# Convenient large-scale differentiation of fully customized monocyte-derived macrophages from human PBMC *in vitro*

## Application Note

### Introduction

Primary human macrophages are difficult to isolate in sufficient amounts from tissue and do not proliferate in culture. In addition, it is commonly accepted that the obtained cells often exhibit significant phenotypical heterogeneity. Monocyte-derived macrophages (MDM) provide an excellent alternative, since human blood monocytes are readily available in large numbers and can be differentiated into macrophages *in vitro*.

The PromoCell Macrophage Base Medium DXF in combination with the Monocyte Attachment Medium were designed as a complete system for easy and cost-efficient differentiation of pure monocyte-derived macrophages directly from peripheral blood mononuclear cells (PBMC) as a starting material (see Figs. 1 and 3). Special equipment as well as prior monocyte purification, e.g. with magnetic beads, is not necessary saving time and costs.

The Macrophage Base Medium DXF is the user-customizable version of the macrophage media product line. It comes without cytokines as a universally applicable MDM culture system featuring a fully user-customizable macrophage differentiation and activation process (see Tab. 1 for suggestions).

As with all of our DXF media series, the Macrophage Base Media DXF exhibit a defined/xeno-free formula. Thus, these media provide a controlled culture environment devoid of all animal component-derived stimuli - a significant benefit in terms of monocytes and macrophages standing for highly reactive immune cells. Therefore, these media properties enable a standardized and customized macrophage differentiation and activation without the influence of undefined or animal-derived components.

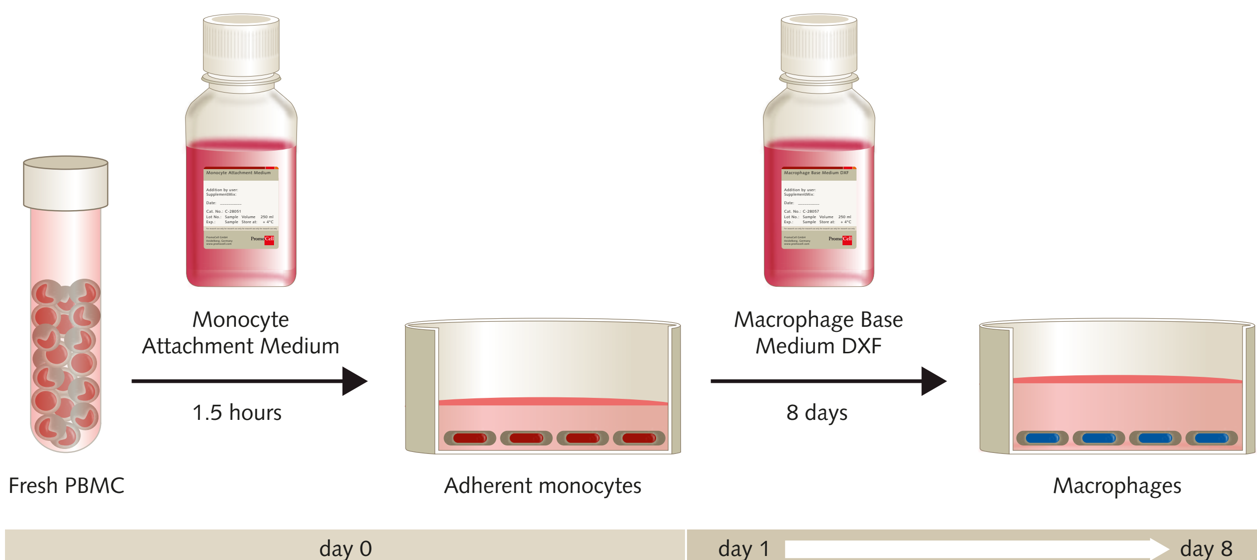


**Fig. 1: Differentiation of monocyte-derived macrophages in 8 days directly from PBMC using the Monocyte Attachment Medium and the Macrophage Base Medium DXF.** Activation of the culture can optionally be performed on day 7.

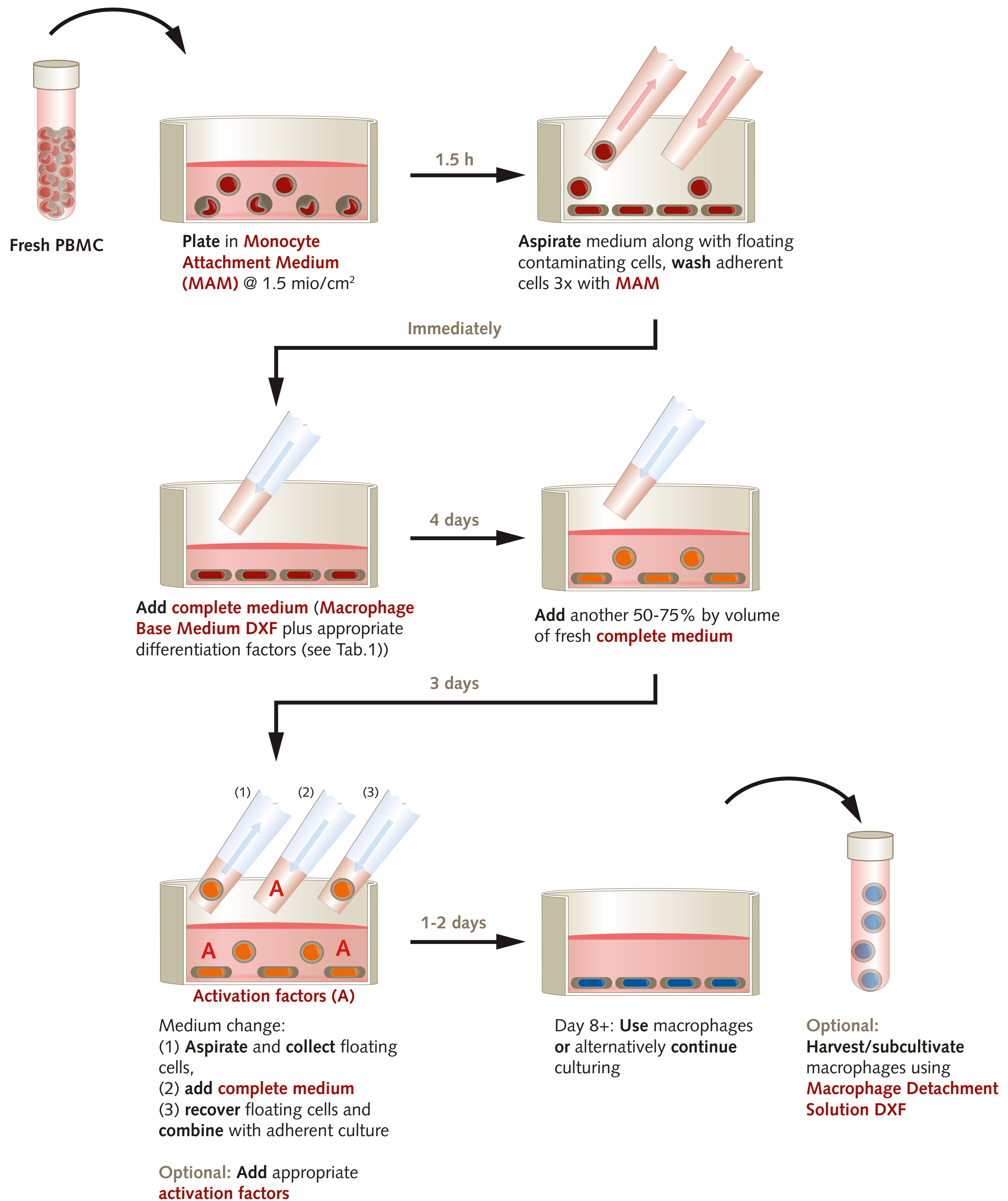


**Fig. 2: Protocol overview using Macrophage Base Medium DXF (8 days).**

**Table 1: Human macrophage activation reference table according to the common framework consensus nomenclature [7] (see also Background, page 6).** 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.

| | Activation state | Former designation | Differentiation factor (day 0+)* | Activator (day 7+) | 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(TGFβ) | M2c | M-CSF | TGF-β1 (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] |

*Use the differentiation factors M-CSF or GM-CSF at 50-100 ng/ml final concentration with the Macrophage Base Medium DXF (= complete medium: see section II., protocol step 4).

*Use aseptic techniques and a laminar flow bench.*

## Macrophage Generation

### I. Materials

- Monocyte Attachment Medium (C-28051)
- Macrophage Base Medium DXF (C-28057)
- Differentiation and activation factors (optional, refer to Tab. 1)
- PBS w/o $Ca^{2+}$/$Mg^{2+}$ (C-40232)
- PBS w/o $Ca^{2+}$/$Mg^{2+}$/2 mM EDTA/0.1% HSA
- Optional: Macrophage Detachment Solution DXF (C-41330, refer to protocol step 11)

*Macrophage Generation Materials*

### II. Monocyte-derived macrophage (MDM) Differentiation Protocol

*Macrophage Differentiation Protocol*

1. **PBMC isolation (day 0)**
   Isolate fresh PBMC from buffy coats using your routine protocol.
   **Note:** Use buffy coats as fresh as possible. Do not use buffy coats older than 24 hours, since this will significantly impair the experimental outcome.

2. **Count the PBMC (day 0)**
   Count the isolated PBMC and resuspend the cells at 100 million PBMC per ml in Monocyte Attachment Medium.

3. **Let the monocytes attach (day 0)**
   Plate freshly isolated PBMC in an appropriate amount of Monocyte Attachment Medium, e.g. 15 ml medium per T-75 flask. Use a seeding density of 1.5 million/cm². Incubate for 1.5 hours at 5% $CO_2$ and 37°C in the incubator without any further manipulation.

4. **Prepare the complete Macrophage Base Medium DXF (day 0)**
   Prepare the Macrophage Base Medium DXF by adding the thawed SupplementMix aseptically to the Basal Medium. Swirl gently to obtain a homogeneous mixture.
   **Note:** In order to promote survival and efficient differentiation of the monocytes in the given defined culture conditions, it is highly recommended to add a cytokine acting as a survival factor to the medium at this point, e.g. M-CSF or GM-CSF at 100 ng/ml. Alternatively, in order to generate non-activated/unpolarized M0 macrophages, add 2% human AB serum to the medium instead of M-CSF or GM-CSF. Macrophage Base Medium DXF with added survival factors (M-CSF, GM-CSF or human AB serum) is referred to as "complete medium" in the following.
   Be aware that M-CSF produces a homogeneous population of differentiated MDM, while GM-CSF, although a stronger-acting survival factor, will yield a more heterogeneous population containing some moDC-like cells (myeloid dendritic cell-like cells) [7].

5. **Wash the adherent cell fraction (day 0)**
   Vigorously swirl the tissue culture vessel and then aspirate the non-adherent cells. Wash the adherent cells, i.e. monocytes, three times with warm Monocyte Attachment Medium by thoroughly swirling the vessel and aspirating the supernatant.

6. **Start the macrophage differentiation (day 0)**
   Add an appropriate amount of complete medium to the cells, e.g. 20 ml per T-75

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*Macrophage Differentiation Protocol*

flask and incubate for 6 days at 37°C and 5% $CO_2$ without medium change.

**Note:** The monocytes differentiate into macrophages under these conditions. If required, activation can be achieved by performing the optional activation step (refer to step 9).

7. **Add fresh complete medium (day 4)**
   Add another 50% to 75% by volume of fresh complete medium to the cells. Incubate the immature macrophages for another 3 days at 37°C and 5% $CO_2$.
   **Note:** Do not remove any of the used medium from the cells, just add the fresh medium.

8. **Medium change (day 7)**
   Aspirate the medium including suspension cells and collect it in a centrifugation tube. Immediately, pipet fresh complete medium to the adherent cells. Centrifuge the cells in the tube for 15 min at 350 x g at room temperature.
   Discard the supernatant and carefully resuspend the cells in a small amount of fresh complete medium. Combine the resuspended cells in the tube with the adherent cells in the fresh complete medium contained in the tissue culture vessel. Incubate at least for another 24 hours at 37°C and 5% $CO_2$.
   **Note:** Adherent as well as suspension cells may be present at this stage.

9. **Optional activation step (day 7)**
   For specific macrophage activation, immediately after the prior media change, supplement the whole volume of the culture with adequate stimuli (refer to Tab. 1 for suggestions).

10. **The macrophages are ready (day 8+)**
    The macrophages may now be used directly in the plates where they reside, e.g. when performing phagocytosis assays. Alternatively, they can be harvested (see instructions in optional step 11). Maintenance of the culture for several weeks is possible by performing weekly medium changes with fresh complete medium.
    **Note:** Typically, macrophages appear as adherent cells with characteristic morphology: prominent nucleus with flatly outspread cytoplasm and multiple pseudopodia.

11. **Optional step: Harvesting/subcultivation of macrophages (day 8+)**
    Aspirate and discard the medium. Wash the adherent macrophages twice with endotoxin-free PBS w/o $Ca^{2+}$/$Mg^{2+}$. Immediately add an appropriate amount of cold (2-8°C) Macrophage Detachment Solution DXF to the cells, e.g. 25 ml per T-75 flask. Seal the tissue culture vessel and incubate cells for 40 min at 2-8°C. If necessary incubate another 20 min at room temperature to enforce cell release from the culture surface. Firmly tap the tissue culture vessel to facilitate cell detachment. Make sure most of the cells have already detached or are only loosely adherent to the surface of the tissue culture vessel. Only then use a cell scraper to dislodge the remaining macrophages.
    Collect the harvested macrophages in centrifugation tubes and dilute 1:1 with PBS/2 mM EDTA/0.1% HSA. Centrifuge cells for 15 minutes at 350 x g at room temperature. Wash the cells twice with PBS/2 mM EDTA/0.1% HSA and count them subsequently. The macrophages are now ready to be used for your experiments.
    **Note:** The percentage of attaching cells after re-seeding depends on the overall health status of the macrophages before detachment and the successful performance of the detachment process itself. Thus, some degree of variation is unavoidable.

## Background

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].
This functional heterogeneity of M2 macrophage functions lead to their allocation into three subgroups, i.e. M2a, M2b and M2c. Indeed, cells of the monocyte/macrophage lineage exhibit extraordinary plasticity in response to endogenous as well as exogenous stimuli potentially even leading to reversal of the initial M1/M2-polarization processes [2]. For example, M2 polarized macrophages can convert to the M1-activated status under certain conditions.

Therefore, it recently became 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 MDM generation (e.g. 100 ng/ml rhu M-CSF). See also Tab. 1.

Defined and xeno-free macrophage culture systems 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.

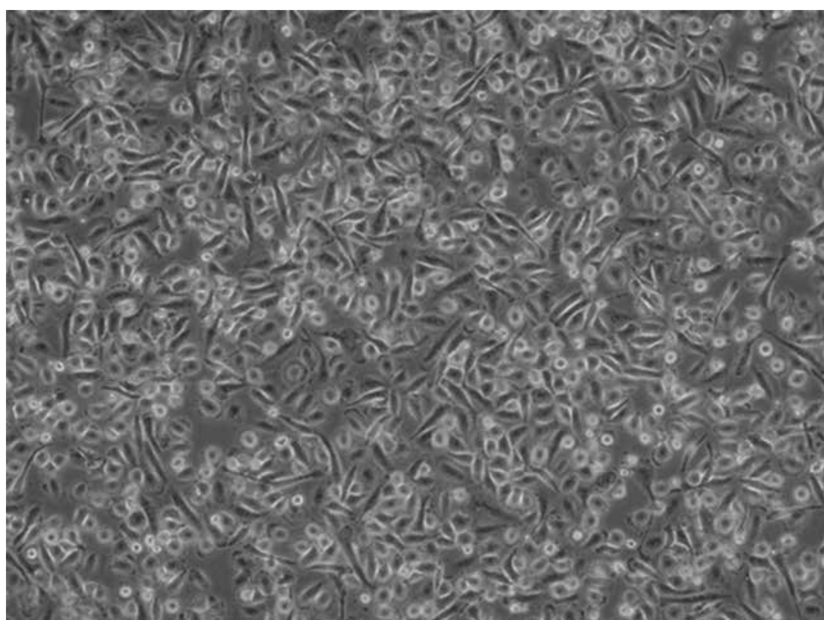

**Fig. 3: Culture of non-activated human monocyte-derived macrophages (MDM) differentiated directly from PBMC as a starting material.** Monocytes were purified using the Monocyte Attachment Medium and differentiated in Macrophage Base Medium DXF containing 100 ng/ml GM-CSF for 10 days.

## Products

| Product | Size | Catalog Number |
|---|---|---|
| Monocyte Attachment Medium (Ready-to-use) | 250 ml | C-28051 |
| Macrophage Base Medium DXF | 250 ml | C-28057 |
| Macrophage Detachment Solution DXF | 250 ml | C-41330 |
| Dulbecco's PBS, w/o $Ca^{2+}$/$Mg^{2+}$ | 500 ml | C-40232 |
| M-CSF (*E.coli*) | 10 µg* | C-60442 |
| M-CSF (Plant) | 10 µg* | C-60442A |
| GM-CSF (*E.coli*) | 10 µg* | C-60420 |
| GM-CSF (Plant) | 10 µg* | C-60420A |
| Dexamethasone | 1 g | PK-CA577-1042-1G |
| IFN-γ (*E.coli*) | 100 µg* | C-60724 |
| IFN-γ (Plant) | 100 µg* | C-60724A |
| IL1-β (*E.coli*) | 10 µg* | C-61120 |
| IL-4 (*E.coli*) | 20 µg* | C-61421 |
| IL-4 (Plant) | 10 µg* | C-61420A |
| IL-10 (*E.coli*) | 10 µg* | C-62012 |
| IL-12 (HEK) | 10 µg* | C-62212 |
| IL-13 (*E.coli*) | 10 µg* | C-62312 |
| TGF-β1 (*E.coli*) | 10 µg* | C-63500 |
| TGF-β1 (CHO) | 10 µg* | C-63503 |
| TGF-β1 (HEK) | 10 µg* | C-63499 |

*bulk sizes (100/250/500 and 1000 µg) are available on request

## Related Products

| Product | Size | Catalog Number |
|---|---|---|
| M1-Macrophage Generation Medium DXF | 250 ml | C-28055 |
| M2-Macrophage Generation Medium DXF | 250 ml | C-28056 |
| PromoFectin-Macrophage | 0.1 ml | PK-CT-2000-MAC-10 |
| PromoFectin-Macrophage | 0.5 ml | PK-CT-2000-MAC-50 |
| TNF-α ELISA Kit, human | 96 tests | PK-EL-63707 |
| TGF-β1 ELISA Kit, human | 96 tests | PK-EL-63506 |
| TGF-β2 ELISA Kit, human | 96 tests | PK-EL-63508 |
| IL-1α ELISA Kit, human | 96 tests | PK-EL-61106 |
| IL-1 beta ELISA Kit, human | 96 tests | PK-EL-61127 |
| IL-6 ELISA Kit, human | 96 tests | PK-EL-61606 |
| IL-8 ELISA Kit, human | 96 tests | PK-EL-61806 |
| IL-10 ELISA Kit, human | 96 tests | PK-EL-62006 |
| IL-12 (p40) ELISA Kit, human | 96 tests | PK-EL-62215 |
| IL-12 (p40+p70) ELISA Kit, human | 96 tests | PK-EL-62207 |
| IL-12 (p70) ELISA Kit, human | 96 tests | PK-EL-62216 |
| IL-18 ELISA Kit, human | 96 tests | PK-EL-62816 |
| IL-23 ELISA Kit, human | 96 tests | PK-EL-63006 |
| IL-27 ELISA Kit, human | 96 tests | PK-EL-62930 |
| RANTES (CCL5) ELISA Kit, human | 96 tests | PK-EL-64130 |
| GRO-alpha (CXCL1) ELISA Kit, human | 96 tests | PK-EL-65430 |
| MIG (CXCL9) ELISA Kit, human | 96 tests | PK-EL-65811 |
| IP-10 (CXCL10) ELISA Kit, human | 96 tests | PK-EL-65520 |
| TNF-alpha ELISA Development Kit (EDK), human | 1000 tests | PK-EL-63720D |
| IL-1α EDK, human | 1000 tests | PK-EL-61112D |

More ELISAs and antibodies for cytokines/chemokines/growth factors are available at www.promokine.info/ELISAs or www.promokine.info/antibodies.

| Product | Size | Catalog Number |
|---|---|---|
| IL-1 beta EDK, human | 10 x 96 tests | PK-EL-61127D |
| IL-6 ELISA Development Kit (EDK), human | 1000 tests | PK-EL-61620D |
| IL-8 EDK, human | 1000 tests | PK-EL-61830D |
| IL-10 EDK, human | 1000 tests | PK-EL-62012D |
| IL-12 EDK, human | 1000 tests | PK-EL-62212D |
| RANTES (CCL5) EDK, human | 1000 tests | PK-EL-64130D |
| GRO-alpha (MGSA, CXCL1) EDK, human | 1000 tests | PK-EL-65430D |
| GRO-beta (MIP-2, CXCL2) EDK, human | 1000 tests | PK-EL-65440D |
| MIG (CXCL9) EDK, human | 1000 tests | PK-EL-65811D |
| IP-10 (CXCL10) EDK, human | 1000 tests | PK-EL-65520D |
| Cell Migration/Chemotaxis Assay Kit (8 µm, 24-well) | 12 Assays | PK-CA577-K909 |
| Cell Migration/Chemotaxis Assay Kit (8 µm, 96-well) | 100 Assays | PK-CA577-K906 |
| Cell Migration/Chemotaxis Assay Kit (5 µm, 24-well) | 12 Assays | PK-CA577-K910 |
| Cell Migration/Chemotaxis Assay Kit (5 µm, 96-well) | 100 Assays | PK-CA577-K907 |
| Cell Migration/Chemotaxis Assay Kit (3 µm, 24-well) | 12 Assays | PK-CA577-K911 |
| Cell Migration/Chemotaxis Assay Kit (3 µm, 96-well) | 100 Assays | PK-CA577-K908 |

More ELISAs and antibodies for cytokines/chemokines/growth factors are available at www.promokine.info/ELISAs or www.promokine.info/antibodies.

## References


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06/2017