Chapter 20

Tools to Investigate the Peroxisome-Dependent Antiviral Response

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Abstract

The importance of peroxisomes in the context of viral infections has been increasingly demonstrated in recent years. The discovery that MAVS localizes at peroxisomes and that peroxisomal and mitochondrial MAVS perform complementing functions within the antiviral response has raised the interest in studying the peroxisome-dependent signaling in the context of infection by different viruses. To that end, specific experimental procedures should be applied, taking into consideration the endogenous localization of MAVS at both organelles. The analysis of peroxisomal MAVS activation requires, hence, the preliminar generation and validation of cell lines where MAVS localizes solely at peroxisomes, as well as other specific cellular tools. Here, we present a detailed protocol to analyse the peroxisome-dependent antiviral response, using virus-specific and virus-unspecific stimuli.

Key words Peroxisomes, Viruses, Antiviral signaling, Innate immune response, MAVS

1 Introduction

In recent years, peroxisomes have emerged as important platforms in the context of viral infections. Different viruses have been shown to modulate peroxisome dynamics to promote the efficient formation of new infectious virus particles [1, 2] (Chap. 19). Furthermore, besides being implicated in immune and inflammation processes [3, 4], peroxisomes have been specifically presented as important platforms for the establishment of the cellular immune response against viral infections [5–7] (Chap. 19).

Upon infection, viral RNA is detected by the cytosolic retinoic inducible gene I (RIG-I)-like receptors (RLR), such as RIG-I or melanoma differentiation-associated protein 5 (MDA5) (Fig. 1). RLR activation induces the oligomerization of the mitochondrial antiviral signaling adaptor protein (MAVS) at peroxisomes and mitochondria, prompting a signaling cascade that culminates with the production of cytokines such as interferons (IFN) and

Michael Schrader (ed.), *Peroxisomes: Methods and Protocols*, Methods in Molecular Biology, vol. 2643,
https://doi.org/10.1007/978-1-0716-3048-8_20,

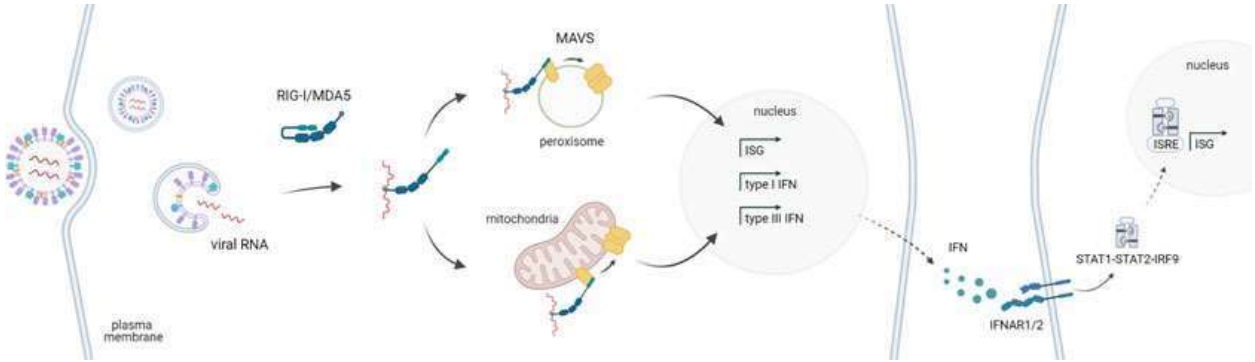

Fig. 1 Schematic representation of the MAVS-dependent antiviral signaling. Upon viral infection and release of viral RNA into the cytoplasm, RIG-I and/or MDA5 recognition of viral components causes a conformational change that exposes the CARD domains and promotes an interaction with MAVS at mitochondria and peroxisomes. Upon interaction, MAVS oligomerizes and activates a downstream signaling cascade that leads to the expression of IFNs and ISGs. IFNs are released into the extracellular space and bind to receptors at the plasma membrane of neighboring cells, activating the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway, stimulating the production of further antiviral factors [8]. (Created with BioRender.com)

IFN-stimulated genes (ISGs). These cytokines will inhibit important steps of the virus particle formation and warn the neighboring cells for the presence of infection [5, 9]. While the specific variances between the signaling pathways downstream the peroxisomal and mitochondrial MAVS have not yet been unraveled, they present different kinetics and lead to different end products. In fact, peroxisomal and mitochondrial MAVS have been shown to perform complementing functions within the antiviral response: while activation of the peroxisomal pathway induces the rapid expression of defense factors providing short-term protection, the mitochondrial pathway activates a signaling cascade with delayed kinetics that amplifies and stabilizes the antiviral response [5]. Peroxisomes have also been suggested as the primary site of initiation of type III IFN expression [6].

The growing interest in the study of the relevance of peroxisomes in the context of the antiviral response demands specific experimental procedures and materials that allow the restricted analysis of the peroxisomal MAVS pathway, without the interference of mitochondrial MAVS. This can be achieved by carefully selecting cell lines, stimuli, and experimental end-product readouts.

The choice of the appropriate cell line for each experimental procedure is largely dependent on the virus under study. Importantly, to specifically analyze the peroxisome-dependent antiviral response, the selected cells should be modified to contain MAVS solely at peroxisomes (MAVS-PEX cells) [2, 5, 6, 10, 11]. Cells with MAVS solely at mitochondria (MAVS-MITO), cells lacking MAVS (MAVS-KO), and wild-type cells (MAVS-WT) should also be prepared and used as controls.

Besides the direct stimulation of the MAVS pathway upon infection with the specific virus of interest, general virus-unspecific stimulations can also be performed to, e.g., specifically analyze the impact of peroxisomal components/mechanisms on the antiviral response, without the influence of a viral infection context. This can be achieved by transfection of synthetic RNA molecules (such as poly(I:C) or 3p-hpRNA) [12–15] that directly interact with RIG-I or MDA-5, or even by overexpression of a constitutively active RIG-I (RIG-I-CARD) [2, 10, 11].

Different readouts can be chosen to analyze the activation of the peroxisomal MAVS signaling. These basically represent the different IFNs (β or γ) and/or ISGs (such as IRF1, IRF3, viperin (*RSAD2*) or IFIT1), or even the response activation in neighboring cells (STAT1 and pSTAT1) (Fig. 1). One other possible readout is the analysis of peroxisomal MAVS oligomerization upon organelle fractionation [2].

Here, we present a detailed and complete experimental procedure to analyze the peroxisome-dependent antiviral response, using two different stimuli and two different methodologies to analyze the readouts resulting from the activation of the antiviral signaling.

2 Materials

2.1 Stimulation of the Peroxisomal MAVS Signaling

As previously explained, these experiments should be performed in cells containing MAVS solely at peroxisomes (MAVS-PEX cells), with MAVS-MITO, MAVS-KO, and MAVS-WT cells being used as controls (*see* **Note 1**).

Here we describe two types of stimulation of the peroxisome-dependent MAVS signaling: infection by an RNA virus, the influenza A virus (IAV), and transfection of 3p-hpRNA (*see* **Note 2**) in A549 cells, alveolar basal epithelial cells permissive to IAV infection.

2.1.1 Transfection of Cultured Mammalian Cells with 3p-hpRNA

1. A549 (ATCC CCL-185TM) MAVS-PEX cells.
2. 3p-hpRNA (5′ triphosphate hairpin RNA) (Invivogen, tlrl-hprna) (*see* **Note 3**).
3. Growth medium: Dulbecco's modified Eagle's medium (DMEM), high glucose (4.5 g/L) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin.
4. 1× Phosphate-buffered saline (PBS) (without Ca^{2+} and Mg^{2+}).
5. Reduced Serum Medium.
6. Lipofectamine 3000 (InvitroGen).
7. 12/24-well plate for cell culture.

2.1.2 Infection of Cultured Mammalian Cells with Influenza A Virus

1. A549 MAVS-PEX cells.
2. Growth medium: Dulbecco's modified Eagle's medium (DMEM), high glucose (4.5 g/L) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin.
3. Reverse-genetics-derived influenza A virus (Puerto Rico/8/1934, H1N1).
4. Infection medium: DMEM, high glucose (4.5 g/L), with pyruvate and without glutamine supplemented with 1% (v/v) glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin.
5. Overlay infection medium: infection medium supplemented with 20% (v/v) FBS.
6. 1× PBS (without Ca2+ and Mg2+).
7. Clean and sterile glass coverslips (12 mm ∅).
8. 12-well plate for cell culture.

2.2 Analysis of the Antiviral Signaling Activation

2.2.1 SDS-PAGE and Immunoblotting

1. SDS-PAGE running buffer: 25 mM Tris base, 192 mM glycine, 0.1% (w/v) sodium dodecyl sulfate (SDS).
2. Towbin transfer buffer: 25 mM Tris base, 192 mM glycine, 10% methanol (v/v).
3. 6× Laemmli SDS-PAGE buffer: 63 mM Tris–HCl (pH 6.8), 12% (w/v) SDS, 30% (v/v) glycerol, 0.06% (w/v) bromophenol blue, 100 mM DTT.
4. Tris-Buffered Saline, 0.05% Tween 20 (TBS-T) (pH 7.4): 19 mM Tris base, 137 mM sodium chloride (NaCl).
5. 0.2% (w/v) Ponceau S in 5% (v/v) acetic acid.
6. 0.45 μm nitrocellulose membrane.
7. 5% Blocking buffer: 5% (w/v) Bovine serum albumin (BSA) in TBS-T (for phosphorylated protein probing) or 5% (w/v) non-fat dry milk in TBS-T (for total protein probing).
8. Antibodies (Table 1).

2.2.2 RNA Isolation and RT-qPCR

I. RNA isolation.
   1. TRIzol.
   2. Chloroform.
   3. Isopropanol.
   4. Ethanol 75%.
   5. Nuclease-free water.
   6. RNase-free filtered tips.

Table 1
List of possible antibodies to be used to assess the peroxisome-dependent antiviral immune response by immunoblotting

Antibody	Company	Reference	Raised in	Species reactivity	Dilution	Buffer
IRF3	Cell Signaling	4302	Rabbit	Human, Mouse, Rat, Monkey	1:1000	TBS-T
IRF1	Cell Signaling	8478	Rabbit	Human, Mouse, Rat	1:1000	TBS-T
MAVS	Santa Cruz	SC-166583	Mouse	Human	1:2000	TBS-T
Viperin	Millipore Sigma	MABF106	Mouse	Human, Mouse, Rat	1:1000	TBS-T
pSTAT1	BioLegend	666,402	Mouse	Human	1:1000	TBS-T
STAT1	Cell Signaling	9172	Rabbit	Human, Mouse, Rat, Monkey	1:2000	TBS-T
NP	GeneTex	GTX636247	Rabbit	Influenza A virus	1:5000	TBS-T
α-Tubulin	Sigma-Aldrich	T9026	Mouse	Bovine, Rat, Yeast, Human, Mouse, Chicken, Fungi, Amphibian	1:2000	TBS-T

II. RT-qPCR.

1. RiboLock RNase inhibitor (RI) (40 U/μL) (Thermo Scientific).
2. RevertAid Reverse transcriptase (RT) (200 U/μL) (Thermo Scientific).
3. 5× RT reaction buffer.
4. DNase I, RNase free (1 U/μL) (Thermo Scientific).
5. 50 mM EDTA.
6. 10× DNase I + $MgCl_2$ buffer.
7. 10 mM dNTP Mix.
8. 10 μM Oligo$(dT)_{15}$ primer.
9. Nuclease-free water.
10. Primers (Table 2).
11. 2× SYBR Green qPCR Master Mix (*see* **Note 4**).
12. 96-well qPCR reaction plates.
13. Sealing Tape for 96-Well qPCR reaction plates.

Table 2
List of possible primers to be used to assess the peroxisome-dependent antiviral immune response by RT-qPCR

Gene	Primer	Sequence
hGAPDH	FW	5′ AAGGTGAAGGTCGGAGTC 3′
	RV	5′ GGGTGGAATCATATTGGAACAT 3′
hIRF1	FW	5′ ATGCCCTCCACCTCTGAAG 3′
	RV	5′ CCACTCCGACTGCTCCAA 3′
hIFNβ	FW	5′ TGGCACAACAGGTAGTAGGC 3′
	RV	5′ TGGAGAAGCACAACAGGAGAG 3′
hRSAD2	FW	5′ ATGAATGCTGGCTACCTAT 3′
	RV	5′ GCTACTCAACTACACTCCTA 3′
hIFIT1	FW	5′ GAATACACAACCTACTAGCCTATG 3′
	RV	5′ TCACCAGACTCCTCACATT 3′

3 Methods

3.1 Stimulation of the Peroxisomal MAVS Signaling

3.1.1 Transfection of Cultured Mammalian Cells with 3p-hpRNA

1. Plate 1.25×10^5 A549 MAVS-PEX cells per well on a 12-well plate in 1 mL of complete growth medium (or 0.63×10^5 A549 MAVS-PEX cells per well of a 24-well plate in 0.5 mL of complete growth medium), and culture overnight. Plan the number of wells to obtain protein and RNA samples for each condition (*see* **Note 5**).
2. Dilute 0.75 μL/0.375 μL (12/24-well) of Lipofectamine 3000 in 50 μL/25 μL (12/24-well) of Opti-MEM (Tube 1). Mix well.
3. Dilute 0.5 μg/0.25 μg of 3p-hpRNA in 50 μL/25 μL (12/24-well) of Opti-MEM (Tube 2).
4. Add 0.75 μL/0.375 μL (12/24-well) of P3000 reagent to Tube 2. Mix well.
5. Add the contents of Tube 2 to Tube 1, and incubate for 10 min.
6. Change the volume of growth medium in each well to 400 μL/200 μL (12/24-well) to achieve a 1 μg/μL final concentration.
7. Add growth medium or the transfection mix (100 μL/50 μL–12/24-well) to each well in a dropwise manner, and incubate for 6 h at 37 °C, 5% CO_2.

3.1.2 Infection of Cultured Mammalian Cells with Influenza A Virus

1. Plate 1.5×10^5 A549 MAVS-PEX cells per well on a 12-well plate in 1 mL of complete growth medium. Add a 12 mm ∅ glass coverslip to one of the wells (used to confirm the

efficiency of infection by immunofluorescence) (*see* **Note 6**), and culture overnight. Plan the number of wells to obtain sufficient protein and RNA samples for each condition (*see* **Note 7**). Consider an extra well to count the cells before infection.

Dilute the virus stock in infection medium to a multiplicity of infection (MOI, number of virions that are added per cell during infection) of 5.

$$\text{e.g. } \frac{\text{MOI (PFU/cell)} \times \text{number of cells}}{\text{virus titer (PFU/mL)}} = \text{virus volume needed to infect one well in mL}$$

2. Remove the growth medium, and wash cells with PBS.
3. Remove PBS, and add 500 μL of infection medium or virus solution.
4. Incubate 10 min at room temperature, shaking gently.
5. Incubate 35 min at 37 °C, 5% CO_2.
6. Add 500 μL of overlay infection medium to all wells.
7. Incubate for the desired times at 37 °C, 5% CO_2.

3.2 Analysis of the Antiviral Signaling Activation

3.2.1 SDS-PAGE and Immunoblotting

I. Sample harvesting.
   1. After washing cells with PBS, add 150 μL/75 μL (12/24-well plate) of 1× Laemmli Buffer to each well, and collect the sample.
   2. Boil the samples at 95 °C for 5 min.
   3. Continue immediately with the SDS-PAGE protocol, or store the samples at −20 °C.

II. SDS-Page.
   1. After boiling for 5 min at 95 °C in 1× Laemmli Buffer, load ¼ or ½ (12/24-well, respectively) of the sample volume (around 35 μg of protein) onto a polyacrylamide gel (4% stacking gel; 10% resolving gel—depends on the molecular weight of the stained proteins).
   2. Run the samples at ~80 V. Increase to ~120 V, after samples reach the resolving gel.

III. Immunobloting.
   1. Wet transfer the samples into a nitrocellulose membrane for 1 h and 30 min at 400 mA.
   2. Rinse the membrane with distilled water.
   3. Stain with Ponceau S solution for 5 min at room temperature.

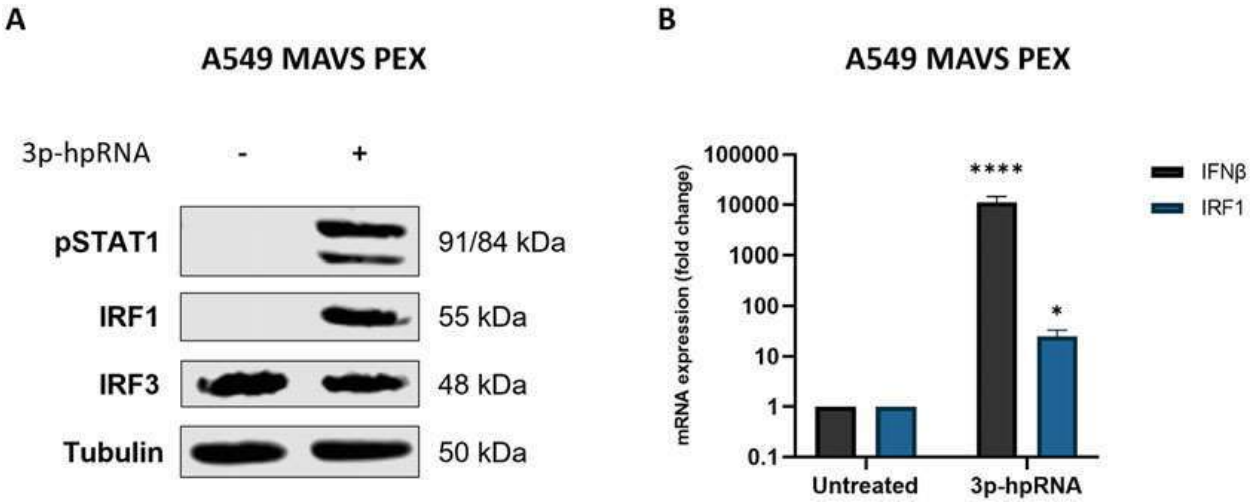

Fig. 2 Analysis of the activation of the peroxisomal MAVS signaling in A549 MAVS-PEX cells treated with 1 μg/mL 3p-hpRNA for 6 h by (**a**) SDS-PAGE and immunoblotting and (**b**) RT-qPCR. Error bars represent mean ± SEM of three independent experiments analyzed using unpaired t-test (*$p < 0.05$, ****$p < 0.0001$)

4. Rinse the membrane with distilled water 3 times, 5 min each, or until the excessive red background is completely removed.
5. Capture the Ponceau S-stained membrane image with an appropriate imaging system.
6. Wash the membrane 3 times, 5 min each with TBS-T.
7. Block the membrane with 5% Blocking buffer for 1 h.
8. Wash the membrane 3 times, 5 min each with TBS-T.
9. Probe for the target antibodies (*see* **Note 8** and Table 1; Fig. 2a includes an example of the expected results).

3.2.2 RNA Isolation and RT-qPCR

I. RNA Isolation (*See* **Note 9**).

a. Homogenization.

1. After washing the cells with PBS, lyse the cells directly in the well by adding 250 μL/150 μL TRIzol (12/24-well), and pass the lysate several times through the pipette to ensure cell disruption.
2. Samples can be stored at −80 °C before proceeding to phase separation.

b. Phase Separation.

1. Incubate the homogenized sample for 5 min at room temperature.
2. Add 200 μL of chloroform per 1 mL TRIzol, here 50 μL/30 μL (12/24-well).
3. Shake tube vigorously 15 times, and incubate 5 min at room temperature.

4. Spin samples 15 min at 12,000 *g*, 4 °C (after centrifugation, the mixture separates into a lower green, phenol-chloroform phase (contains protein), an interphase (DNA) and a colorless upper aqueous phase (RNA)).
5. Transfer the aqueous phase to a fresh tube (be careful to not disrupt the other phases).
6. Store lower organic phases for further protein isolation (optional).

c. RNA Purification.

1. Add 500 µL isopropanol per 1 mL TRIzol used for initial homogenization (vortex in low speed just to mix the samples), here 125 µL/75 µL (12/24-well).
2. Incubate for 10 min at 4 °C.
3. Spin samples 15 min at 12,000 *g* and 4 °C (a gel-like RNA pellet is supposed to be formed).
4. Remove supernatant (leave approximately 50 µL to ensure that you do not disrupt the pellet).
5. Wash pellet with 1 mL of 75% ethanol per 1 mL of TRIzol (vortex in low speed), here 250 µL/150 µL (12/24-well).
6. Incubate 5 min at room temperature, and spin for 15 min at 17,000 *g* and 4 °C.
7. Discard almost all the supernatant, and wash again with 75% ethanol with the same volume as in the previous step.
8. Remove all supernatant without disturbing the pellet, and air-dry for 10 min (be careful to not overdry).
9. Add 20 µL of nuclease-free water warmed at 55 °C (do not resuspend as it will damage the RNA).
10. Warm samples in a thermoblock for 10 min at 55 °C (to facilitate solubility). From this step onwards, RNA should be kept on ice to avoid degradation.
11. Quantify the RNA samples using a spectrophotometer.
12. Proceed with DNase treatment and cDNA synthesis, or store at −80 °C (*see* **Note 10**).

II. DNase I Treatment (*See* **Note 10**).

1. Prepare a microcentrifuge tube for each sample with 0.5 µg of RNA, and add nuclease-free water up to 8.5 µL.
2. Add 1 µL of 10× DNase I + $MgCl_2$ buffer to each tube.
3. Add 0.5 µL of DNase I, RNase free (1 U/µL) to each tube.
4. Mix gently.
5. Incubate tubes at 37 °C for 30 min.
6. Add 1 µL of 50 mM EDTA to each tube to stop the reaction.

7. Mix gently.
8. Incubate tubes at 65 °C for 10 min.
9. Proceed to cDNA synthesis.

III. cDNA Synthesis.

1. Prepare the cDNA synthesis reaction master mix (multiply by the number of samples and compensate for pipetting errors):
   - 10 μM Oligo$(dT)_{15}$ primer—2.8 μL.
   - 10 mM dNTPs—0.5 μL.
   - 5× RT Buffer—4 μL.
   - RiboLock RNase inhibitor (RI)—0.5 μL.
   - RevertAid Reverse transcriptase (RT)—0.5 μL.
   - Nuclease-free water—0.7 μL.
2. Add 9 μL of the master mix to the RNA (final volume 20 μL).
3. Mix gently.
4. Start the cDNA synthesis reaction in a thermocycler using the following protocol: 10 min at room temperature, 60 min at 40 °C, and 10 min at 70 °C.
5. Proceed to qPCR reaction or store cDNA at −20 °C.

IV. qPCR Reaction.

1. Prepare a qPCR reaction master mix for each gene of interest (including the housekeeping gene) with the following components (consider that duplicates or triplicates should be performed, as well as a blank for each gene, and an extra 10% of the volume should be added to compensate pipetting errors):
   - 2× SYBR Green qPCR Master Mix—10 μL.
   - Forward Primer (10 μM) (*see* **Note 11**)—0.5 μL.
   - Reverse Primer (10 μM) (*see* **Note 11**)—0.5 μL.
   - Nuclease-free water—7 μL.
2. Pipette 18 μL of the master mix into each well of the 96-well plate.
3. Add 2 μL of each cDNA sample (in duplicate or triplicate) or 2 μL of nuclease-free water (blank wells).
4. Seal the plate.
5. Spin the plate at 800 *g* for 2 min at 4 °C.
6. Perform the qPCR reaction in a real-time PCR system using the protocol shown in Fig. 3 (*see* **Note 12**; Fig. 2b includes an example of the expected results).

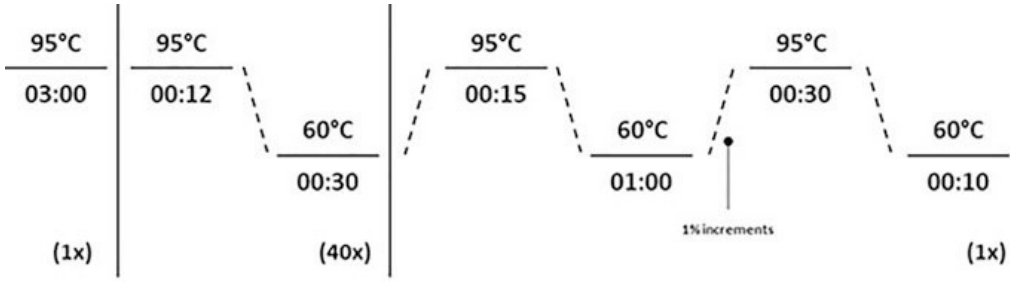

Fig. 3 RT-qPCR cycling parameters. Initial denaturation step is followed by 40 cycles of denaturation, annealing, and extension steps. After the thermocycling reaction, the melting curve phase is performed with continuous measurement of fluorescence

4 Notes

1. MAVS PEX and MAVS-MITO cells can be generated by retrovirally transducing MAVS-KO cells with constructs that lead to the expression of MAVS variants that localize solely at each one of the organelles (as in [5, 6]). Assuring transgene expression at background level is of utmost importance as MAVS overexpression leads to protein oligomerization and activation of downstream signaling. A thorough characterization and validation of MAVS expression levels and the antiviral immune response in the desired cells should be carried out before proceeding.
2. This virus-unspecific stimulus was chosen in detriment of poly (I:C) as the latter may also lead to stimulation of the toll-like receptor pathway [12]. It was furthermore preferred against the overexpression of RIG-I-CARD as the latter involves transfection of a DNA molecule and may, hence, induce the activation of the STING pathway [16, 17].
3. Avoid repeated freeze-thaw cycles of 3p-hpRNA, as it will lose efficacy.
4. Concentration of ROX (an internal reference dye that provides a constant fluorescent signal for sample normalization) needed for your Real-Time PCR system must be assessed before acquiring high or low ROX mix.
5. Confirm the confluency of the cells before proceeding to the transfection or infection reactions preparation (should be around 80–90%).
6. Immunofluorescence can be performed by staining with an antibody that recognizes the IAV nucleoprotein (NP) (*see* Table 1). At 8 h post-infection, NP should be mainly localized in the cytoplasm.
7. After storing RNA samples at −80 °C, these should be quantified after every freeze-thaw cycle to account for RNA degradation.

8. Due to the high number of antibodies being probed, it would be wise to let the gel run until an extensive band separation is obtained, facilitating the membrane cutting for parallel immunoblotting. An efficient stripping buffer should be used to sequentially probe the membrane with antibodies targeting proteins with similar size (kDa).
9. The use of commercial RNA extraction kits may be used as an alternative to the TRIzol manual RNA extraction method described here.
10. A DNase treatment of the isolated RNA must be carried out to ensure complete removal of contaminating DNA, as it can serve as a template to produce false positives. In fact, this is particularly important for Interferon-β (IFN-β) transcripts, as the assay primers for IFN-β do not span exon-exon boundaries.
11. For each analyzed gene by SYBR Green qPCR, a primer efficiency test that should render results between 90% and 110% must be carried out.
12. Always verify the melt curves of the qPCR reactions to assure analysis of the correct transcript amplification.

Acknowledgments

The authors acknowledge support from the European Union through the Horizon 2020 program (H2020-WIDESPREAD-2020-5 ID-952373) and from the Portuguese Foundation for Science and Technology (FCT) (PTDC/BIA-CEL/31378/2017; CEECIND/03747/2017; SFRH/BD/148516/2019 and UIDB/04501/2020).

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