DHRS9 is a Stable Marker of Human Regulatory Macrophages

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Abbreviations:

DC, dendritic cell
DHRS9, dehydrogenase/reductase 9
FCS, fetal calf serum
GC, glucocorticoid
GMP, Good Manufacturing Practice
HABS, human AB serum
IDO, indoleamine 2,3-dioxygenase
IVIg, intravenous immunoglobulin
LPS, lipopolysaccharide
mAb, monoclonal antibody
M-CSF, macrophage colony-stimulating factor (CSF1)
MDSC, myeloid-derived suppressor cell
Mo-DC, monocyte-derived dendritic cell
Mreg, regulatory macrophage
Mφ, macrophage
pAb, polyclonal antibody
RA, retinoic acid
SDR, short-chain dehydrogenase reductase
Treg, regulatory T cell
ABSTRACT

Background: The human regulatory macrophage (Mreg) has emerged as a promising cell type for use as a cell-based adjunct immunosuppressive therapy in solid organ transplant recipients. In this brief report, dehydrogenase/reductase 9 (DHRS9) is identified as a robust marker of human Mregs.

Materials and Methods: The cognate antigen of a mouse monoclonal antibody raised against human Mregs was identified as DHRS9 by immunoprecipitation and MALDI-MS sequencing. Expression of DHRS9 within a panel of monocyte-derived macrophages was investigated by quantitative PCR, immunoblotting and flow cytometry.

Results: DHRS9 expression discriminated human Mregs from a panel of in vitro derived macrophages in other polarisation states. Likewise, DHRS9 expression distinguished Mregs from a variety of human monocyte-derived tolerogenic antigen-presenting cells in current development as cell-based immunotherapies, including Tol-DC, Rapa-DC, DC-10 and PGE2-induced MDSC. A subpopulation of DHRS9-expressing human splenic macrophages was identified by immunohistochemistry. Expression of DHRS9 was acquired gradually during in vitro development of human Mregs from CD14⁺ monocytes and was further enhanced by IFN-γ treatment on day 6 of culture. Stimulating Mregs with 100 ng/ml lipopolysaccharide for 24-hours did not extinguish DHRS9 expression. Dhrs9 was not an informative marker of mouse Mregs.

Conclusion: DHRS9 is a specific and stable marker of human Mregs.
INTRODUCTION

Several immunoregulatory cell-based products are presently being investigated as adjunct immunosuppressive agents in early-phase clinical trials in solid organ transplantation(1). One particularly promising candidate cell type is the regulatory macrophage (Mreg). The human Mreg represents a unique state of macrophage polarisation, which is distinguished from other activation states by a constellation of cell-surface markers and potent T cell suppressor function(2). Human Mregs suppress mitogen-stimulated T cell proliferation in vitro through interferon-gamma (IFN-γ)–induced indoleamine 2,3-dioxygenase (IDO) activity, as well as contact-dependent deletion of activated T cells(3). In addition, Mregs drive the development of activated induced regulatory T cells that, in turn, suppress the proliferation and activity of effector T cells (Riquelme-P et al, unpublished). Human Mregs derive from CD14+ peripheral blood monocytes when cultured in the presence of M-CSF and high concentrations of heat-inactivated human serum for more than 4 days prior to stimulation with IFN-γ. A proprietary GMP-compliant process for manufacturing a therapeutic product, known as Mreg_UKR, containing human Mregs has been established at a commercial pharmaceutical manufacturing facility in Germany(4). Now, Mreg_UKR is being investigated in a Phase-I/II trial as a means of promoting immune regulation in kidney transplant recipients with the objective of safely minimising maintenance immunosuppression (clinicaltrials.gov: NCT02085629).

With the objective of discovering novel markers of human Mregs, mouse monoclonal antibodies were raised against human Mregs. In this brief report, we identify dehydrogenase/reductase 9 (DHRS9) as the antigen recognised by one such Mreg-reactive monoclonal antibody (ASOT1). Within a panel of differently polarised monocyte-derived macrophages, expression of DHRS9 mRNA and protein was essentially restricted to Mregs. LPS stimulation did not extinguish DHRS9 expression by Mregs; therefore, DHRS9 behaves as a relatively specific and stable marker of in vitro generated human regulatory macrophages.
METHODS

Generation of human monocyte-derived macrophages. Mregs and IFN-γ-Mφ were generated according to previously described methods(2) from peripheral blood leucocytes obtained as a by-product of thrombocyte collection from healthy donors. Briefly, CD14+ monocytes were isolated from Ficoll-prepared PBMC by positive-selection with anti-CD14 microbeads (Miltenyi, Bergisch-Gladbach) and were then plated in 6-well Cell+ plates (Sarstedt, Nürnberg) at 10⁵ cells/cm² in RPMI-1640 (Lonza, Cologne) supplemented with 10% heat-inactivated human AB serum (Lonza), 2 mM Glutamax (Invitrogen, Karlsruhe), 100 U/mL penicillin (Lonza), 100 µg/mL streptomycin (Lonza), and rhM-CSF (R&D Systems, Wiesbaden-Nordenstadt) at 25 ng/ml carried on 0.1% human albumin (CSL-Behring, Hattersheim-am-Main). On day 6 of culture, cells were stimulated for a further 18-24 hours with 25 ng/mL rhIFN-γ (Chemicon, Billerica, MA). IFN-γ-stimulated macrophages (IFN-γ-Mφ) were generated by cultivating CD14+ monocytes under identical conditions to Mregs expect that human serum was replaced with 10% heat-inactivated fetal calf serum (FCS) (Biochrom, Berlin). Macrophages (Mφ) in other defined states of polarisation (5) were generated from positively-isolated CD14+ monocytes according to protocols adapted from the literature (6-9) and previously published methods (2) (see Table 1 for a summary of culture conditions). The tolerogenic monocyte-derived therapeutic cell products(10) shown in Fig.2F-G were prepared during The ONE Study workshop from CD14+ monocytes isolated by CliniMACS from leucapheresis products from 6 healthy, male donors (Table 1).

Flow cytometry. Cells were stained for flow cytometry following standardised protocols, in brief, surface staining was performed at 4°C in staining buffer (DPBS/1% BSA/0.02% NaN₃/10% FcR-block, Miltenyi) for 60 minutes using the directly-conjugated antibodies listed in Table 2. In all cases, saturating antibody concentrations were used and dead cells were excluded by 7-AAD staining (BD Biosciences). Data were captured with a Calibur cytometer (BD Biosciences) and analysed with FlowJo (Tree Star Inc).
Western blotting, immunoprecipitation and protein sequencing. Gel electrophoresis and immunoblotting were performed per conventional methods. Protein A/G sepharose (Sigma-Aldrich) was used to immunoprecipitate the antigen of ASOT1, which was then sequenced by MALDI-MS (Proteome Factory, Berlin).

Immunocytochemistry and histology. Cytological specimens were stained as previously described elsewhere (11). Tissue sections were stained with anti-DHRS9 mAb (clone 3C6) following established protocols and were then evaluated by a qualified clinical histopathologist.

PCR. RNA was isolated using RNeasy Plus kits (Qiagen). SuperScript-III (Invitrogen) was used for reverse-transcription reactions. qPCR was performed with a LightCycler real-time PCR system using the FastStart DNA Master SYBR Green I kit (Roche Diagnostics). DHRS9 primer sequences and cycling conditions are presented in Table 3. DHRS9 signals were normalised against GAPDH mRNA expression. ALDH1A1, ALDH1A2, BCO1, BCO2 and CD1C were amplified using predesigned primer pairs (QuantiTect, Qiagen) per the manufacturer’s recommendations. PCR specificity was confirmed sequencing of amplicons (MWG Biotech).

RESULTS

DHRS9 expression uniquely identifies Mregs amongst comparator macrophages

Although the CD14<sup>low</sup> CD16<sup>low</sup> TLR2<sup>−</sup> and CD163<sup>low</sup> cell-surface phenotype distinguished human Mregs from a variety of differently polarised monocyte-derived macrophages, this specification relies upon the absence of marker expression in Mregs (Fig.1). To identify positive markers of Mregs, a series of monoclonal antibodies (mAb) were generated by vaccinating mice with human Mreg lysates. Screening these mAb by immunocytochemistry identified a mAb clone (ASOT1) that reacted strongly with Mregs, but
not other monocyte-derived macrophages (Mφ), including resting Mφ, LPS + IFNγ-stimulated Mφ, IL-4-stimulated Mφ and immunoglobulin (Ig)-stimulated Mφ (Fig. 2A). By immunoprecipitation and MALDI-MS sequencing of its antigen, ASOT1 was shown to recognise dehydrogenase/reductase 9 (DHRS9), a little-studied retinol dehydrogenase of the short-chain dehydrogenase/reductase (SDR) family of NAD(P)(H)-dependent oxidoreductases (12-14) (Fig. 2B). Quantitative PCR confirmed that high-level expression of DHRS9 mRNA expression was restricted to Mregs within the panel of comparator macrophages (Fig. 2C). A rabbit polyclonal antibody (pAb) generated in-house against a synthetic peptide (CTDPENVKRTAQWVKNQVGEKG) corresponding to an N-terminal epitope of DHRS9 reacted against a protein of ~35 kDa immunoprecipitated by ASOT1 (Fig. 2D). Since a commercially-available mAb (clone 3C6, Abnova) against DHRS9 also reacted with the same immunoprecipitated protein, it was concluded that both ASOT1 and our custom-made rabbit pAb recognised DHRS9. Using our custom-made rabbit pAb for immunoblotting, DHRS9 protein expression was shown to be unique to Mregs within a panel of comparator human macrophages (Fig. 2E). DHRS9 expression was not diminished after stimulation of Mregs with 100 ng/ml LPS for 24 hours (Fig. 2C&E). To ascertain whether DHRS9 expression is a unique property of Mregs or a common characteristic of tolerogenic monocyte-derived cells, DHRS9 gene and protein expression was examined in samples obtained from The ONE Study Workshop on ‘Tolerogenic Monocyte-derived Antigen Presenting Cells.’ DHRS9 mRNA expression was significantly higher in Mregs than immature mo-DC, tolerogenic DC (Tol-DC) (15), Rapamycin-treated DC (Rapa-DC) (16,17), IL-10 conditioned DC (DC-10) (18-20) or PGE2-induced MDSC (21,22) (Fig. 2F). Likewise, at the protein level, DHRS9 expression was greater in Mregs than any of these comparator monocyte-derived cells (Fig. 2G).
Factors affecting DHRS9 expression in human macrophages

DHRS9 expression increased steadily throughout the transition of monocytes to Mregs in culture and was further induced by IFN-γ stimulation on day 6 (Fig. 3A). Treatment of resting macrophages with IL-4, plate-bound Ig, dexamethasone, IFN-γ or LPS + IFN-γ did not induce DHRS9 expression to the same level as Mregs (Fig.2C&E). Reanalyzing publicly available microarray data(23) revealed an induction of DHRS9 in glucocorticoid-stimulated Mφ and IFN-γ-stimulated Mφ, but no significant up-regulation under 26 other treatment conditions, which are meant to encompass all known states of human monocyte-derived macrophage polarisation (Fig.3B). By contrast, 14 treatment conditions led to >2-fold down-regulation of DHRS9 expression. Notably, glucocorticoid-induced DHRS9 mRNA expression was weak compared to DHRS9 expression in Mregs at the mRNA (Fig.2C) and protein level (Fig.2E). To characterise DHRS9 expression by Mregs at the single-cell level, a flow cytometry method was developed for detecting DHRS9 expression using mAb 3C6 (Fig.3C). DHRS9 signal was continuously distributed in Mreg populations, indicating that expression was not restricted to a subset of cells within Mreg cultures (Fig.3D). DHRS9 expression was greater in Mregs than in either resting Mφ or IFNγ-stimulated Mφ. Consistent with results from qPCR and microarray analyses, a very low-level of DHRS9 expression was observed in IFNγ-stimulated Mφ.

Human Mregs express enzymes involved in retinoid metabolism

The SDR family of retinol dehydrogenases is responsible for conversion of retinol to retinal, which is further metabolised to retinoic acid (RA) by retinal dehydrogenases(14,24) (Fig.4A): expression of ALDH1A1 and ALDH1A2 mRNA was detectable in Mregs (Fig. 4B). Retinol is liberated from β-carotene through the action of beta-carotene monooxygenases: Expression of BCO2, but not BCMO1, was also detected in Mregs (Fig. 4B). Accordingly, Mregs express the necessary apparatus to convert retinol and β-carotene to RA. CD1D mRNA
expression, which is upregulated by RA through RARα activation(25) was also detected in Mregs.

**DHRS9**<sup>+</sup> macrophages are naturally present in human spleen

Methods for producing human Mregs as a cell-based therapy have been optimised for generating a phenotypically homogeneous and stable population of cells. By contrast, macrophage subsets isolated from tissues tend to be much more heterogeneous. For this reason, in vitro derived human Mregs must be regarded as an artificial cell population; nevertheless, it may be interesting and informative to ask what naturally occurring macrophage subsets are most like in vitro derived Mregs. To ascertain whether DHRS9-expressing macrophages normally exist in tissues, an immunohistochemical staining procedure for fixed specimens was established using mAb 3C6. Soref and colleagues previously reported DHRS9 expression by stratified aquamous epithelium of human skin, which was confirmed using our staining method and reagents (data not shown). In earlier work, our group tracked ex vivo generated, radio-labelled allogeneic human Mregs after intravenous administration to a prospective kidney transplant recipient. These studies showed Mregs trafficked in blood through the lung to accumulate in spleen, liver and bone marrow(3). Therefore, human spleen sections were stained for DHRS9, revealing a minor population of DHRS9-expressing cells with typical macrophage morphology that appeared to be most prevalent in the subcapsular red pulp (Fig.5). Although it is not possible to conclude that these splenic red pulp macrophages are a physiological counterpart of the in vitro derived human Mreg, the existence of a naturally-occurring DHRS9<sup>+</sup> macrophage population suggests that DHRS9 expression by ex vivo generated Mregs is not purely an artefact of cell culture.

**Dhrs9** expression is not up-regulated in mouse Mregs

To ascertain whether Dhrs9 expression could also be used as a marker of mouse Mregs, a previously published microarray dataset was reinterrogated(26). This dataset
comprised triplicate whole-genome gene-expression profiles from CD11b+ Ly6C+ Ly6G− bone marrow monocytes, monocyte-derived Mregs, monocyte-derived DC (mo-DC) and 8 other differently treated macrophage populations from C57BL/6 mice (Fig.6). Dhrs9 expression was not up-regulated in Mregs or in any other comparator population. Moreover, no other SDR-family member was selectively up-regulated in Mregs, nor any comparator macrophage type. Accordingly, Dhrs9 expression is not useful for distinguishing mouse Mregs from other monocyte-derived cell types(27).

DISCUSSION

Here, we identify DHRS9 expression as a relatively specific and stable marker of in vitro generated human Mregs, which could be detected by qPCR, immunoblotting, immunohistochemistry and flow cytometry. Within the panel of comparator monocyte-derived macrophages used for this study, DHRS9 expression was principally restricted to Mregs. DHRS9 expression remained stable after LPS stimulation, which shows that certain aspects of the Mreg phenotype are refractory to repolarising signals. Hence, DHRS9 expression appears to be a unique and relatively stable characteristic of human Mregs as a ‘cell type’(28,29).

Yet, the conditions leading to DHRS9 expression in human Mregs are not fully defined, but glucocorticoid and IFN-γ appear to enhance DHRS9 expression to some degree. We hypothesise that presently undefined components of serum influence the in vitro development of Mregs from monocytes. In future experiments, assaying DHRS9 expression should facilitate screening of serum-derived factors for Mreg-inducing or –suppressing activity. The functional implications of DHRS9 expression by human Mreg are not clear. Although Mregs express the necessary retinoid metabolising enzymes, we cannot directly infer that Mregs generate RA. Nevertheless, it is well-known that certain tissue-resident macrophage populations responsible for maintaining tissue homeostasis and preventing
constitutive inflammation, such as those in the gut, suppress T cell reactions and induce Tregs through production of RA(30). We speculate that ex vivo generated human Mregs may act through similar pathways when administered to patients.

In conclusion, DHRS9 is a relatively stable and specific marker of human Mreg. The ability to positively identify DHRS9-expressing macrophages as Mregs should greatly facilitate future in vitro studies with these cells and may be useful in searching for a naturally occurring counterpart.

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FIGURE LEGENDS

Figure 1: **Comparative phenotyping of Mregs and other human macrophages.** Low or absent expression of CD14, CD16, TLR2 and CD163 discriminated human Mregs from a panel of differently stimulated human monocyte-derived macrophages, including resting Mφ, LPS + IFNγ-stimulated Mφ, IL-4-stimulated Mφ, immunoglobulin (Ig)-stimulated Mφ and glucocorticoid (GC)-stimulated Mφ. Values represent mean ± SD of n=6 donors.

Figure 2: **DHR9S expression distinguishes human Mregs from monocyte-derived macrophages and dendritic cells.** (A) In immunocytochemistry, the ASOT1 mAb recognised an antigen expressed by Mregs, but not comparator macrophages. (B) An antigen of ~35 kD was specifically immunoprecipitated by ASOT1 and was subsequently identified by MALDI-MS as DHR9S. (C) Strong DHR9S mRNA expression was detected in Mregs, but not comparator macrophage types (n=6; mean ± SD). (D) ASOT1 precipitated an antigen which was also recognised by an anti-DHR9S rabbit pAb (generated in-house) and a mouse mAb (clone 3C6, Abnova), confirming that ASOT1 recognises DHR9S. (E) Immunoblotting with our custom-made rabbit anti-DHR9S pAb demonstrated that DHR9S protein expression distinguishes Mregs from comparator macrophages. (F) DHR9S mRNA expression distinguished human Mregs from a panel of tolerogenic monocyte-derived therapeutic cell products. (G) DHR9S protein was detected in human Mregs but not other tolerogenic monocyte-derived therapeutic cell products using a commercial rabbit anti-DHR9S pAb (ab98155, Abcam).

Figure 3: **Factors affecting DHR9S expression in human macrophages.** (A) DHR9S mRNA expression increased steadily over 7 days of culture as human monocytes transitioned to Mregs and was further induced by IFN-γ stimulation on day 6 (n=6; mean ± SD). (B) Pattern of DHR9S expression in a publicly available microarray dataset encompassing 29
differently stimulated human monocyte-derived macrophages (Xue, J. et al, Immunity; GEO accession: GSE47189). >2-fold up-regulation of DHRS9 was elicited by treatment with either 1 μM dexamethasone or 200 IU/ml IFN-γ. (LPS, lipopolysaccharide; GC, glucocorticoid; HDL, high-density lipoprotein; IC, immune complex; P3C, Pam3CSK4; PGE2, Prostaglandin E2; LA, lauric acid; OA, oleic acid; LiA, linoleic acid; SA, steric acid; PA, palmitic acid; TPP, TNF + PGE2 + P3C). (C) Optimisation of an intracellular staining method for detection of DHRS9 expression in human macrophages using PE-conjugated anti-DHRS9 mAb clone 3C6. (D) Quantification of DHRS9 expression in human Mregs, IFN-γ Mφ and resting Mφ by flow cytometry.

**Figure 4: Human Mregs express enzymes involved in retinoid metabolism.** (A) A schematic overview of retinoid metabolism. DHRS9 is a member of the short-chain dehydrogenase/reductase (SDR) family of NAD(P)(H)-dependent oxidoreductases that catalyse interconversion of retinol and retinal. (B) Mregs expressed ALDH1A1, ALDH1A2 and BCO2 mRNA, as well as the RA-responsive gene, CD1D (n=3). (C) in (i) airway epithelium, (ii) skin, and (iii) spleen. The arrow indicates a DHRS9-expressing macrophage. (Original magnification 400x.)

**Figure 5: Identification of DHRS9+ Mφ in the subcapsular red pulp of human spleen.**

Immunohistochemical staining of 3 μm, H&E-counterstained sections of fixed, paraffin-embedded human spleen revealed a population of DHRS9+ macrophages. (A) DHRS9+ Mφ detected with mAb 3C6 appeared to be most prevalent in the subcapsular red pulp. Original magnification, 20x. (B) Negative control. Original magnification, 20x. (C) A high density of DHRS9+ macrophages were detected in the subcapsular splenic red pulp using mAb 3C6. Original magnification, 40x. (D) DHRS9 staining with mAb 3C6 was restricted to cytoplasm of cells with typical macrophage morphology. Original magnification, 40x.
Figure 6: **DHRS9 is not a marker of mouse Mregs.** Microarray analysis of SDR-family gene expression in monocyte-derived macrophages and DCs from C57BL/6 mice (Riquelme, P. et al, Molecular Therapy; GEO accession: GSE32690). No differential expression of DHRS9 or other SDR family members was observed between mouse Mregs and comparator cell types.
Figure 2

A

| Condition       | ASOT1 without IFN-γ | Resting Mφ | LPS + IFN-γ Mφ | IFN-γ Mφ | IL-4 Mφ |
|-----------------|---------------------|------------|----------------|----------|---------|
| Mreg            |                     |            |                |          |         |
| IgG1 control    |                     |            |                |          |         |

B

Western Ab: Control Ig, ASOT1
IP Ab: Control Ig, ASOT1

C

Relative DHRS9 mRNA Expression

D

Western Ab: Rabbit pAb anti-DHRS9, Mouse mAb anti-DHRS8
IP Ab: Control Ig, ASOT1, Control Ig, ASOT1

E

DHRS9
β-Actin

F

Log-transformed DHRS9 mRNA expression

G

DHRS9
β-Actin
Figure 6

Log$_2$ expression values

- Mreg
- Mreg without IFN-γ
- LPS-treated Mreg
- Resting Mφ
- LPS + IFN-γ Mφ
- IFN-γ Mφ
- IL-4 Mφ
- Ig Mφ
- GC Mφ
- mo-DC
- Monocyte

Dhrs9 Dhrs13 Dhrs11 Dhrs7b Dhrs7 Dhrs4 Dhrs3 Dhrs1
Table 1: Summary of the methods used to generate different monocyte derived cells

| Cell Type | Medium | Stimulation (last 18h) | Culture (days) | Ref. |
|-----------|--------|------------------------|----------------|------|
| Mreg      | RPMI 1640, 10% HABS, 5 ng/ml M-CSF. | 25 ng/ml | 7 | (2) |
| Mreg wo IFNγ | RPMI 1640, 10% HABS, 5 ng/ml M-CSF. | none | 7 | (2) |
| Resting Mφ | RPMI 1640, 20% FCS, 100 ng/ml M-CSF. On day 6, 5% FCS. | none | 7 | (2, 6) |
| LPS+IFN-γ Mφ | RPMI 1640, 20% FCS, 100 ng/ml M-CSF. On day 6, 5% FCS. | 20 ng/mL rhIFN-γ + 100 ng/ml LPS | 7 | (2, 6) |
| IFN-γ Mφ | RPMI 1640, 10% FCS, 100 ng/ml M-CSF. | 20 ng/mL rhIFNγ | 7 | (2, 7) |
| IL-4 Mφ | RPMI 1640, 20% FCS, 100 ng/ml M-CSF. On day 6, 5% FCS. | 20 ng/mL rhIL-4 | 7 | (2, 6) |
| GC Mφ | RPMI 1640, 20% FCS, 100 ng/ml M-CSF. On day 6, 5% FCS. | 10^{-7} M dexamethasone | 7 | (2, 9) |
| Ig Mφ | IVIg coated wells, RPMI 1640, 10% FCS, 100 ng/ml M-CSF. On day 6, 5% FCS. | 100 ng/ml LPS | 7 | (2) |
| iDC | IMDM, 10% FBS, 1000 U/ml rhGM-CSF, 1000 U/ml rhIL-4. | none | 6 | (21) |
| Tol-DC | AIM-V, 100 U/ml rhGM-CSF | none | 6 | (15) |
| Rapa-DC | AIM-V, 1000 U/ml rhGM-CSF, 1000 U/ml rhIL-4, from d2 10 ng/ml Rapamycin. | none | 7 | (16, 17) |
| DC-10 | RPMI 1640, 10% FBS, 100 ng/ml rhGM-CSF, 10 ng/ml rhIL-4, 10 ng/ml rhIL-10. | none | 7 | (18-20) |
| MDSC | IMDM, 10% FBS, 1000 U/ml rhGM-CSF, 1000 U/ml rhIL-4, 10^{-6}M PGE_{2} | none | 6 | (21, 22) |
Table 2: Antibodies used for flow cytometry

| Antigen | CD14 | Isotype | CD16 | Isotype | CD163 | Isotype | CD282 | Isotype |
|---------|------|---------|------|---------|-------|---------|-------|---------|
| Clone   | MφP9 | 27-35   | 3G8  | 679.1Mc7| GHI/61| MOPC-21 | TL2.1 | eBM2a   |
| Isotype | mlG2b| mlG2b   | mlG1 | mlG2b   | mlG1  | mlG2a   | mlG2a | mlG2a   |
| Conjugate| APC  | APC     | APC  | APC     | PE    | PE      | FITC  | FITC    |
| Test vol. (ul) | 5.00  | 40.00   | 10.00 | 10.00   | 20.00 | 20.00   | 5.00  | 5.00    |
| Supplier | BD   | BD      | BC   | BC      | BD    | BD      | eBio  | eBio    |
| Cat. #   | 345787| 555745  | B00845| IM2475U | 556018| 555749  | 11-9922| 11-4724 |
| Status   | CE/IVD| RUO     | ASR  | RUO     | RUO   | RUO     | RUO   | RUO     |
### Table 3: Primer sequences and cycling conditions

| Gene | Primer sequences (5'-3') | Annealing Temp. (°C) | Amplicon length (bp) |
|------|--------------------------|----------------------|----------------------|
| DHRS9 | TGACCGACCAGAGAATGTCAA GCCGGGAACACCAGCATTATT | 60 | 101 |
| GAPDH | TTGCCATCAATGACCCCTTCA CGCCCACCTTGATTTTGGGA | 57 | 173 |