PromoCell®

Standardized culture of assay-ready and fully functional human primary macrophages

Application Note

Background

Primary human macrophages (Mφ) are difficult to isolate from tissue in sufficient amounts with a homogeneous phenotype and do not proliferate in culture.

Human monocyte-derived macrophages (hMDM) are highly pure, readily available, and therefore provide an excellent alternative, although their differentiation *in vitro* is time-consuming. However, the distinct cell loss observed during detachment of this strongly adherent cell type as well as the low rate of re-attachment after subculture significantly flaw experimental design and flexibility in daily routine. As macrophage research is gaining significance constantly, there is an increasing demand for less time-consuming and easy to handle applications.

Being part of our unique range of macrophage culture products, cryopreserved human macrophages are now available as a reliable source of standardized cells in a ready-to-use format allowing for full experimental flexibility. The frozen macrophages are produced in our well-proven M1/M2- Macrophage Generation Media XF and are available as fully qualified M1- (hMDM-GMCSF(-)) or M2- (hMDM-MCSF(-)) polarized cells. The macrophages can be seeded into all kinds of multiwell-plates, dishes, and flasks. After plating, the macrophages can be maintained as biologically functional, adherent cultures (see Figs. 1 and 2) for several weeks. Optionally, user-customizable activation of the cells can be performed (please refer to the culture protocol, Fig. 3 and Tab. 1).

Since we provide a comprehensive range of macrophage media compatible with our cryopreserved cells, a complete and versatile solution for macrophage-related research is now at hand.

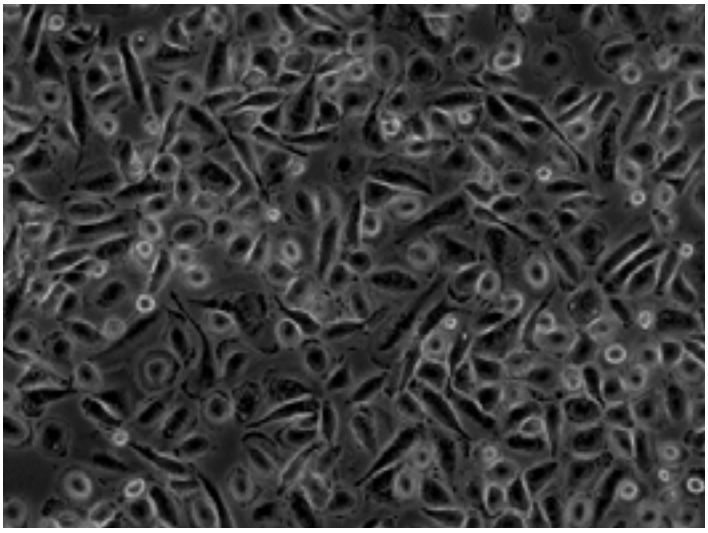

Fig. 1: Morphology of cryopreserved macrophages in culture.
Image shows hMDM-GMCSF(-) 24 hours after plating at 100.000 cells/cm². A healthy culture with the typical cellular morphology of non-activated mature Mφ-M1 is established from the cryopreserved cells. 200x magnification.

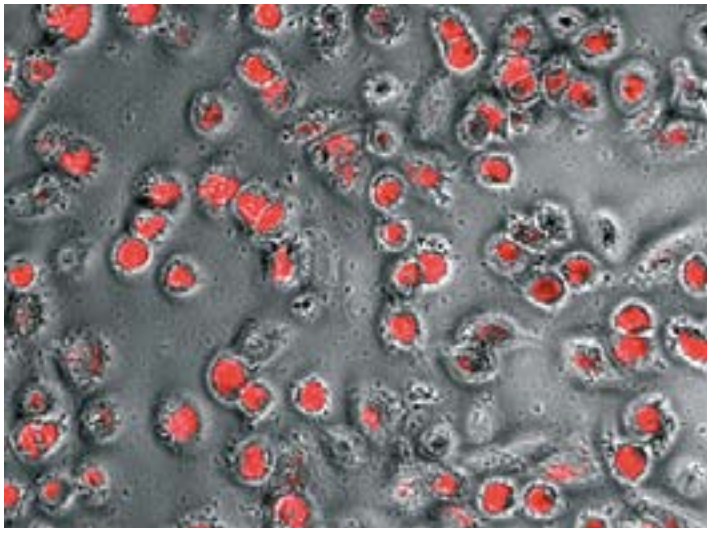

Fig. 2: "All you can eat": Our cryopreserved macrophages in action.
Demonstration of the strong phagocytic activity of hMDM- MCSF(-) using pHrodo™-labeled E. coli. Red fluorescence indicates large numbers of bacteria ingested by the macrophages. Plating density: 140.000 cells/cm², 200x magnification.

Macrophage Nomenclature

Macrophages are tissue-resident professional phagocytes and antigen-presenting cells (APC), which differentiate from circulating peripheral blood monocytes. They perform important active and regulatory functions in innate as well as adaptive immunity [8]. Indeed, macrophages are involved in the outcome of many diseases, e.g., allergic and autoimmune disorders, cancer, diabetes, atherosclerosis, rheumatoid arthritis and metabolic syndrome [9].

Traditionally, activated macrophages of different phenotypes have routinely been classified into M1 and M2 macrophages. The "classically activated" M1 macrophages comprise immune effector cells with an acute inflammatory phenotype. These are highly aggressive against bacteria and produce large amounts of lymphokines [10]. In contrast, the "alternatively activated" anti-inflammatory M2 macrophages comply with various regulatory functions of many kinds including regulation of immunity, maintenance of tolerance and tissue repair/wound healing [8, 10].

Indeed, it is now common sense that the traditional M1/M2 model of macrophage polarization/activation is not satisfactory to reflect the whole complexity of activation states of this highly plastic cell lineage [11]. As a consequence, a group of international macrophage experts published a common framework proposal for macrophage activation nomenclature [7]. This new system stipulates the designation of *in vitro* macrophage activation states according to the stimulus used (e.g. 20 ng/ml recombinant human (rhu) IL-4) in combination with clear disclosure of differentiation factors employed for hMDM generation (e.g. 100 ng/ml rhuM-CSF). See also Tab. 1.

Easy-to-handle, serum- and xeno-free macrophage culture systems devoid of ill-defined stimuli in combination with the published guidelines for unified experimental standards for *in vitro* macrophage activation constitute essential corner points for purposeful and effective progress in macrophage-related research.

	Activation state	Former designation	Differentiation factor (day 0+)	Activator (day 1+)	Activation process reference
M1	M(IFN-γ)	M1	GM-CSF (or M-CSF)	IFN-γ (50 ng/ml)	[1]
	M(LPS+IFN-γ)	M1	GM-CSF (or M-CSF)	IFN-γ (50 ng/ml) + LPS (10 ng/ml)	[1]
	M(LPS)	M1	GM-CSF (or M-CSF)	LPS (100 ng/ml)	[1]
	M(-)	M1, non-activated	GM-CSF	-	[2]
	M(-)	M0 / Mφ	2% human AB serum	-	[1, 3]
	M(-)	M2, non-activated	M-CSF	-	[2]
	M(GC)	M2c	M-CSF	DEX (100 nM)	[2]
	M(TGFb)	M2c	M-CSF	TGF-b1 (20 ng/ml)	[2]
	M(IL-10)	M2c	M-CSF	IL-10 (10 ng/ml)	[4]
	M(IC+LPS)	M2b	M-CSF	IgG (immobilized) + LPS (100 ng/ml)	[5]
M2	M(IL-4)	M2a	M-CSF	IL-4 (20 ng/ml)	[4, 5]
	TAM	M2-like	tumor microenvironment	tumor microenvironment	[6]

Table 1: Human macrophage activation reference table according to the common framework consensus nomenclature [7]. *The published differentiation factor/activator combinations are listed to serve as a basic guidance. Specific effects of activation on macrophages should be tested in comparison to the most appropriate non-activated M(-)-baseline variant as a control. DEX = dexamethasone, IC = immune complexes, IFN = interferon, IgG = immunoglobulin G, GC = glucocorticoids, (G)M-CSF = (granulocyte/) macrophage colony stimulating factor, IL = interleukin, LPS = lipopolysaccharide, TAM = tumor associated macrophages, TGF = transforming growth factor.*

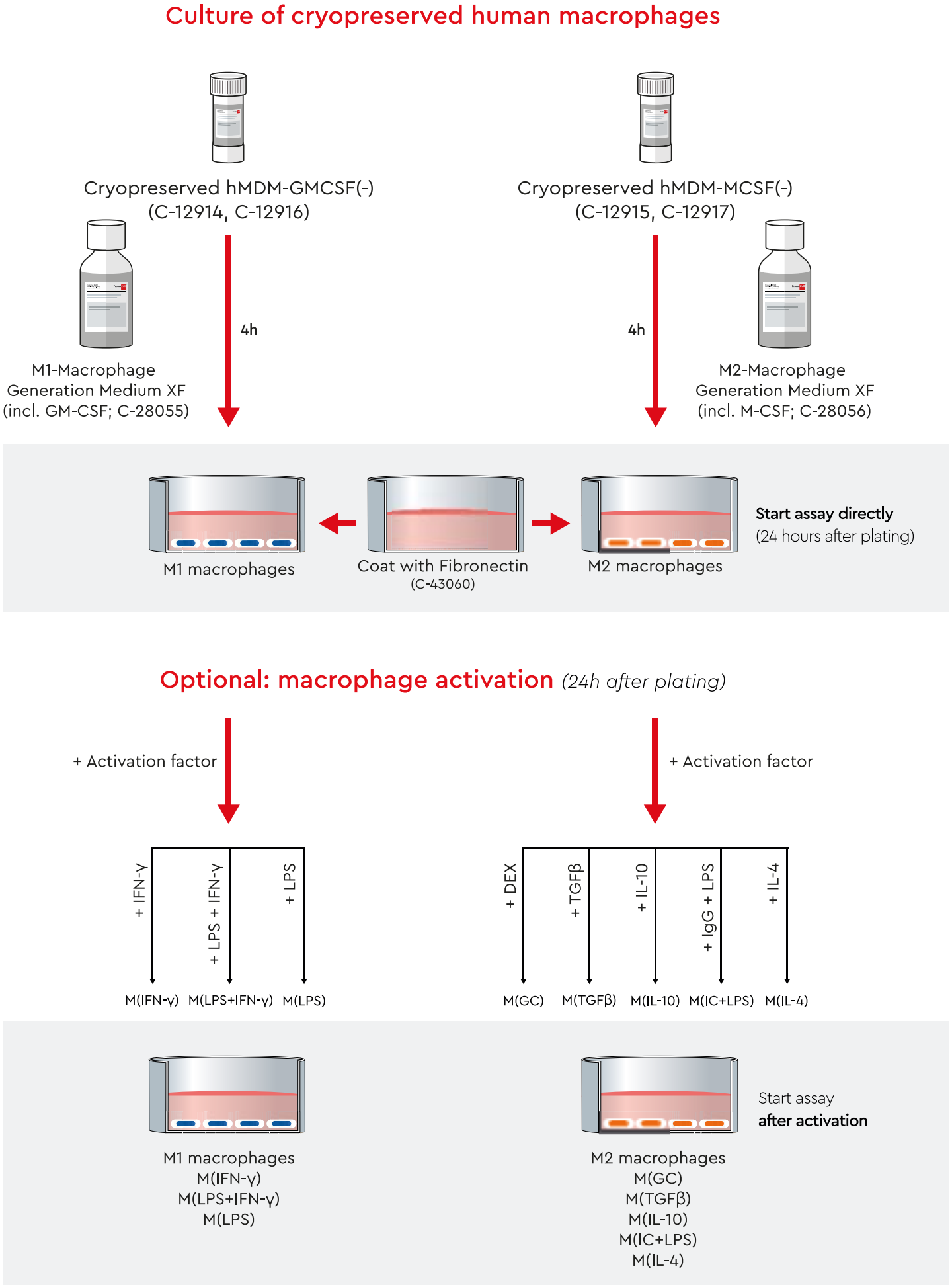

Fig. 3: Culture of cryopreserved human macrophages.
The macrophages can be directly used for experiments after thawing and plating. Optional: Activation of polarized M1/M2 macrophages before assay. See Tab. 1 for more activation details.

For more information see the application notes at www.promocell.com/scientific-resources/application-notes/.

Protocoll for culture of cryopreserved human macrophages

I. Culture Protocol

Materials

- Cryopreserved hMDM-GMCSF(-) (C-12914, C-12916) or hMDM-MCSF(-) (C-12915, C-12917)
- M1- or M2-Macrophage Generation Medium XF (C-28055 or C-28056)
- Human fibronectin solution
- PBS w/o Ca^{2+}/Mg^{2+} (C-40232)
- 70% Ethanol
- Optional: human serum AB for long-term cultures of hMDM-MCSF (see step 6)

Use aseptic techniques and a laminar flow bench.

1

Coat the culture vessel with human fibronectin

Coat the culture vessel with human fibronectin according to the instruction manual of the supplier. Use approximately 100 µl of the diluted fibronectin solution (10 µg/ml) per cm^2 of culture surface.

Note: Commercially available fibronectin-coated plasticware can also be used.

2

Prepare the complete medium and pre-equilibrate in the coated culture vessel

Prepare the M1/M2-Macrophage Generation Medium XF according to the instruction manual. Add an appropriate amount of medium (300–400 µl/cm^2, e.g. 3 ml per 6-well or 8 ml per T-25 flask) to the fibronectin-coated vessel and pre-equilibrate for at least 30 minutes at 37°C and 5% CO_2 before seeding the cells.

Note: Use freshly prepared medium for best results. Macrophage Generation Medium XF with added cytokines used for plating of cryopreserved macrophages must not be older than one week at the time of plating. Discard complete medium older than two weeks.

3

Thaw the cryopreserved macrophages

Place 14 ml of fresh M1/M2-Macrophage Generation Medium XF (tempered at 2–8°C) in a 15 ml conical tube and keep it under the laminar flow bench. This will be used as the thawing medium. Do not prewarm.
Remove the cryovial from the liquid nitrogen container and immediately place it on dry ice – even for short transportation.
Under a laminar flow bench, briefly twist the cap a quarter turn to relieve pressure, then retighten. Immerse the vial into a water bath (37°C) up to the height of the screw cap for 2 minutes. Ensure that no water enters the thread of the screw cap. Thoroughly rinse the cryovial with 70% ethanol under a laminar flow bench. Then, aspirate the excess ethanol from the thread area of the screw cap.

Note: Work swiftly once the cells are thawed.

4

Equilibrate and count the thawed macrophages

Open the vial and transfer the cells to the 15 ml tube containing the thawing medium using a 2 ml serological pipet. Avoid to introduce air bubbles during pipetting. Mix by a single gentle inversion and let the cells recover for 20 minutes at room temperature under the laminar flow bench. After the recovery period determine the viable cell count using your standard method. Centrifuge the cells for 15 minutes at 350 x g at room temperature. Aspirate the supernatant except for 50–100 µl. Loosen the cell pellet by grasping the top of the tube and firmly flicking its bottom 2–3 times. Then, resuspend the cells at 1 million cells/ml in fresh ambient tempered M1/M2-Macrophage Generation Medium XF using a serological pipet.

Note: Adhere to the stated centrifugation speed and time.

5

Plate the thawed macrophages

Immediately after thawing, plate the cell suspension at 100.000–200.000 cells/cm² in the fibronectin-coated culture vessels containing the pre-equilibrated M1/M2-Macrophage Generation Medium XF. Leave the cells untouched in the incubator to allow for attachment of the hMDM to the culture surface for at least 4 hours (4–24 hours).

Note: A 70–90% confluent cell monolayer of hMDM-GMCSF (Mφ-M1) is routinely obtained with a seeding density of 100.000 cells/cm², while the smaller sized hMDM-MCSF (Mφ-M2) require approx. 200.000 cells/cm².

6

Replace the medium

Change the medium 4–24 hours after plating using approx. 300-400 µl fresh medium per cm² of culture surface. Discard residual non-adherent cells. Allow the hMDM to stay in culture for at least 24 hours after seeding before starting your experiments. Optional activation of the polarized macrophages may be performed 24 hours after thawing. In the following time, change the culture medium every two to three days. The macrophages can be maintained in culture for several weeks.

Note: As compared to GM-CSF, M-CSF acts as a weaker long-term macrophage survival factor. Accordingly, the integrity of M2 macrophage cultures (hMDM-MCSF) to be main-tained for over one week may optionally be improved by addition of human serum AB from day 5–7 onwards without affecting the polarization status of the cells. Simply add 2% (v/v) of human serum AB to fresh complete M2-Macrophage Generation Medium XF and filter aseptically through a 0.22 µm filter in order to remove the serum-associated lipid precipitate.

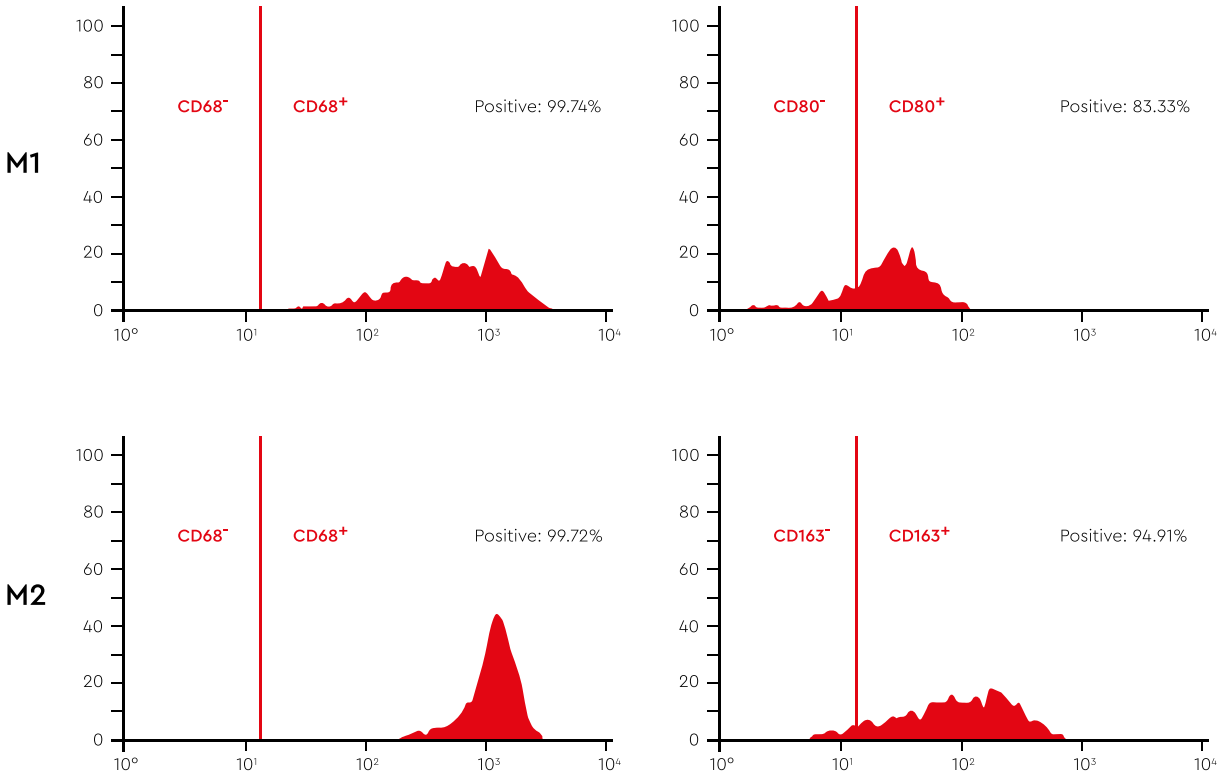

Fig. 4: Exemplary flow cytometric analysis of cryopreserved human M1/M2 macrophages.
*The M1/M2 macrophages were grown as an adherent culture on fibronectin coated culture vessels in the corresponding M1- or M2-Macrophage Generation Medium XF and were analyzed 1–3 days after thawing. The adherent cells were detached by using the Macrophage Detachment Solution XF (C-41330). M1 macrophages exhibit a CD68⁺ (99,74%) and CD80⁺ (83,33%) marker expression profile, typical for M1 macrophages (**upper row**). M2 macrophages exhibit a CD68⁺ (99,72%) and CD163⁺ (94,91%) marker expression profile, typical for M2 macrophages (**lower row**).*

Products

Cells	Recommended Maintenance Medium*	Plating density**	Marker	Size	Catalog Number
Human M1 Macrophages (GM-CSF), monocyte-derived, single donor (hMDM-GMCSF(-))	C-28055	100.000 cells per cm^2	$CD80^+$, $CD68^+$	1.5 mio / 5 mio	C-12914 / C-12916
Human M2 Macrophages (M-CSF), mono-cyte-derived, single donor (hMDM-MCSF(-))	C-28056	200.000 cells per cm^2	$CD163^+$, $CD68^+$	1.5 mio / 5 mio	C-12915 / C-12917

*** The plated cells do not proliferate.**
**** Recommeded plating density for obtaining an adherent cell monolayer with 70–90% confluency.**

Media	Size	Catalog Number
M1-Macrophage Generation Medium XF	250 ml	C-28055
M2-Macrophage Generation Medium XF	250 ml	C-28056

Related Products

Media & Reagents	Size	Catalog Number
Monocyte Attachment Medium (Ready-to-use)	250 ml	C-28051
Macrophage Base Medium XF	250 ml	C-28057
Macrophage Detachment Solution XF	250 ml	C-41330
Dulbecco's PBS, without Ca^{2+}/Mg^{2+}	500 ml	C-40232
Cryo-SFM	30 ml/125 ml	C-29910/C-29912

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