubations, place the empty humidified chamber back into the +30 °C incubator to maintain the temperature. Leave PBST on the sample(s) after the last wash.
5. Immediately proceed to the next step.

Preparation of amplification primer mix

1. Thaw Amplification Primer at room temperature.
2. After thawing, vortex, centrifuge briefly, and place on ice.
3. Prepare the amplification primer mix on ice in a 1.5 mL DNA low binding tube according to table 9.

Table 9: Volumes for preparation of amplification primer mix for one well of MACSwell Four and MACSwell One Small Imaging Frames.

Amplification primer mix	Volume required for one well of a MACSwell Four Imaging Frame	Volume required for one well of a MACSwell One Small Imaging Frame
RNase-free water	197.5 µL	395 µL
Buffer A	50 µL	100 µL
Amplification Primer	2.5 µL	5 µL
Total volume	250 µL	500 µL

Amplification Primer hybridization

1. Gently remove 1× PBST from the sample(s).
2. Add amplification primer mix to the well.
3. Heat the sample(s) by placing them directly onto a heat block set to +37 °C for 30 minutes. To maintain temperature, gently cover the MACSwell with aluminum foil during amplification primer hybridization.
4. Wash the sample(s) three times with 1× PBST for 2 minutes each. For MACSwell Four Imaging Frames, add 500 µL per well, for MACSwell One Small Imaging Frames, add 1 mL per well. Leave 1× PBST on the sample(s) after the last wash.

Preparation of amplification mix

1. Thaw Amplification Buffer at room temperature.
2. After thawing, vortex, centrifuge briefly, and place on ice.
3. Prepare amplification mix on in 1.5 mL DNA low binding tube according to table 10.
   ▲ **Note:** Amplification Enzyme is temperature-sensitive. Always keep the enzyme at –20 °C. Do not vortex.

Table 10: Volumes for preparation of amplification mix for one well of MACSwell Four and MACSwell One Small Imaging Frames.

Amplification mix	Volume required for one well of a MACSwell Four Imaging Frame	Volume required for one well of a MACSwell One Small Imaging Frame
Amplification Buffer	237.5 µL	475 µL
Amplification Enzyme	12.5 µL	25 µL
Total volume	250 µL	500 µL

Amplification

1. Gently remove 1× PBST from the sample(s).
2. Add amplification mix to the well.
3. Place sample(s) into a humidified chamber and seal with parafilm.
4. Incubate at +30 °C overnight (16–22 hours).
5. End of day 3. Immediately proceed to day 4.

Day 4: Fixation

Preparation of Fixation Reagent

1. Thaw Fixation Reagent at room temperature.
2. Vortex and centrifuge briefly.

Preparation of fixation reagent working solution

- Dilute Fixation Reagent 1:1000 according to table 11.

Table 11: Preparation of fixation reagent working solution.

Reagent	Volume (µL)
Fixation Reagent	2
DMSO	1998
Total volume	2000

Preparation of fixation mix

- Prepare fixation mix according to table 12.

Table 12: Preparation of fixation mix for one well of MACSwell Four and MACSwell One Small Imaging Frames.

Fixation mix	Volume required for one well of a MACSwell Four Imaging Frame	Volume required for one well of a MACSwell One Small Imaging Frame
Nuclease-free water	155 µL	310 µL
10× PBS	25 µL	50 µL
DMSO	25 µL	50 µL
1:1000- diluted Fixation Reagent	45 µL	90 µL
Total volume	250 µL	500 µL

Fixation

1. Remove the sample(s) from the +30 ºC incubator.
2. Gently remove amplification mix from the sample(s).
3. Wash the sample(s) one time for 5 min with 1× PBST. For MACSwell Four Imaging Frames, add 500 µL per well, for MACSwell One Small Imaging Frames, add 1 mL.
   ▲ **Note:** Be very gentle with pipetting before fixation.
4. Gently remove 1x PBST from the sample(s).
5. Gently add fixation mix to the well.
6. Incubate the sample(s) for 1 hour at room temperature.
7. Remove fixation mix from the sample(s).
8. Wash sample(s) three times for 2 min with 1× PBST. For MACSwell Four Imaging Frames, add 500 µL per well, for MACSwell One Small Imaging Frames, add 1 mL.
   ▲ **Note:** After fixation, sample(s) can be stored for a maximum of 5 days at +4 °C before proceeding with the MACSima Experiment. To store sample(s), add sufficient 1× PBST to the well(s) and close the MACSwell Imaging Frame with the lid. Place the sample(s) in the humidified chamber and seal them with parafilm.
9. Proceed to the MACSima Experiment.

Preparation of the MACSima Experiment

Preparation of RNAsky Detection Plate and RNAsky Release Reagent

1. Thaw the RNAsky Detection Plate and the RNAsky Release Reagent for at least 30 minutes at room temperature protected from light.
2. After thawing, vortex the RNAsky Detection Plate until no precipitate is present at the bottom of the plate.
   ▲ **Note:** There must be no precipitation present at the bottom of the plate before loading it onto the instrument.

Pre-staining of nuclei using DAPI

1. Right before the start of the MACSima Experiment, perform a DAPI pre-staining.
2. Prepare DAPI pre-staining solution according to table 13.
   ▲ **Note:** The optimal dilution factor of DAPI Staining Solution may vary depending on the sample type and might need to be adapted.
3. Remove 1× PBST from the sample(s) by pipetting.

4. Add DAPI pre-staining solution and incubate for 10 minutes in the dark at room temperature.
5. Remove DAPI pre-staining solution.
6. Gently wash the sample(s) three times with MACSima Running Buffer. For MACSwell Four Imaging Frames, add 475 µL per well, for MACSwell One Small Imaging Frames, add 950 µL per well.
7. Remove MACSima Running Buffer.
8. Add MACSima Running Buffer to each well depending on the used MACSwell Imaging Frame. For MACSwell Four Imaging Frames, add 475 µL per well, for MACSwell One Small Imaging Frames, add 950 µL.
   ▲ **Note:** Make sure that the initial sample volume is correct before loading the sample into the MACSima System.
9. Immediately proceed to the next step.

Table 13: Preparation of DAPI pre-staining solution for one well of MACSwell Four and MACSwell One Small Imaging Frames.

DAPI pre-staining solution	Volume required for one well of a MACSwell Four Imaging Frame	Volume required for one well of a MACSwell One Small Imaging Frame
DAPI Staining Solution	50 µL	100 µL
MACSima Running Buffer	200 µL	400 µL
Total volume	250 µL	500 µL

Loading the MACSima System

- Refer to the MACSima System user manual for detailed information on how to use the instrument.

1. Start the MACSima System and software.
2. Refer to the user manual and follow the instructions of the MACSima Software.
3. Load the respective experiment configuration.
4. Unpack the RNAsky Detection Plate from the pouch.
   ▲ **Note:** Do not remove the sealing or add buffer to the RNAsky Detection Plate. The MACSima System will handle the content automatically.
5. Load the Detection Plate, the components of the RNAsky Support Kit, and the MACSwell Imaging Frame(s) containing the prepared sample(s) without the lid into the instrument as instructed by the MACSima Software.
   ▲ **Note:** Be sure to load the plate in the correct orientation.
   ▲ **Note:** Remove the lids of the reagent vials and ensure that the MACSima Mixing Vials are open and empty before placing them into the instrument.
6. Start the experiment by following the instructions of the MACSima Software. For RNAsky Run setup, click on **Refine Autofocus** and choose **Image Contrast Detection** from the drop-down menu for each individual ROI that has been selected. Adjust exposure time as needed.
7. After the experiment has finished, follow the instructions of the MACSima Software. Discard the Detection Plate and the reagent vials.