

January 2020

# EYEPRIM™ Handbook

Guideline for sample processing and analyses applications.



[WWW.EYEPRIM.COM](http://WWW.EYEPRIM.COM)



EYEPRIM™ is a trademark of Opia Technologies S.A.S., Paris, France.

This product is covered by European patent EP2226014 as well as additional patents and pending international patent applications owned by Opia Technologies.

Consult instructions for use. EYEPRIM™ is a Class I sterile medical device according to the directive 93/42/CEE, CE 0051 (IMQ).

## CONTENTS

<b>ABOUT OPIA TECHNOLOGIES.....</b>	<b>5</b>
<b>QUALITY CONTROL.....</b>	<b>5</b>
<b>TECHNICAL ASSISTANCE.....</b>	<b>5</b>
<b>INTRODUCTION .....</b>	<b>6</b>
<b>PREREQUISITES ON THE SAMPLING MEMBRANE .....</b>	<b>6</b>
<b>CELL FIXATION / PRESERVATIVE MEDIA.....</b>	<b>7</b>
<b>TREATMENT OF SAMPLES.....</b>	<b>9</b>
CYTOLOGY .....	9
<i>Staining</i> .....	9
<i>Immunofluorescence</i> .....	12
<i>3D-cytology</i> .....	15
RNA EXTRACTION.....	16
FLOW CYTOMETRY .....	18
<b>TABLE OF FIGURES .....</b>	<b>21</b>
<b>TABLE OF DIAGRAMS AND TABLES.....</b>	<b>21</b>
<b>TABLE OF PROTOCOLS .....</b>	<b>21</b>
<b>BIBLIOGRAPHY .....</b>	<b>22</b>

## About Opia Technologies

Founded in January 2010 and based at the Vision Institute in Paris, OPIA Technologies is a company dedicated to the development of an innovative portfolio of medical devices for the ocular surface.

Our aim is to provide innovative and reliable sampling tools in order to further introduce the bio-molecular analysis of the ocular surface and to improve the care of patients with ocular surface diseases.

The first product developed by OPIA is EYEPRIM™, a device to perform conjunctival impressions of the living eye, enabling further analysis of cells and biomarkers to help diagnose several ocular surface disorders such as dry eye, allergies or infections.

## Quality Control

EYEPRIM™ is manufactured in accordance with OPIA Technologies and its suppliers' Quality Management System certified ISO 13485. Each lot is tested against predetermined specifications to ensure consistent product quality.

## Technical assistance

OPIA Technologies is very attentive to the proper use of its products. If you have any questions or experience any difficulties with EYEPRIM™ use, please do not hesitate to contact us.

Since EYEPRIM™ is a new device, our customers are a major source of information for its use. This information is helpful to other customers as well as the researchers at OPIA Technologies. We therefore encourage you to contact us for any suggestions about product performance, new applications or techniques.

For technical assistance and more information please contact us at [sales@opiatech.com](mailto:sales@opiatech.com).

You may also visit our FAQ page on EYEPRIM™ website at [www.eyeprim.com](http://www.eyeprim.com).

## Introduction

EYEPRIM™ is a sampling medical device that enables the harvesting of conjunctival epithelial cells and associated biomarkers from the eye. The analysis of such samples is potentially useful in clinical practice for diagnosis, follow-up and treatment of ocular surface disorders.

EYEPRIM™ is the first sterile, validated, and reliable tool to practice the sampling method traditionally called impression cytology, or in a more proper way: conjunctival impression.

From an original idea of professor Baudouin (M.D., professor and chair of ophthalmology, Quinze-Vingts Hospital), EYEPRIM™ solves all the issues related to this sampling technique and provides great sampling performances, in a reliable and reproducible manner, independent of the user (*Roy et al. Assessment of a new device for conjunctival impression. Abstract ARVO 2012*).

Thanks to its performances, EYEPRIM™ offers new possibilities in terms of analyses of conjunctival samples.

This Handbook aims to guide you in your practice. The information herein comes from literature, but also from our users' feedback. This Handbook will be updated with any information we consider relevant, coming from our research or from any customer willing to share with us his experience with EYEPRIM™.

The latest version of this handbook is available on [www.eyeprim.com](http://www.eyeprim.com).

## Prerequisites on the sampling membrane

The sampling surface of EYEPRIM™ is a Polyethersulfone membrane, similar to the one quoted in the literature for conjunctival sampling since the beginning of year 2000.

This membrane was selected for its performances in terms of cell collection, which is particularly interesting for analyses requiring a large number of cells such as PCR or flow cytometry based assays.

However, this membrane is also quite suitable for cytological analysis and immunocytochemistry, provided that you use proper instruments.

In this handbook, we will give you as much information as you need to help you to adapt your practice.

## Cell fixation / preservative media

The choice of the medium in which the membrane will be immersed after the sampling depends mostly on the analysis planned. However, this is not the only matter. You need to ask yourself a number of questions to guide the final choice.

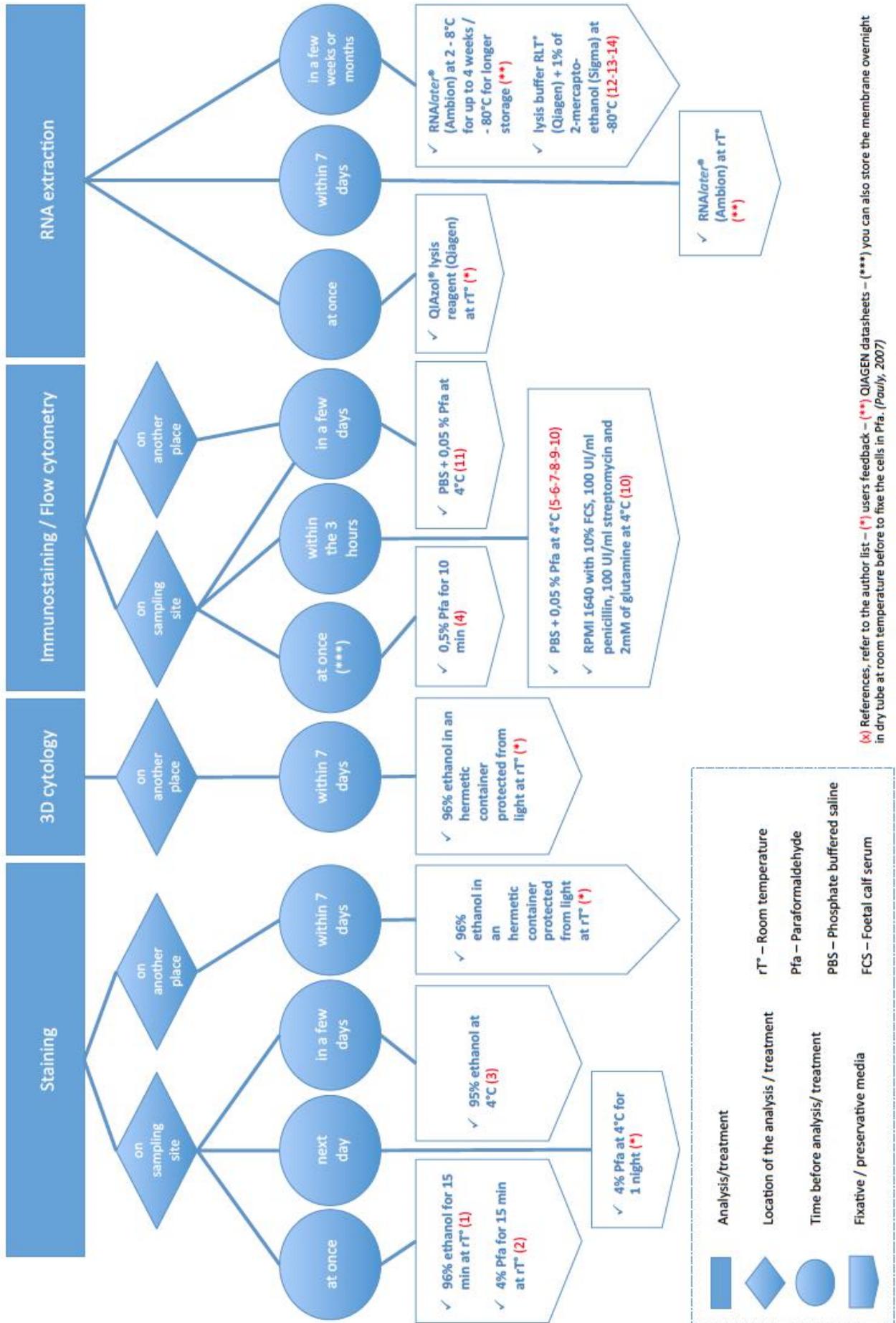
- How long will the sample remain in the medium before the analysis?
- Where will the analysis be performed? Will it be in a different location than the sampling?
- Does the sample need to be transported? How far?

The diagram 1 can help you in your reflection. Data come from literature (refer to the author list below) and users feedback. For all the analyses mentioned in the diagram 1, you will find tips and protocols in the following chapters of this handbook.

### Author list for diagram 1:

- |   |                                   |
|---|-----------------------------------|
| (1) <i>Peral and Pintor, 2008.</i>        | (8) <i>Gicquel et al. 2007.</i>   |
| (2) <i>Adams, 1979.</i>                   | (9) <i>Baudouin et al. 2008.</i>  |
| (3) <i>Nelson et al. 1983.</i>            | (10) <i>Barabino et al. 2010.</i> |
| (4) <i>De Nicola et al. 2005.</i>         | (11) <i>Brignole et al. 2000.</i> |
| (5) <i>Bourcier et al. 2000.</i>          | (12) <i>Corrales et al. 2003.</i> |
| (6) <i>Brignole-Baudouin et al. 2004.</i> | (13) <i>Corrales et al. 2009.</i> |
| (7) <i>Baudouin et al. 2005.</i>          | (14) <i>Corrales et al. 2011.</i> |

Diagram 1: Choice of the fixative according to the sample process plan



## Treatment of samples

Once conjunctival samples collected according to EYEPRIM™ instruction for use (the latest version of instruction for use can be found on [www.eyeprim.com](http://www.eyeprim.com)) and immersed in the proper medium, they must be prepared for analysis. In the following chapters, you will find protocols or tricks that will help you in getting the most out of the samples for applications such as cytology, flow cytometry, or PCR analysis. The information comes from the literature and from our experience together with feedback from our users. The proposed protocols are not absolute rules, but have been used successfully. They can be adapted to suit your practice. Do not hesitate to give us your suggestions.

### Cytology

The observation of conjunctival specimens by microscopy after staining of the cells is the oldest technique for the analysis of conjunctival impression. It is widely used nowadays in clinical practice and research, especially for the diagnosis and study of dry eye. Cytological analysis techniques, from the most traditional (light microscopy) to the most innovative (3D cytology), are feasible from EYEPRIM™ samples. Find below what you need to know before you carry out these analyses.

### Staining

Although cellulose membranes are the most often used because of their property of being made transparent, EYEPRIM™ Polyethersulfone membrane is not an obstacle to optical microscopy. A suitable microscope, which reflects light (top-down lighting to illuminate the specimen) instead of transmission light (bottom-up illumination) should be used. This will allow you to observe cell shape, size, the nucleus-to-cytoplasm ratio, and nuclear chromatin condensation on your sample (*Mrugacz et al., 2008*).

The standard staining techniques that can be used with EYEPRIM™ membrane are listed in table 1. Figures 1 to 7 come from the literature and show you examples of what you will be able to see on the membrane after staining.

**Table 1: Standard staining techniques for samples collected on Polyethersulfone membranes.**

Staining technique	Applicable	Not applicable	No information	Reference
PAS-hematoxylin	X			<i>Mrugacz et al. (2008)</i>
PAS-GIEMSA	X			<i>Calonge et al. (2004)</i>
Hematoxylin - Eosin	X			<i>Mrugacz et al. (2008)</i>
May-Grunwald-Giemsa	X			<i>Mrugacz et al. (2008)</i>
Feulgen	X			<i>Mrugacz et al. (2008)</i>
PAS-Gill's modified Papanicolou	X			<i>Martínez-Osorio et al. (2009)</i>
Cresyl violet			X	/
Alcian Blue			X	/
Modified Wright's stain (Diff-Quick)			X	/

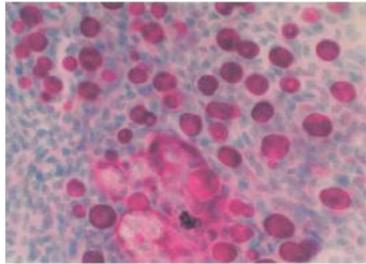


Figure 1: Polyethersulfone filter and PAS-Giemsa staining, X40. Conjunctival impression cytology specimen from a patient diagnosed of mild blepharitis. Although this sample can be considered normal, there is, however, a high density of goblet cell secretions (PAS positive dots); in addition a sheet of PAS positive material can be observed, somehow obscuring the view of the normal epithelial cells. *From Calonge et al. (2004)*

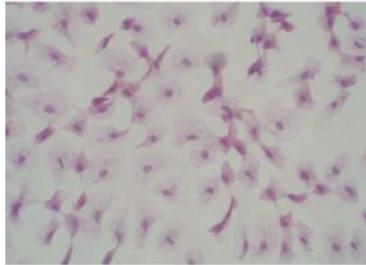


Figure 2: Polyethersulfone filter and PAS-Giemsa staining, X40. This conjunctival impression cytology belongs to a patient diagnosed of aqueous-deficient dry eye, in addition to meibomian gland disease (evaporative dry eye). Goblet cells are absent. The cytoplasm of epithelial cells is larger and the nucleocytoplasmic ratio is between 1:4 and 1:6. *From Calonge et al. (2004)*



Figure 3: Polyethersulfone filter and PAS-Giemsa staining, X40. This conjunctival impression cytology from a contact lens wearer with mild dry eye shows typical snake-like chromatin arrangements in all cell nuclei. *From Calonge et al. (2004)*

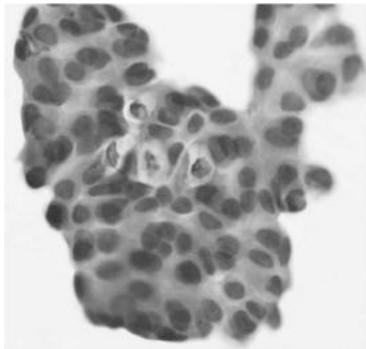


Figure 4: Polyethersulfone filter and Hematoxylin-Eosin staining, X40. Conjunctival impression cytology from a normal subject: the correct picture of small, round and closely packed, contiguous epithelial cells. These cells have similar shape and sizes with eosinophilic stained cytoplasm, and numerous oval goblet cells with light cytoplasm are seen among the cells. *From Mrugacz et al. (2008)*

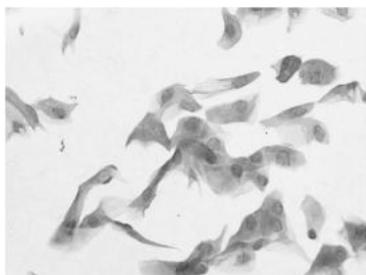


Figure 5: Polyethersulfone filters and Hematoxylin-Eosin staining, X40. Conjunctival impression cytology from a patient with Cystic Fibrosis. The epithelial cells are more separated and show morphological alterations. Some of them exhibit changes in the N/C ratio, others are less regular in shape (more elongated or polygonal), and show less eosinophilic stained cytoplasm. *From Mrugacz et al. (2008)*

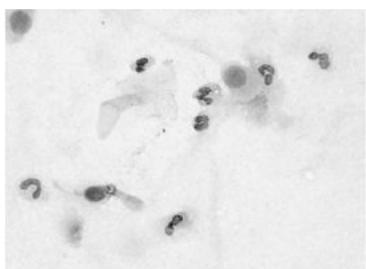
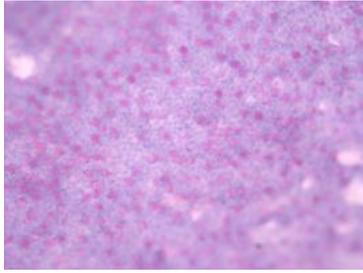


Figure 6: Polyethersulfone filter and May-Grunwald-Giemsa staining, X200. Conjunctival impression cytology from a patient with Cystic Fibrosis. There is the presence of neutrophils. *From Mrugacz et al. (2008)*



**Figure 7: Polyethersulfone filter and PAS-hematoxylin staining, X20. Conjunctival impression cytology on rabbit. Data on file (2012)**

We suggest you the following protocol (protocol 1) for PAS-hematoxylin staining on EYEPRIM™ membranes. You can also find alternative protocols for PAS-hematoxylin staining in literature (Adams, 1979 ; Nelson et al. 1983 ; Murube and Rivas, 2003 ; Bolzan et al. 2005 ; Peral and Pintor, 2008 ; Garcia et al. 2012)

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#### **Protocol 1: PAS-hematoxylin staining (Fig. 7)**

##### **Material:**

- Distilled water
- 5% periodic acid solution (Sigma P7875)
- Tap water
- Schiff reagent (DiaPath C0622)
- Hematoxylin (DiaPath C0283)

##### **1. Rinse 3 times the sample in distilled water for 1 min.**

*Note: this is a valid step for a prior fixation in 4% paraformaldehyde. If the sample is an alcoholic fixative (such as ethanol or methanol solution) you shall rather put the sample 5 min in distilled water in order to rehydrate it.*

##### **2. Oxidize in 5% periodic acid solution (Sigma P7875) for 15 min.**

##### **3. Wash 2 times in tap water for 2 minutes.**

##### **4. Place in distilled water for 5 minutes. 3 times.**

##### **5. Place in Schiff reagent (DiaPath C0622) for 30 minutes. Protected from light.**

##### **6. Wash in distilled water for 10 minutes.**

##### **7. Counterstain in hematoxylin (DiaPath C0283) for 1 minute.**

##### **8. Wash three times in distilled water for 5 minutes.**

##### **9. Dry and coverslip using a mounting medium.**

*Note : After drying the sample and before to coverslip, you can also clear the sample in xylene (2 successive baths of 5 min each, Bolzan et al. 2005).*

## Immunofluorescence

Immunofluorescence on impression cytology samples was historically practiced on transparent membranes. Once again, the opacity of the EYEPRIM™ membrane is not an obstacle to this technique, as long as you proceed with a confocal microscope in order to avoid the fluorescence coming from the membrane itself (*Calonge et al. 2004*).

You can find in literature several examples of immunostaining performed directly on Polyethersulfone membranes. These examples are shown in Table 2.

**Table 2: Immunostaining applied directly on polyethersulfone membranes for confocal fluorescence microscopy analysis.**

Antibodies	Counterstain	Revealed structures	Reference	Figure
- Anti-HLA DR - Anti-MUC5AC	- Propidium iodide	- Dendritic cells - Goblet cells - Nucleic acids	<i>Brignole-Baudouin (2003)</i>	<i>Fig. 9, 10</i>
- Anti-muscarinic type 2 receptor	- Propidium iodide	- Epithelial cells - Nucleic acids	<i>Calonge et al. (2004)</i>	<i>Fig. 11</i>
- Phalloïdin (conjugated with Axela 488) or anti-vimentin	- Propidium iodide	- Epithelial cells (cytoskeleton) - Nucleic acids	<i>De Nicola et al. (2005)</i>	<i>Fig. 12</i>
- (FITC)-conjugated mouse IgG1 pan cytokeratin - (PE)-conjugated mouse IgG1 anti HLA-DR	- Propidium iodide	- Epithelial cells - HLA-DR expression on epithelial cells - Nucleic acids	<i>Gicquel et al. (2007)</i>	<i>Fig. 8</i>

The sample treatment before immunocytological analysis of specimens by confocal microscopy consists in 3 key steps: tissue fixation, cell permeabilization (only for intracellular labelling) and last, cell labelling.

For morphological analysis of epithelial cells by immunofluorescence, we suggest you use the protocol 2 from literature. (*De Nicola et al. 2005*)

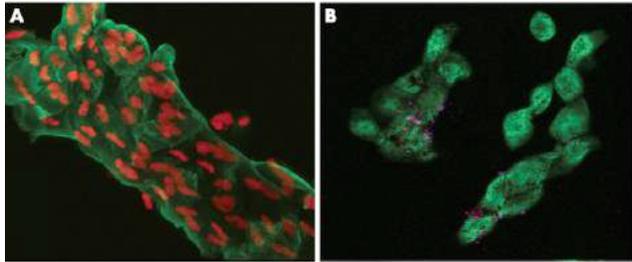


Figure 8: (Polyethersulfone filters and immunostaining) Laser confocal images of conjunctival epithelium from impression cytology specimens harvested in a burned patient. (A) Cytokeratin staining of the epithelial cells appears in green. Counterstaining with propidium iodide reveals snake-like chromatin. (B) (double immunostaining) Cytokeratin appears in green. HLA-DR in red/purple. *From Gicquel et al. (2007)*

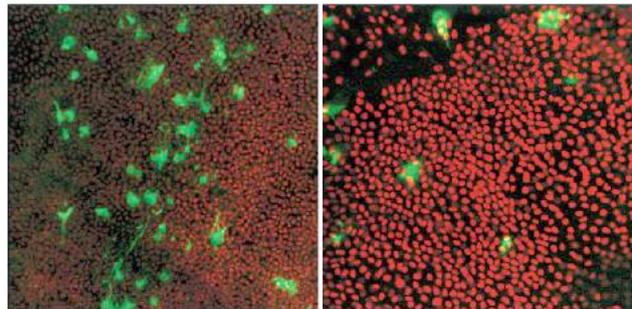


Figure 9: (Polyethersulfone filters and immunostaining) Confocal microscope images of conjunctival epithelium showing a scarcity of goblet cells in a case of dry eye (right image). Anti-MUC5AC staining of goblet cells appears in green, counterstaining with propidium iodide reveals nucleus in red. *From Brignole-Baudouin (2003)*

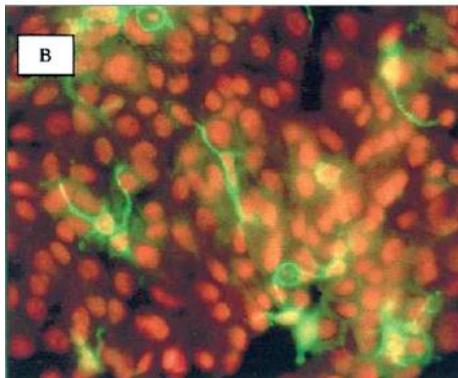


Figure 10: (Polyethersulfone filter and immunostaining) Anti-HLA DR staining of dendritic cells appears in green, counterstaining with propidium iodide reveals nucleus in red. *From Brignole-Baudouin (2003)*

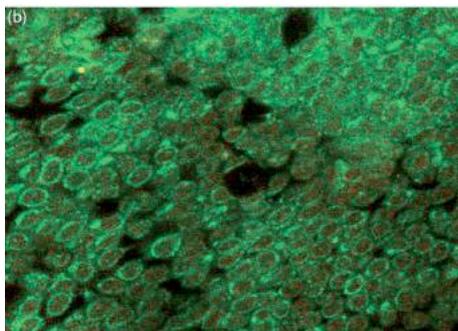


Figure 11: (Polyethersulfone filter and immunostaining) Confocal microscope image of conjunctival impression cytology specimen from a normal donor immunostained with antibody anti-muscarinic type 2 receptor. Immunostaining was performed directly on the filter paper. Positive green immunostaining can be seen around the red counterstained nuclei with propidium iodide (X63). *From Calonge et al. (2004)*

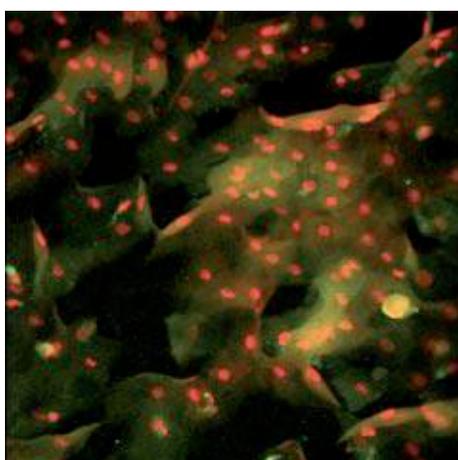


Figure 12: (Polyethersulfone filter and immunostaining) Polyhedral conjunctival epithelial cells in squamous metaplasia. Phalloidin staining of epithelial cells actin appears in green, counterstaining with propidium iodide reveals nucleus in red. *From De Nicola et al. (2005)*

**Material:**

- Paraformaldehyde 0,5%
- Phosphate-buffered saline (PBS, pH 7,2)
- Triton X-100® (Sigma) 0,1 %-diluted
- Phalloidin conjuguated Alexa 488 (200 UI/ml, Molecular Probes, dilution 1:50)
- Propidium Iodide (Immunotech, Marseille)
- Antifade medium (Vectashield H1000, Vector Laboratories, Burlingame)
- Confocal microscope (Nikon PCM 2000)

**Step 1: Fixation**

- 1. Put the sample in Paraformaldehyde 0,5% for 10 minutes.**
- 2. Wash in PBS.**

**Step 2: Permeabilization**

- 3. Place in Triton X-100® 0,1% diluted for 5 minutes.**

*Note: you can skip this step if you don't want to address intracellular targets. You can also skip this step if sample was fixed in medium with a permeabilizing effect on cells membranes, such as cooled acetone (-20°C) or cooled methanol (-20°C).*

*Be careful if you want to address both intracellular and extracellular targets; Triton X100 destroys membrane-associated antigens.*

- 4. Wash in PBS.**

*Note: This protocol does not contain a step for blocking non-specific sites. If your labelling procedure require such a step, Peral and Pintor suggest to treat the sample with blocking solution (PBS, bovine serum albumin 3%, fetal bovine solution 5%) for 1 hour at 37° before starting the labelling step. (Peral and Pintor, 2008)*

**Step 3: Labelling**

- 5. Incubate the sample with Phalloïdin conjuguated-Alexa 488 for 1 hour at room temperature.**
- 6. Wash 2 times in PBS.**
- 7. Counterstain with Propidium Iodide.**

**Step 4: Mounting and observation**

- 8. Add 1 drop of antifade medium and coverslip.**
- 9. Observe the sample by confocal microscopy directly on the membrane.**

### 3D-cytology

3D cytology is a patented analysis method for the study of goblet cells and goblet cells secretions. It allows better characterization of the goblet cells behaviour according to three criteria: the goblet cells density, mucin cloud height (MCH) and spread mucin thickness (SMT) (Peral and Pintor, 2008). This technique is realized on EYEPRIM™ conjunctival impression sample, using a Laser Scanning Microscope.

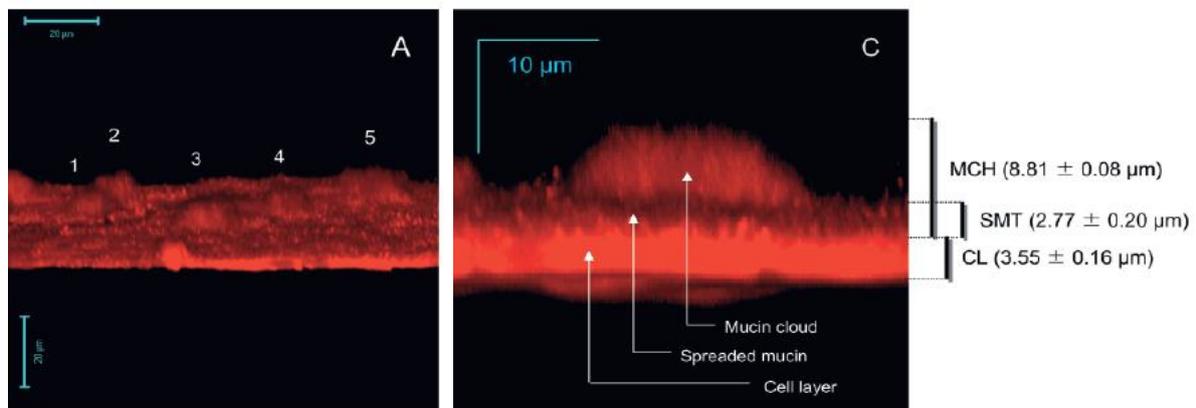


Figure 13: A. Conjunctival landscape containing the secretion of 5 goblet cells from a healthy individual. C. Details of cell 5, where the average parameters from the mucin cloud are shown. The MCH and the SMT represent the mean values for a control patient. From Peral and Pintor (2008)

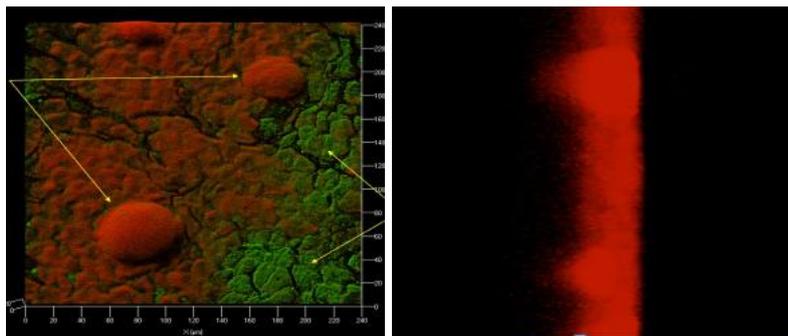


Figure 14: 3D cytology on a conjunctival sample collected with EYEPRIM™. Left picture shows epithelial cells and goblet cells. Right picture shows the mucin clouds of two goblet cells.

If you are interested in such analysis, please contact Opia Technologies or OcuPharm Diagnostics. We will provide you with a sampling KIT, including the cost of the analysis.

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Madrid 28037  
Spain  
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Refer to the section *Cell fixation / preservative media* to choose the best fixative medium to use for the transport of your sample.

A conjunctival sample is considered by the law as an infectious biological substance category B. Be careful to follow the packing instructions P650 for the transport (triple packaging).

## RNA extraction

EYEPRIM™ has especially great yield performances for ribonucleic acids (RNA) and allows the measurement of several biomarkers from a single membrane.

The protocol 3 below is based on QIAGEN protocol (RNeasy® Lipid Tissue Handbook) and on the experience of several users of EYEPRIM™. You can adapt it to your practice. Obviously, many alternatives are possible.

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### Protocol 3: RNA extraction protocol for impression cytology samples obtained by EYEPRIM™.

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#### Material:

- RNAlater® (Ambion)
- QIAzol® (Qiagen)\*
- QIAshredder® homogenizer unit (Qiagen)
- RNeasy® Mini spin column (Qiagen)\*
- Buffer RW1\*
- Buffer RPE\*
- RNase-free water\*
- 1,5 ml and 2 ml tubes\*
- Chloroform
- 70% ethanol
- Microcentrifuge
- Vortex mixer

*Note: The equipment marked with \* is supplied in the RNeasy® Lipid Tissue kit (Qiagen).*

#### **Step 1: Stabilization of the sample**

You can either stabilize the sample in RNAlater® or directly proceed to the lysis of the cells putting the sample in QIAzol® reagent. The sample must be totally immersed (at least 10 volumes per 1 volume of tissue or 10 µl reagent per 1 mg tissue). For 1 membrane, 0,7 ml of reagent seems to be sufficient.

#### **Step 2: Preservation**

Samples in RNAlater® can be kept at room temperature (15-25°C) up to 1 week (up to 1 day at 37°C, up to 4 weeks at 2-8°C). For longer preservation, first incubate the tissue overnight in the reagent at 2-8°C, and transfer it to -80°C for storage.

For samples in QIAzol®: transfer samples to -80°C for storage immediately.

*Note: Don't forget to thaw the sample before to proceed to step 3.*

#### **Step 3: RNA extraction**

##### ***For samples in QIAzol®:***

- a. Samples (include the membrane) are vortexed for 2 min.
- b. Transfer cell lysate into a QIAshredder homogenizer unit (Qiagen) and centrifuge for 2 minutes at full speed in a microcentrifuge to homogenize.

- c. Add 0.2 ml chloroform per 1 ml QIAzol® lysis Reagent pipetted in step b. Securely cap the tube containing the homogenate, and shake it vigorously for 15 s.
- d. Place the tube containing the homogenate on the bench top at room temperature for 2–3 min.
- e. Centrifuge at 12,000 x g for 15 min at 4°C. After centrifugation, heat the centrifuge to room temperature (15–25°C) if the same centrifuge will be used in the later steps.
- f. Transfer the upper, aqueous phase to a new tube. Add 1 volume (usually 600 µl) of 70% ethanol, and mix thoroughly by vortexing. Do not centrifuge. Proceed immediately to step g.

*Note: Precipitates may be visible after addition of ethanol. Resuspend precipitates completely by vigorous shaking, and proceed immediately to next step.*

- g. Transfer up to 700 µl of the sample to an RNeasy® Mini spin column placed in a 2 ml collection tube. Close the lid gently, and centrifuge for 30 s at 8000g at room temperature. Discard the flow-through. Reuse the collection tube. If the sample volume exceeds 700 µl, centrifuge successive aliquots in the same RNeasy® spin column. Discard the flow-through after each centrifugation.
- h. Add 700 µl Buffer RW1 to the RNeasy® spin column. Close the lid gently, and centrifuge for 30 s at 8000g (10,000 rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in next step.
- i. Add 500 µl Buffer RPE to the RNeasy® spin column. Close the lid gently, and centrifuge for 30s at 8000g (10,000 rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in next step.
- j. Add 500 µl Buffer RPE to the RNeasy® spin column. Close the lid gently, and centrifuge for 2 min at 8000 g (10,000 rpm) to wash the spin column membrane. The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.
- k. Place the RNeasy® spin column in a new 2 ml collection tube, and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min.
- l. Place the RNeasy® spin column in a new 1.5 ml collection tube. Add 50 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at 8000g (10,000 rpm) to elute the RNA.

***For samples in RNAlater®:***

Remove the samples (still on the membrane) from RNAlater® using forceps and transfer it to 0,7 ml QIAzol® Lysis Reagent. Be sure to remove any excess reagent or crystals that may have formed. Then follow the protocol above.

*Alternative extraction procedures: It is possible to use the lysis buffer RLT® (Qiagen) instead of QIAzol. Follow the RNeasy Mini protocol (Qiagen) for animal tissues. The homogenize step can use QIAshredder unit.*

Find below (table 3) a list of biomarkers measured by reverse transcription polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qRT-PCR) from conjunctival impression samples, found in literature.

**Table 3: Main biomarkers measured in literature by RT-PCR or qRT-PCR from conjunctival impression on polyethersulfone membranes.**

<b>Biomarkers</b>	<b>References</b>
<b>MUC1</b>	<i>Corrales et al. (2003) ; Corrales et al. (2009) ; Corrales et al. (2011)</i>
<b>MUC2</b>	<i>Corrales et al. (2003) ; Corrales et al. (2009) ; Corrales et al. (2011)</i>
<b>MUC4</b>	<i>Corrales et al. (2003) ; Corrales et al. (2009) ; Corrales et al. (2011)</i>
<b>MUC5AC</b>	<i>Corrales et al. (2003) ; Corrales et al. (2009) ; Corrales et al. (2011)</i>
<b>MUC7</b>	<i>Corrales et al. (2003) ; Corrales et al. (2009) ; Corrales et al. (2011)</i>
<b>MUC13</b>	<i>Corrales et al. (2009)</i>
<b>MUC15</b>	<i>Corrales et al. (2009)</i>
<b>MUC16</b>	<i>Corrales et al. (2009)</i>
<b>MUC17</b>	<i>Corrales et al. (2009)</i>
<b>IL-1</b>	<i>Narayanan et al. (2006)</i>
<b>IL-6</b>	<i>Narayanan et al. (2006)</i>
<b>IL-8</b>	<i>Narayanan et al. (2006)</i>
<b>ICAM-1</b>	<i>Narayanan et al. (2006)</i>
<b>GRO-β</b>	<i>Narayanan et al. (2006)</i>
<b>TRAIL</b>	<i>Narayanan et al. (2006)</i>
<b>Ephrin A5</b>	<i>Narayanan et al. (2006)</i>
<b>EMMPRIN</b>	<i>Huet et al. (2011)</i>
<b>MMP-9</b>	<i>Huet et al. (2011)</i>

## Flow cytometry

The analysis of conjunctival impression specimens by flow cytometry provides quantitative information on the populations of cells sampled. This technique is most often performed on polyethersulfone membranes. EYEPRIM™ is therefore particularly suitable for this analysis method. Moreover, the analysis can be performed from a single membrane thanks to great cells collection performances.

The cells labelling protocol for flow cytometry is similar to the one described in the immunofluorescence section. The difference lies in the cells extraction from the membrane, which is a key step in the preanalytical phase for flow cytometry.

The protocole 4 proposed below comes from the Dry Eye WorkShop report (2007) enriched by our users' feedbacks.

**Material:**

- Phosphate buffered saline with 0.05% paraformaldehyde
- Phosphate buffered saline
- Centrifuge
- Clean tubes
- Specific and negative control antibodies

**Step 1: Fixation**

**1. Dip the sample into Phosphate buffered saline with 0.05% paraformaldehyde.**

The tubes have to be kept at 4°C before and after impression collection in order to avoid sample degradation during the phase of fixation. Under this condition the filters with the conjunctival specimens can be stored several days and sent to the laboratory in cold-conditioned containers before being processed for flow cytometry analyses.

*Note: Barabino et al. showed that the choice of the fixative influences the cells extraction from the membrane. If you can analyse the sample within the 3 hours from the cells collection, always prefer a cell culture medium RPMI 1640 with 10% Foetal Calf Serum, 100 UI/ml penicillin, 100 UI/ml streptomycin and 2mM of glutamine rather than a Phosphate buffered saline with 0,05% Paraformaldehyde. In both cases the sample must be stored at 4°C. (Barabino et al. 2010)*

*Note: For more options in the choice of fixative, refer to section Cells fixation/ preservative media.*

**Step 2: Cell extraction**

**2. Extract cells by gentle manual agitation with a pipette tip.**

*Note: The extraction time varies from one publication to the other from 10-15 minute (Barabino et al. 2010) to 30 minutes (Gicquel et al. 2007, Bourcier et al. 2000, Brignole et al. 2000). One of our users did it in only 1 minute using the RPMI medium.*

*It seems also that adding a quarter volume of PBS with 0,5% BSA in the medium facilitates cell detachment thanks to protein enrichment of the environment.*

**3. Transfer the cell suspension into clean tube and centrifuge in PBS with 1% BSA at 1600 rpm for 5 minutes.**

**Step 3: Immunolabelling**

**4. Incubate with specific and negative control antibodies.**

Indirect or direct immunofluorescence procedures may be used. Simple or multi-color analysis can be performed commonly using 2 to 4 antibodies conjugated with different fluorochromes.

*Note: In case of intracellular target, you must permeabilize the membranes before step 3. Refer to protocol 2 step 2.*

**5. Centrifuge in PBS at 1600 rpm for 5 minutes.**

**6. Resuspend in PBS before flow cytometry analysis.**

Find below (table 4) a list of biomarkers exploited by flow cytometry from conjunctival impression samples, found in literature.

**Table 4: Main biomarkers measured by flow cytometry from conjunctival impression in literature.**

<b>Biomarkers</b>	<b>References</b>
<b>CD3</b>	<i>Barabino et al. (2010)</i>
<b>CD4</b>	<i>Barabino et al. (2010)</i>
<b>CD8</b>	<i>Barabino et al. (2010)</i>
<b>CD14</b>	<i>Barabino et al. (2010)</i>
<b>CD19</b>	<i>Barabino et al. (2010)</i>
<b>CD20</b>	<i>Barabino et al. (2010)</i>
<b>CD40</b>	<i>Bourcier et al. (2000) ; Brignole et al. (2000)</i>
<b>CD40 L</b>	<i>Bourcier et al. (2000) ; Brignole et al. (2000)</i>
<b>CD45</b>	<i>Baudouin et al. (2005)</i>
<b>CD56</b>	<i>Barabino et al. (2010)</i>
<b>CK19</b>	<i>Barabino et al. (2010)</i>
<b>CCR4</b>	<i>Baudouin et al. (2005) ; Baudouin et al. (2008)</i>
<b>CCR5</b>	<i>Baudouin et al. (2005) ; Gulati et al. (2006) ; Baudouin et al. (2008)</i>
<b>TNF-<math>\alpha</math></b>	<i>Liang et al. (2006)</i>
<b>TNFR-1</b>	<i>Liang et al. (2006)</i>
<b>Fas</b>	<i>Brignole et al. (2000)</i>
<b>APO2-7</b>	<i>Brignole et al. (2000)</i>
<b>HLA-DR</b>	<i>Bourcier et al. (2000) ; Brignole et al. (2000) ; Baudouin et al. (2005) ; Gicquel et al. (2007) ; Mrugacz et al. (2007) ; Baudouin et al. (2008) ; Barabino et al. (2010)</i>

## Table of figures

<i>Figure 1: Polyethersulfone filter and PAS-Giemsa staining</i> .....	p.10
<i>Figure 1: Polyethersulfone filter and PAS-Giemsa staining</i> .....	p.10
<i>Figure 3: Polyethersulfone filter and PAS-Giemsa staining</i> .....	p.10
<i>Figure 4: Polyethersulfone filter and Hematoxylin-Eosin staining</i> .....	p.10
<i>Figure 5: Polyethersulfone filters and Hematoxylin-Eosin staining</i> .....	p.10
<i>Figure 6: Polyethersulfone filter and May–Grunwald–Giemsa staining</i> .....	p.10
<i>Figure 7: Polyethersulfone filter and PAS-hematoxylin staining</i> .....	p.11
<i>Figure 8: (Polyethersulfone filters and immunostaining)</i> .....	p.13
<i>Figure 9: (Polyethersulfone filters and immunostaining)</i> .....	p.13
<i>Figure 10: (Polyethersulfone filter and immunostaining)</i> .....	p.13
<i>Figure 11: (Polyethersulfone filter and immunostaining)</i> .....	p.13
<i>Figure 12: (Polyethersulfone filter and immunostaining)</i> .....	p.13
<i>Figure 13: 3D cytology. Mucin cloud spread and height. From Peral and Pintor (2008)</i> .....	p.15
<i>Figure 14: 3D cytology on a conjunctival sample collected with EYEPRIM</i> .....	p.15

## Table of diagrams and tables

<i>Diagram 1: Choice of the fixative according to sample process plan</i> .....	p.8
<i>Table 1: Standard staining techniques for samples collected on Polyethersulfone membranes</i> .....	p.9
<i>Table 2: Immunostaining applied directly on polyethersulfone membranes for confocal fluorescence microscopy analysis</i> .....	p.12
<i>Table 3: Main biomarkers measured in literature by RT-PCR or qRT-PCR from conjunctival impression on polyethersulfone membranes</i> .....	p.18
<i>Table 4: Main biomarkers measured by flow cytometry from conjunctival impression in literature</i> .....	p.20

## Table of protocols

<i>Protocol 1: PAS-hematoxylin staining</i> .....	p.11
<i>Protocol 2: Immunostaining to observe epithelial cells morphology by confocal microscopy</i> .....	p.14
<i>Protocol 3: RNA extraction protocol for impression cytology samples obtained by EYEPRIM</i> .....	p.16
<i>Protocol 4: Preanalytical steps for the analysis of an EYEPRIM™ conjunctival sample by flow cytometry</i> .....	p.

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