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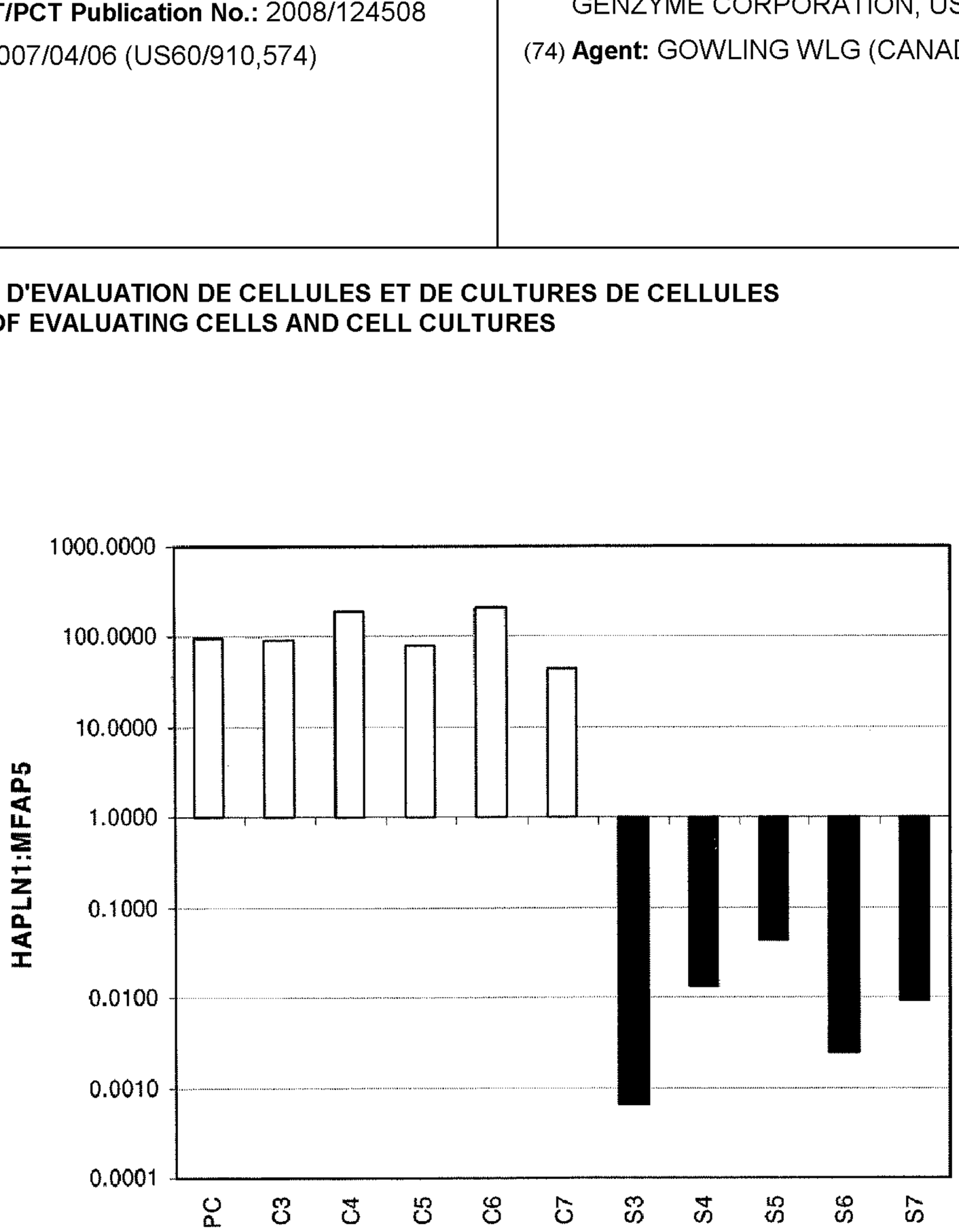
(54) **Titre : PROCEDES D'EVALUATION DE CELLULES ET DE CULTURES DE CELLULES**
(54) **Title: METHODS OF EVALUATING CELLS AND CELL CULTURES**



(57) **Abrégé/Abstract:**
Methods of evaluating the composition of a cell culture (e.g., to distinguish chondrocytes from fibroblasts) and methods for evaluating the phenotype of an individual cell (e.g., as a chondrocyte) are disclosed. The methods may be used, for example, for

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(57) **Abrégé(suite)/Abstract(continued):**
assessing chondrocyte cultures used for treatment of cartilage defects. In some embodiments, the invention involves identifying cell culture composition or the identity of a cell based on expression level of a fibroblast marker. In other embodiments, the invention involves comparing expression levels of at least one chondrocyte marker and at least one fibroblast marker in a cell culture sample or in an individual cell. In illustrative embodiments, the chondrocyte marker is hyaluronan and proteoglycan link protein 1 (HAPLN1), and the fibroblast marker is microfibrillar associated protein 5 (MFAP5).

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(54) Title: METHODS OF EVALUATING CELLS AND CELL CULTURES

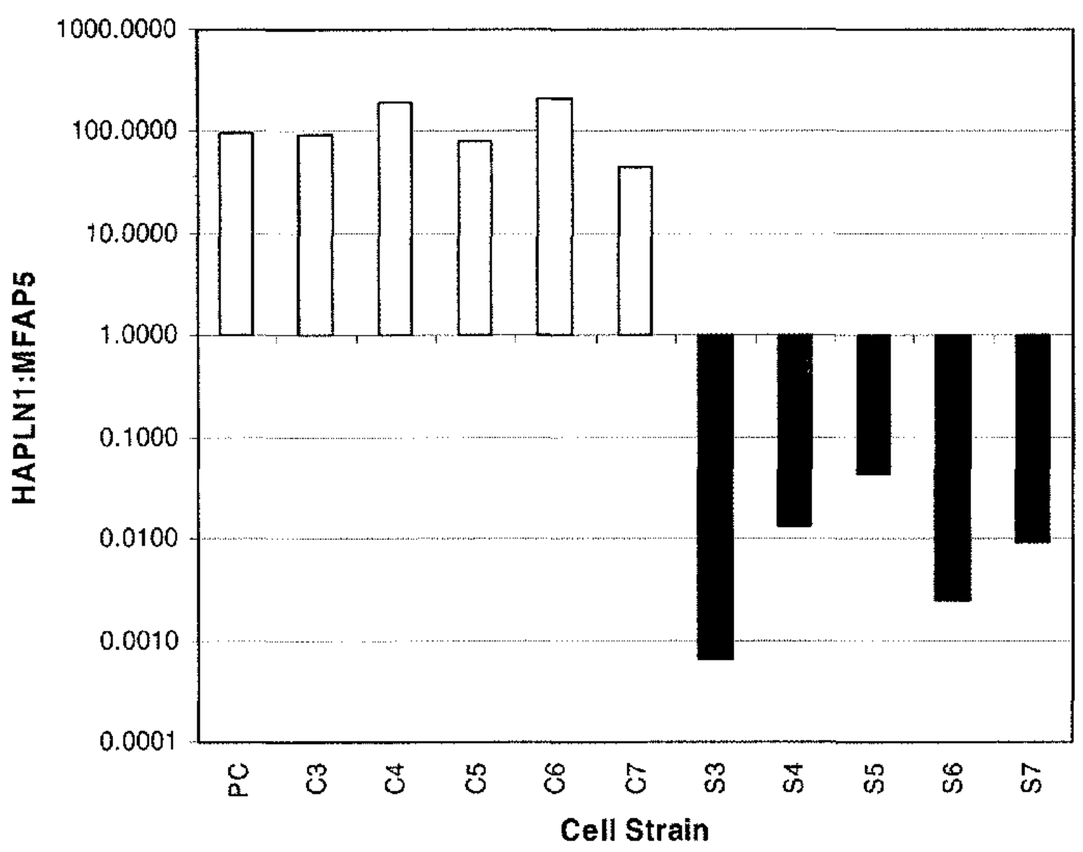

Fig. 9

(57) Abstract: Methods of evaluating the composition of a cell culture (e.g., to distinguish chondrocytes from fibroblasts) and methods for evaluating the phenotype of an individual cell (e.g., as a chondrocyte) are disclosed. The methods may be used, for example, for assessing chondrocyte cultures used for treatment of cartilage defects. In some embodiments, the invention involves identifying cell culture composition or the identity of a cell based on expression level of a fibroblast marker. In other embodiments, the invention involves comparing expression levels of at least one chondrocyte marker and at least one fibroblast marker in a cell culture sample or in an individual cell. In illustrative embodiments, the chondrocyte marker is hyaluronan and proteoglycan link protein 1 (HAPLN1), and the fibroblast marker is microfibrillar associated protein 5 (MFAP5).

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METHODS OF EVALUATING CELLS AND CELL CULTURES

[0002] This invention relates to methods of determining the composition of a cell culture, more particularly, to methods of distinguishing between chondrocytes and fibroblasts.

[0003] Injuries to articular cartilage have poor rates of repair, in part due to the lack of blood supply in cartilage tissue (Basad et al., In: Hendrich et al., Cartilage Surgery and Future Perspectives, Thieme Verlag, 49-56 (2003)). Trauma to knee joints can result in, for example, chondral and osteochondral lesions, and such injuries may progress to osteoarthritis (Brittberg et al., New England Journal of Medicine, 331(14): 889-895 (1994)). In severe cases of osteoarthritis, a total knee replacement may be needed. However, the artificial prostheses used in knee replacements have limited lifetimes, thus knee replacements are not optimal remedies, particularly for non-elderly patients (Brittberg et al., *supra*).

[0004] In some cases, articular cartilage injuries may be repaired by autologous chondrocyte implantation (Brittberg et al., Clin. Orthopaed. Rel. Res., 367S: S147-S155 (1999)). In this procedure, chondrocytes are harvested from a patient, expanded in cell culture to increase the number of chondrocytes, and then implanted back into the injury site of the patient. The chondrocytes are covered with a flap of periosteal tissue to seal the chondrocytes into the injury site. Although the cultured chondrocytes have a tendency to de-differentiate in culture, in a successful implant, de-differentiated chondrocytes preserve their re-differentiation potential and will re-differentiate into chondrocytes that produce a hyaline cartilaginous tissue upon implantation.

[0005] In a modified technique known as matrix-induced autologous chondrocyte implantation (MACI® implantation procedure), cultured chondrocytes are loaded onto a collagen matrix before they are implanted into the patient (Basad et al., *supra*). In addition, the collagen matrix can be fixed with fibrin glue rather than suturing, making it a simpler surgical technique.

[0006] Different techniques and media can be used to culture chondrocytes. Examples of serum-free media for chondrocyte culture and methods for isolation and propagation of chondrocytes are described, for example, in US Patent Nos. 6,150,163 and 7,169,610, and in US Provisional Patent Application No. 60/805,307.

[0007] Fibroblasts or fibroblast-like cells (such as synoviocytes) may be co-isolated with chondrocytes and, thus, co-propagated in a cell culture in the course of preparing chondrocyte implants. Chondrocytes are known to take on a fibroblastic appearance when they de-differentiate in culture (Benya and Shaffer, Cell, 30: 215-224 (1982)). Nevertheless, they maintain their differentiation potential, i.e., they are able to re-express a chondrocytic phenotype upon implantation. As a result, it can be difficult to distinguish cultured de-differentiated chondrocytes from co-cultured fibroblasts or fibroblast-like cells based on appearance.

[0008] In addition, gene expression patterns in cultured, de-differentiated chondrocytes are different from those of native cartilage chondrocytes. For example, many markers that are highly expressed in native cartilage chondrocytes are expressed at reduced levels in cultured chondrocytes (Binette et al., J. Orthopaed. Res., 16: 207-216 (1998)). Accordingly, expression of such a chondrocyte marker may not necessarily distinguish a de-differentiated chondrocyte from cells of other types that may be present in the cell culture. Furthermore, many known fibroblast markers are expressed in both de-differentiated chondrocytes and native cartilage chondrocytes, albeit at different levels. Accordingly, the expression level of such a fibroblast marker may not necessarily indicate whether cells present in the sample are de-differentiated chondrocytes, fibroblasts, or fibroblast-like cells.

[0009] There is a need for methods of identifying chondrocytes, fibroblasts and fibroblast-like cells, particularly, methods applicable to cell culture.

SUMMARY OF THE INVENTION

[0010] In certain aspects, the methods of the invention provide methods of evaluating the composition of a cell culture (e.g., to distinguish chondrocytes from fibroblasts) and methods for evaluating the phenotype of an individual cell (e.g., as a chondrocyte). The methods of the invention may be used, for example, for assessing chondrocyte cultures used for the treatment of cartilage defects. In some embodiments, the invention involves identifying cell culture composition or the identity of a cell based on expression level of a fibroblast marker. In other embodiments, the invention involves comparing expression levels of at least one chondrocyte marker and at least one fibroblast marker in a cell culture sample or in an individual cell. In illustrative embodiments, the chondrocyte marker is hyaluronan and proteoglycan link protein 1 (HAPLN1), and the fibroblast marker is microfibrillar associated protein 5 (MFAP5).

[0011] The invention is based, at least in part, on the identification of MFAP5 as a cell phenotype marker that is highly expressed in certain non-chondrocytic cell types, such as fibroblasts and synoviocytes, while being expressed at significantly lower levels in chondrocytes. The invention is further based, at least in part, on the finding that the expression level ratios of MFAP5 and a chondrocyte marker, such as HAPLN1, is a reliable indicator of the cell phenotype in cultures derived from cartilage biopsies. While under some conditions it may be preferable to use both types of markers (i.e., fibroblast and chondrocyte markers) in order to confirm the composition of cell culture or the phenotype of an individual cell, the invention also provides embodiments in which determining the normalized expression level of the MFAP5 marker alone may be sufficient for that purpose.

[0012] In some embodiments, the fibroblast marker is other than MFAP5 and is such that its normalized expression levels are lower in chondrocytes than in fibroblasts. In some embodiments, the fibroblast marker is such that its normalized expression levels are lower in chondrocytes (e.g., primary and/or passaged chondrocytes) than in fibroblasts and/or synoviocytes. In some embodiments, the fibroblast marker is expressed at least 2-, 5-, 8-, 10-fold lower, or less, in chondrocytes than in fibroblasts and/or synoviocytes.

[0013] Thus, in one aspect, the invention provides a method of evaluating the composition of a cell culture (Method 1), e.g., cell culture that tentatively contains chondrocytes; and a method of evaluating the phenotype of an individual cell (Method 2). In the embodiments of Method 1, the expression level of a respective marker is determined as the average expression level of that marker in a plurality of cells (e.g., culture sample). In the embodiments of Method 1, the composition of a cell culture may be evaluated as a whole to determine whether it contains chondrocytes. In the embodiments of Method 2, the expression level of a marker is determined as the expression level of that marker in the individual cell being evaluated. Thus, while Method 1 identifies the composition of the cell culture, Method 2 identifies the phenotype of an individual cell, e.g., whether or not the cell is a chondrocyte.

[0014] In some embodiments, Method 1 comprises:

a) obtaining a plurality of cells from a cell culture;

b) determining the average expression level of a fibroblast marker of the invention in a plurality of cells from the cell culture; and

c) determining the composition of the culture based on the expression level;

wherein the expression level below a predetermined threshold indicates that the cell culture contains chondrocytes. Alternatively, the expression level above a predetermined threshold indicates that the cell culture does not contain chondrocytes (e.g., the culture does not comprise at least 50%, 55%, 60%, 65%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or more chondrocytes).

[0015] In some embodiments, Method 1 involves comparing expression levels of a fibroblast marker (MFAP5 or another fibroblast marker) and a chondrocyte marker (e.g., HAPLN1) to a control or to each other. In some embodiments, the fibroblast marker and the chondrocyte marker are such that the ratio of their expression levels (chondrocyte marker to fibroblast marker) in primary and/or passaged chondrocytes is equal to, or greater than, 5, 10, 20, 30, 50, 75, 100 or more times the expression ratio in cultured fibroblasts.

[0016] In particular, in some embodiments, Method 1 comprises:

a) obtaining a plurality of cells from a cell culture;

b) determining the average expression level of a chondrocyte marker in the plurality of cells;

c) determining the average expression level of a fibroblast marker in the plurality of cells; and

d) determining the composition of the culture based on the average expression level of the chondrocyte marker and the average expression level of the fibroblast marker.

In some embodiments, the culture is identified as containing chondrocytes if the expression level of the chondrocyte marker is above a predetermined threshold, while the expression level of the fibroblast marker is below a predetermined threshold. Alternatively, the expression level of the fibroblast marker above a predetermined threshold indicates that the cell culture does not contain chondrocytes (e.g., the culture does not comprise at least 50%, 55%, 60%, 65%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or more chondrocytes).

[0017] In some embodiments, the step of determining the culture composition includes comparing the average expression levels of the chondrocyte marker and the average expression level of the fibroblast marker. In some embodiments, the markers' expression levels are compared relative to each other (thus, the thresholds may be defined, e.g., as a given difference between the expression levels of two markers, or a ratio thereof). For example, in some embodiments, a ratio of a chondrocyte marker (e.g., HAPLN1) expression level to that of a fibroblast marker (e.g., MFAP5), which is greater than a predetermined threshold, e.g., 0.25, 0.55, 1, 2, 2.2, 5, 10, 25, 50 or more, indicates that the cell culture contains chondrocytes.

[0018] In some embodiments of Method 1, the expression levels of chondrocyte and fibroblast markers are determined at the RNA level, e.g., by a standard curve method of quantitative RT-PCR or by a comparative C_T method of quantitative RT-PCR (which measures the difference in the number of threshold cycles required for the fibroblast marker and the chondrocyte markers).

[0019] In a related aspect, the invention provides a method of evaluating the phenotype of an individual cell (Method 2), e.g., using flow cytometry or single-cell RT-PCR. The method is useful for identifying individual cells from a cell culture, including a cell culture derived from cartilage or synovium, a chondrocyte

culture, a fibroblast culture, synoviocyte culture, or any other appropriate culture. The method is also useful for identifying individual cells derived from any appropriate biological samples in which it is desirable to identify individual cells, including cartilage samples, synovium samples, fibroblast samples, etc. The fibroblast and chondrocyte markers in Method 2 may be chosen and evaluated as described for Method 1.

[0020] In some embodiments, Method 2 comprises

a) determining the expression level of a fibroblast marker of the invention in the cell; and

b) determining the phenotype of the cell based on the expression level of the fibroblast maker;

wherein the expression level below a predetermined threshold indicates that the cell is a chondrocyte. Alternatively, the expression level above a predetermined threshold indicates that the cell is not a chondrocyte (e.g., a fibroblast or a synoviocyte). In some embodiments, Method 2 comprises:

a) determining the expression level of a chondrocyte marker in the cell;

b) determining the expression level of a fibroblast marker in the cell; and

c) evaluating the phenotype of the cell based on the expression level of the chondrocyte marker and the expression level of the fibroblast marker.

In some embodiments, the cell is identified as a chondrocyte if the expression level of the chondrocyte marker is above a predetermined threshold level, while the expression level of the fibroblast marker is below a predetermined threshold level. Alternatively, the cell is not a chondrocyte if the expression level of the chondrocyte marker is below a predetermined threshold level, while the expression level of the fibroblast marker is above a predetermined threshold level. In some embodiments, step c) of evaluating the phenotype of the cell includes comparing the expression levels of the chondrocyte marker and the expression level of the fibroblast marker.

[0021] Additional aspects of the invention will be set forth in the description that follows.

BRIEF DESCRIPTION OF THE FIGURES

[0022] **Figure 1** is a flow diagram illustrating stages in an exemplary manufacturing process used for producing cultured chondrocytes from chondrocyte biopsies.

[0023] **Figure 2** depicts HAPLN1 expression levels in several cell strains as determined by a standard curve method of RT-PCR. Expression levels were normalized to 18S ribosomal RNA. The expression level in primary chondrocytes (PC) was scaled to 1; other ratios were scaled accordingly. Cell strains used are listed in Table 2.

[0024] **Figure 3** depicts expression levels of MFAP5 in the same cell strains as shown in Figure 2, as determined by a standard curve method of RT-PCR. Expression levels were normalized to 18S ribosomal RNA. The expression level in primary chondrocytes (PC) was scaled to 1; other ratios were scaled accordingly.

[0025] **Figure 4** depicts the ratios of HAPLN1 and MFAP5 expression levels from Figures 2 and 3. The ratio in primary chondrocytes (PC) was scaled to 1; other ratios were scaled accordingly.

[0026] **Figure 5** depicts the ratios of HAPLN1 and MFAP5 expression levels in the same strains as shown in Figure 2. The expression levels were determined by a comparative C_T method of RT-PCR, and the ratios were calculated as $2^{(C_{T,MFAP5} - C_{T,HAPLN1})}$.

[0027] **Figure 6** depicts HAPLN1 expression levels in a number of additional chondrocyte and synoviocyte strains. The expression levels were determined by a standard curve method of RT-PCR and normalized to 18S ribosomal RNA. The expression level in primary chondrocytes (PC) was scaled to 1; other ratios were scaled accordingly. Cell strains used are listed in Table 3.

[0028] **Figure 7** depicts MFAP5 expression levels in the same strains as shown in Figure 6. The expression levels were determined by a standard curve method of RT-PCR and normalized to 18S ribosomal RNA. The expression level in primary chondrocytes (PC) was scaled to 1; other ratios were scaled accordingly.

[0029] **Figure 8** depicts the ratios of HAPLN1 and MFAP5 expression levels from Figures 6 and 7. The ratio in primary chondrocytes (PC) was scaled to 1; other ratios were scaled accordingly.

[0030] **Figure 9** depicts the ratios of HAPLN1 and MFAP5 expression levels in the same strains as shown in Figure 6. The expression levels were determined by a comparative C_T method of RT-PCR, and the ratios were calculated as $2^{(C_{T,MFAP5} - C_{T,HAPLN1})}$.

[0031] **Figure 10A** depicts the ratios of HAPLN1 and MFAP5 expression levels in the same strains as shown in Figures 2 and 6, as well as additional chondrocyte, synoviocyte, and dermal fibroblast strains identified in Table 4. The expression levels were determined by a comparative C_T method of RT-PCR using custom-designed primers and probes as described in Example 3. The HAPLN1:MFAP5 ratios were calculated as $2^{(C_{T,MFAP5} - C_{T,HAPLN1})}$. **Figure 10B** depicts the ratios of HAPLN1 and MFAP5 expression levels in additional cell strains identified in Table 5. The expression levels were determined using the same methods as described for Figure 10A.

[0032] **Figure 11** shows a comparison between expression level ratios for HAPLN1 and MFAP5 in monolayer and collagen-scaffold cultures. Cell strains used are listed in Table 7. HAPLN1 and MFAP5 expression levels were determined by a standard curve method of RT-PCR. Expression levels were normalized to 18S ribosomal RNA. The ratio in monolayer culture of primary chondrocytes (PC) was scaled to 1; other ratios were scaled accordingly.

[0033] **Figure 12** shows a comparison between expression level ratios for HAPLN1 and MFAP5 in the monolayer and collagen-scaffold cultures using the same strains as shown in Figure 11. The expression levels of HAPLN1 and MFAP5 were determined by a comparative C_T method of RT-PCR, and the ratios were calculated as $2^{(C_{T,MFAP5} - C_{T,HAPLN1})}$.

[0034] **Figure 13** depicts the change in the expression level ratios for HAPLN1 and MFAP5 as a function of culture level. Three synoviocyte strains were cultured from primary culture (culture level 1) through fourth passage (culture level 5), as shown in the figure. The expression levels of HAPLN1 and MFAP5 were determined by a comparative C_T method of RT-PCR, and the ratios were calculated as $2^{(C_{T,MFAP5} - C_{T,HAPLN1})}$.

[0035] **Figures 14A and 14B** depict the change in the expression level ratios for HAPLN1 and MFAP5 as a function of culture level. In Figures 14A and 14B, chondrocyte strains were sampled from cartilage (culture level 0) and then cultured from primary (culture level 1) through second passage (culture level 3), as shown in the figure. The expression levels were determined by a comparative C_T method of RT-PCR using custom-designed primers and probes as described in the Example 5. The HAPLN1:MFAP5 ratio was calculated as $2^{(C_{T,MFAP5} - C_{T,HAPLN1})}$.

[0036] **Figure 15** depicts ratios of expression levels of HAPLN1 and MFAP5 in cultures of mixed populations of chondrocytes and synoviocytes. Three trials were conducted, each with varying proportions of the two cell types. The expression levels were determined by a comparative C_T method of RT-PCR using custom-designed primers and probes as described in Example 6. The HAPLN1:MFAP5 ratios were calculated as $2^{(C_{T,MFAP5} - C_{T,HAPLN1})}$.

[0037] **Figure 16** depicts molar ratios of expression levels of HAPLN1 and MFAP5 in various cell strains, which are listed in Table 12, using absolute copy numbers of the markers as determined by an absolute quantitation method. RT-PCR was performed as described in Example 3, except that 2µl of cDNA was used per 13 µL PCR reaction. Standard curves were prepared from synthetic HAPLN1 and MFAP5 RNA transcript standards run at 10^3, 10^4, and 10^5 copies/reaction. The quantities of HAPLN1 and MFAP5 mRNA copies present in each test sample were determined from these standard curves.

DETAILED DESCRIPTION OF THE INVENTION

[0038] The invention is based, at least in part, on the identification of MFAP5 as a gene that is highly expressed in certain non-chondrocytic cell types, such as fibroblasts and synoviocytes, while being expressed at significantly lower levels in chondrocytes. Accordingly, in some embodiments the invention provides methods of using MFAP5 as a cell phenotype marker. MFAP5 is a serine-threonine-rich protein that binds to fibrillins and was reported to be involved in the stabilization of type I procollagen (Lemaire et al., Arthritis & Rheumatism, 52(6): 1812-1823 (2005)). The nucleotide and amino acid sequences of human MFAP5 can be found under GenBank® Accession No. NM_003480; its nucleotide

sequence is also provided as SEQ ID NO:1. In addition to, or in place of MFAP5, other fibroblast markers can also be used in the methods of the invention, as described below.

[0039] Accordingly, in one aspect, the invention provides a method of evaluating the composition of a cell culture comprising chondrocytes (Method 1) and a method of evaluating the phenotype of an individual cell (Method 2).

[0040] In the embodiments concerning Method 1, the expression level of a respective marker is determined as the average expression level of that marker in a plurality of cells. In the embodiments of Method 1, the composition of a cell culture may be evaluated as a whole to determine whether it contains chondrocytes. In the embodiments of Method 2, the expression level of a marker is determined as the expression level of that marker in an individual cell being evaluated. Thus, while Method 1 identifies the composition of cell culture, Method 2 identifies the phenotype of an individual cell, e.g., whether or not the cell is a chondrocyte.

[0041] In some embodiments, the fibroblast marker is other than MFAP5 and is such that its normalized expression levels are lower in chondrocytes than in fibroblasts. In some embodiments, the fibroblast marker is such that its normalized expression levels are lower in chondrocytes (e.g., primary chondrocytes, cultured de-differentiated chondrocytes) than in fibroblasts (e.g. dermal fibroblasts) and/or synoviocytes. In some embodiments, the fibroblast marker is expressed at least 2-, 5-, 8-, 10-fold lower, or less, in chondrocytes than in fibroblasts and/or synoviocytes. Such additional markers can be identified using, e.g., gene array analysis, as described in, e.g., Leung et al., Trends in Genetics, 19(11): 649 -659 (2003).

[0042] In some embodiments, Method 1 comprises determining the expression level of a fibroblast marker of the invention in a plurality of cells from a cell culture, wherein the expression level below a predetermined threshold indicates that the cell culture contains chondrocytes. Alternatively, the expression level above a predetermined threshold indicates that the cell culture does not contain chondrocytes (e.g., the culture does not comprise at least 50%, 55%, 60%, 65%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or more chondrocytes).

[0043] In illustrative embodiments, the fibroblast marker is MFAP5, and a higher-than-threshold expression of MFAP5 by the cell culture indicates that the culture contains a substantial number of non-chondrocytes. In some embodiments, the predetermined threshold level is 1) equal to or less than that of MFAP5 expression in pure fibroblast cultures (e.g., 2-, 3-, 4-, or 5-fold lower) or 2) equal to or greater than (e.g., 2-, 3-, 4-, or 5-fold greater) the level of MFAP5 expression in pure chondrocyte cultures (e.g., primary chondrocytes obtained from cartilage biopsies). For fibroblast markers other than MFAP5, the predetermined threshold can be analogously selected based on the expression levels of the respective marker in pure fibroblasts and/or chondrocytes. The "predetermined" level does not need to be chosen prior to determining marker expression levels and may be chosen after expression levels are determined, based for example, on the statistical analysis of the expression results.

[0044] The plurality of cells from the culture under evaluation may be represented by a sample or an aliquot obtained from that culture. For example, in case of cultures grown on collagen matrices, punch sampling can be used as described in the Examples. The plurality of cells, typically, will contain at least the number of cells sufficient to conduct a given method of expression analysis, or more. For example, for PCR as few as 10-1,000 cells are usually sufficient, but a lower number can also be used.

[0045] In some embodiments, Method 1 and Method 2 involve comparing expression levels of a fibroblast marker (MFAP5 or another fibroblast marker) and a chondrocyte marker (e.g., HAPLN1 or another chondrocyte marker) to a control or to each other. The order in which the expression levels of either marker are determined can vary. For example, one can first determine the expression level of a chondrocyte marker and then determine the expression level of a fibroblast marker, or vice versa. In some embodiments, the expression levels of both types of markers can be determined simultaneously.

[0046] Examples of some chondrocyte markers useful in the methods of the invention, including their GenBank™ accession numbers and SEQ ID NOs, are provided in Table 1. Thus, in some embodiments, the chondrocyte marker is chosen from HAPLN1, MGP, EDIL3, WISP3, AGC1, COMP, COL2A1, COL9A1, COL11A1, LECT1, S100B, CRTAC1, SOX9, and NEBL.

Table 1: Examples of chondrocyte markers

Marker Name	GenBank™ Accession No.	SEQ ID NO	Reference
hyaluronan and proteoglycan link protein 1 (HAPLN1)	NM_001884	SEQ ID NO:2	Buckwalter et al., J. Biol. Chem., 259(9): 5361-5363 (1984)
matrix Gla protein (MGP)	NM_000900	SEQ ID NO:3	Monroe et al., Nat Genet., 21(1): 142-4 (1999)
GF-like repeats and discoidin I-like domains 3 (EDIL3)	NM_005711	SEQ ID NO:4	Genes Dev., 12(1): 21-33 (1998)
WNT1 inducible signaling pathway protein 3 (WISP3)	NM_003880	SEQ ID NO:5	Kutz et al., Mol. Cell. Biol., 25(1): 414-21 (2005)
aggrecan 1 (AGC1)	NM_001135	SEQ ID NO:6	Roughley et al., Eur. Cell Mater.,11: 1-7 (2006)
cartilage oligomeric matrix protein (COMP)	NM_000095	SEQ ID NO:7	Song et al., J. Hum. Genet., 48(5): 222-5 (2003)
type II collagen (COL2A1)	NM_001844	SEQ ID NO:8	Nishimura et al., Hum. Mutat., 26(1): 36-43 (2005)
type IX collagen (COL9A1)	NM_001851	SEQ ID NO:9	Czarny-Ratajczak et al., Am. J. Hum. Genet., 69(5): 969-80 (2001))
type XI collagen (COL11A1)	NM_001854	SEQ ID NO:10	Poulson et al., J. Med. Genet., 41(8):e107

			(2004)
leukocyte cell derived chemotaxin 1 protein (LECT1)	NM_007015	SEQ ID NO:11	Hiraki et al., Eur. J. Biochem., 260(3): 869-78 (1999)
S100 calcium binding protein beta (S100B)	NM_006272	SEQ ID NO:12	Steffansson et al., Nature, 295(5844): 63-4 (1982)
cartilage acidic protein 1 (CRTAC1)	NM_018058	SEQ ID NO:13	Steck et al., Biochem. J., 353: 169-174 (2001)
SRY-box 9 protein (SOX9)	NM_000346	SEQ ID NO:14	Kou and Ikegawa, J. Biol. Chem., 279(49): 50942-8 (2004)
nebulette (NEBL)	NM_006393	SEQ ID NO:15	Grogan et al., Arth. & Rheum., 56(2): 586-95 (2007)

[0047] Additional chondrocyte markers can be identified using, e.g., gene array analysis, as described in, e.g., Leung et al., Trends in Genetics, 19(11): 649-659 (2003). Generally, a chondrocyte marker is a gene or protein whose normalized expression levels are higher in chondrocytes (e.g., primary chondrocytes, cultured de-differentiated chondrocytes) than in fibroblasts (e.g. dermal fibroblasts) and/or synoviocytes. In some embodiments, the chondrocyte marker is expressed at least 2, 4, 5, 8, 10, 50, 75, 100 times or greater in chondrocytes than in fibroblasts and/or synoviocytes.

[0048] In some embodiments, the fibroblast marker and the chondrocyte marker are chosen in such a way that the ratio of their expression levels in primary chondrocytes and/or in passaged chondrocytes is equal to or greater than 5, 10, 20, 30, 50, 75, 100 or more times that in dermal fibroblasts and/or synoviocytes.

[0049] In particular, in some embodiments, Method 1 comprises:

a) obtaining a plurality of cells from a cell culture;

b) determining the average expression level of a chondrocyte marker in the plurality of cells;

c) determining the average expression level of a fibroblast marker in the plurality of cells; and

d) determining the composition of the culture based on the average expression level of the chondrocyte marker and the average expression level of the fibroblast marker.

In some embodiments, the culture is identified as containing chondrocytes if the expression level of the chondrocyte marker is above a predetermined threshold, while the expression level of the fibroblast marker is below a predetermined threshold. Alternatively, the culture does not contain chondrocytes (e.g., the culture does not comprise at least 50%, 55%, 60%, 65%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or more chondrocytes) if the expression level of the chondrocyte marker is below a predetermined threshold, while the expression level of the fibroblast marker is above a predetermined threshold level.

[0050] In further embodiments of Method 1, the invention comprises a method of evaluating the composition of a cell culture, said method comprising:

a) obtaining a cartilage biopsy from a mammal;

b) isolating cells from the biopsy;

c) culturing cells isolated in step b) in a cell culture;

d) obtaining a sample of the cell culture;

e) determining the expression levels of MFAP5 and HAPLN1 in one or more cells from the sample; and

f) determining the composition of the culture based on the expression levels of MFAP5 and HAPLN1.

[0051] In some embodiments, the step of determining the culture composition comprises comparing the average expression levels of the chondrocyte marker and the average expression level of the fibroblast marker. In some such embodiments, the cell culture is evaluated as containing chondrocytes when the ratio of HAPLN1 expression to that of MFAP5 is greater than 0.25. In particular embodiments, this ratio indicates that the cell culture contains at least 50% chondrocytes.

[0052] In some embodiments, the markers' expression levels are compared relative to each other (thus, the thresholds may be defined, e.g., as a given difference between the expression levels of two markers or a ratio thereof).

For example, in some embodiments, a ratio of a chondrocyte marker (e.g., HAPLN1) expression level to that of a fibroblast marker (e.g., MFAP5), which is greater than a predetermined threshold, e.g., 0.25, 0.55, 1, 2, 2.2, 5, 10, 25, 50 or more, indicates that the cell culture contains chondrocytes.

[0053] In some embodiments of Method 1, the expression levels of chondrocyte and fibroblast markers are determined at the RNA level, e.g., by a standard curve method of quantitative RT-PCR or by a comparative C_T method of quantitative RT-PCR (which measures the difference in the number of threshold cycles required for the fibroblast marker and the chondrocyte markers).

[0054] In a related aspect, the invention provides a method of evaluating the phenotype of an individual cell (Method 2), e.g., using flow cytometry. The method is useful for identifying individual cells from a cell culture, including a cell culture derived from cartilage or synovium, a chondrocyte culture, a fibroblast culture, synoviocyte culture, or any other appropriate culture. The method is also useful for identifying individual cells derived from any appropriate biological samples in which it is desirable to identify individual cells, including cartilage samples, synovium samples, fibroblast samples, etc. In some embodiments, Method 2 comprises determining the expression level of a fibroblast marker of the invention in the cell, wherein the expression level below a predetermined threshold indicates that the cell is a chondrocyte. Alternatively, the expression level above a predetermined threshold indicates that the cell is not a chondrocyte (e.g., the cell is a fibroblast or a synoviocyte). In some embodiments, Method 2 comprises:

a) determining the expression level of a chondrocyte marker in the cell;
b) determining the expression level of a fibroblast marker in the cell; and
c) evaluating the phenotype of the cell based on the expression level of the chondrocyte marker and the expression level of the fibroblast marker.

In some embodiments, the cell is identified as a chondrocyte if the expression level of the chondrocyte marker is above a predetermined threshold level, while the expression level of the fibroblast marker is below a predetermined threshold level. The fibroblast and chondrocyte markers in the embodiments of Method 2 may be chosen and evaluated as described for Method 1 above.

[0055] Flow cytometry can be performed using commercially available antibodies or such antibodies may be prepared as described in, e.g., Linsenmeyer et al., Biochem. Biophys. Res. Com., 92(2): 440-6 (1980).

[0056] Cells and cultures being evaluated by the methods of this invention may be obtained from any biological sample, including any tissue, cell culture, or other material, that may or may not contain chondrocytes. In some embodiments, the cells or cultures being evaluated are of mammalian, particularly human, origin. In some embodiments, the cell culture is grown from cells released from a cartilage biopsy. For example, in autologous chondrocyte implantation, cartilage cells for the procedure are normally cultured from a cartilage biopsy of the patient receiving the implant. Carticel® autologous chondrocyte product (Genzyme Corporation, Cambridge, MA) is an example of a cultured chondrocyte product. In some embodiments of the invention, the cell culture comprises a collagen matrix loaded with chondrocytes. Such chondrocytes may be obtained from a cartilage biopsy and cultured prior to being loaded on the matrix, e.g., as used in the MACI® implant product. The method of the invention is useful for identifying, and/or confirming identify of cells loaded onto the collagen support prior to implanting the matrix.

[0057] To illustrate an example of the utility of the cell culture determination method, reference is made to Figure 1. This figure illustrates the steps involved in producing a cultured chondrocyte product for autologous chondrocyte implantation, such as using Carticel® autologous chondrocytes, or for producing a cultured chondrocyte product for the MACI® implantation procedure. In step 1, a cartilage biopsy from a patient undergoing autologous chondrocyte implantation is shipped for processing (step 2). The biopsy material is digested at step 3 to release and harvest chondrocytes from the cartilage. The released cells are plated in tissue culture flasks and expanded in primary culture at step 4, and if necessary, subcultured. Once the cells reach an adequate number, they can be, optionally, cryopreserved at step 5 until the patient is ready to receive the implant. Once the patient is ready to receive the cells, they are thawed and plated into tissue culture flasks and grown to prepare an assembly culture (step 6).

[0058] For use in an autologous chondrocyte implant, if a sufficient number of cells are obtained in the assembly culture, the cells are centrifuged to a

cell pellet and resuspended in shipping medium, which is the "final product" such as the Carticel® autologous chondrocyte product (step 8). This "final product" is subjected to a number of QC tests, including for example, a sterility test, a cell viability test, an endotoxin test, a mycoplasma test, and a culture composition test (step 9 "QC identity" as described herein) to ensure that the cultured cells contain a sufficient number of chondrocytes. If the cultured cells pass all QC tests, they are shipped (step 10) to the patient for implantation (step 11).

[0059] Alternatively, when the assembly culture from step 6 is to be used in a MACI® implant, the cells are resuspended in culture medium, seeded onto a collagen scaffold, and cultured for 4 days (step 7). At the end of the culture period, the cells are rinsed with shipping medium to produce a final product for MACI® implants. This product is also subjected to the QC tests outlined above. Accordingly, whether the final product is a suspension of cultured chondrocytes, such as Carticel® autologous chondrocytes, or the final product is a scaffold-seeded product for MACI® implants, the method of the invention is useful as a lot identification assay or lot release assay, to confirm the composition of a cell culture as containing chondrocytes prior to shipment of the culture. For example, the "QC identity" (step 9) can be performed at any step prior to the final product assembly, e.g., before step 4, 5, 6, 7, or 8.

[0060] Many methods of determining gene or protein expression levels are known to persons skilled in the art, e.g., as described in Sambrook et al. (eds.) Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, 1989; Current Protocols in Molecular Biology (Ausubel et al. (eds.) New York: John Wiley and Sons, 1998). Examples of such methods include polymerase chain reaction (including absolute quantitation by PCR, real time PCR (RT-PCR) and qRT-PCR, multiplex or singleplex PCR), single cell PCR, northern blot assays, nuclease protection assays, in situ hybridization assays, immunohistochemistry assays, immunocytochemistry assays, electrophoresis assays such as gel or capillary, Western blot assays, ELISAs, immunoprecipitation assays, chromatography based assays such as HPLC or gel chromotography, mass spectrometry assays, RNase protection assays, flow cytometry assays, DNA methylation assays, and histone modification analysis assays.

[0061] In all methods of the invention, expression levels, at the RNA or at the protein level, can be determined using any suitable method, including any one of conventional methods. RNA levels may be determined by, e.g., quantitative RT-PCR (e.g., TaqMan™ RT-PCR or RT-PCR), Northern blotting, or any other method for determining RNA levels, or as described in the Examples. Protein levels may be determined, e.g., by using Western blotting, ELISA, flow cytometry, enzymatic activity assays, or any other method for determining protein levels. Expression levels may be scaled and/or normalized per total amount of RNA or protein in the sample and/or a control, which may typically be a housekeeping gene such, as beta-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH), or 18S ribosomal RNA, etc.). Normalization is typically done to account for variability in the amount of protein, DNA, or RNA input. For example, in the Examples, expression levels are normalized to 18S ribosomal RNA using a standard curve.

[0062] In illustrative embodiments, the expression levels of the fibroblast and chondrocyte markers are determined using RT-PCR, either by a standard curve or by a comparative C_T method for relative quantification. In some embodiments, absolute quantitation of marker copy numbers can be determined by preparing standard curves using known amounts of the markers. The general methods for conducting such assays are described, e.g., in Real-Time PCR Systems: Applied Biosystems 7900HT Fast Real-Time PCR System and 7300/7500 Real-Time PCR Systems, Chemistry Guide, Applied Biosystems, 2005, Part No. 4348358 Rev. E.

[0063] In the case of comparing two markers using the comparative C_T method, the amount of the ratio of expression levels of a fibroblast marker to that of a chondrocyte marker can be calculated as $(1+ E)^{\wedge}(C_{T,f} - C_{T,c})$, wherein $C_{T,f}$ is the number of the fibroblast marker threshold cycles, $C_{T,c}$ is the number of threshold cycles of the chondrocyte marker, assuming that efficiency of amplification (E) is the same for both markers and the starting amount of both markers is normalized to the same amount of endogenous control (e.g., as in two duplicate samples). In the case of $E \approx 1$, as illustrated in the Examples, the ratio can be approximated as $2^{\wedge}(C_{T,f} - C_{T,c})$. Otherwise, the calculations can be performed as described in Appendix A of Real-Time PCR Systems: Applied

Biosystems 7900HT Fast Real-Time PCR System and 7300/7500 Real-Time PCR Systems, Chemistry Guide, Applied Biosystems, 2005, Part No. 4348358 Rev. E.

[0064] Further embodiments of the invention are illustrated in the following examples, which are intended to be exemplary and not intended to be limiting on the scope of the invention.

EXAMPLES

Example 1: Expression of HAPLN1 and MFAP5 in Chondrocytes, Synoviocytes and Fibroblasts

[0065] *Cell Isolation and culture*--Human chondrocyte cultures were isolated from cartilage using the method for producing Carticel® autologous chondrocytes or the Protease method for producing cultured chondrocytes. Using the method for producing Carticel® autologous chondrocytes, cartilage tissue was trimmed of bone and synovium and subjected to a first digestion where tissue was enzymatically treated in collagenase solution for 18 hours at 37°C. Cells released from the first digestion were plated in tissue culture flasks with fetal bovine serum (FBS) and gentamicin containing medium (EGHXX). The cells were then subjected to a second digestion where remaining tissue from the first digestion was treated with a collagenase/trypsin solution for 2.5 hours at 37°C. Cells released from the second digestion were plated in tissue culture flasks with EGHXX. Tissue pieces remaining after the second digestion were plated in tissue culture flasks with EGHXX. Using the Protease isolation method, cartilage tissue was trimmed of bone and synovium and subjected to a first digestion in Pronase E (Sigma-Aldrich Inc., St. Louis, MO) solution for 1.5 hours at 37°C. The Pronase solution was then removed and a second digestion of the cartilage was performed in collagenase solution for 18 hours at 37°C. The released cells were then plated in tissue culture flasks with EGHXX. After isolation, the cell culture methods were the same for cells obtained from either isolation method. Primary cell cultures were re-fed fresh EGHXX every 2 to 4 days. When the primary culture flasks reached 50% to 80% confluence, they were trypsinized to a single cell suspension, neutralized with EGHXX to inactivate trypsin, and a cell count was performed. The resulting cell suspension was then either sampled, further

expanded by subculturing, or cryopreserved for long term storage. The subculture of the primary culture is referred to as the secondary culture, or first passage. Subsequent subcultures are referred to as the second passage, third passage, fourth passage, etc. Subculturing was performed by plating cells in tissue culture flasks with EGHXX and-re-feeding with fresh EGHXX every 2 to 4 days. When the subcultures reached 80% to 100% confluence, they were trypsinized to a single cell suspension, neutralized with EGHXX to inactivate trypsin, and a cell count was performed. The resulting cell suspension was then either sampled, further expanded, or cryopreserved for long term storage.

[0066] Human synoviocyte cultures (synovium derived cell cultures, also known as synovial fibroblasts) S1 and S2 were obtained from Cell Applications Inc. (San Diego, CA) as cryopreserved primary cultured cells. The synoviocytes were plated in tissue culture flasks with EGHXX medium and cultured using the method for producing Carticel® autologous chondrocytes as described above. Human dermal fibroblast cultures were purchased from Cell Applications Inc. as cryopreserved-primary cultured cells. The dermal fibroblasts were cultured using the method for producing Carticel® autologous chondrocytes as described above.

[0067] The cell cultures used in this Example are listed in Table 2.

Table 2: Cell cultures used in RT-PCR Analysis (Example 1)

Cell Culture	Cell Type	Type of Cell Culture
PC	Chondrocyte	Primary culture
C1	Chondrocyte	Second Passage
C2	Chondrocyte	Second Passage
S1	Synoviocyte	Second Passage
S2	Synoviocyte	Second Passage
F1	Dermal Fibroblast	Second Passage
F2	Dermal Fibroblast	Second Passage

[0068] *RNA and cDNA preparation*--RNA was isolated from cell cultures using the TRI-spin procedure (Reno et al., Biotechniques 22: 1082-6 (1997)). Isolated RNA concentrations were determined spectrophotometrically. For the preparation of cDNA from samples PC, C1, C2, S1, S2, F1, and F2, the First

Strand Synthesis Kit (Roche, Indianapolis, IN), using random hexamer primers was run according to the manufacturer's instructions. The resulting cDNA was stored at -20°C or -80°C until analysis.

[0069] *Gene expression analysis*--Gene expression analysis was performed using quantitative real time RT-PCR, using either a standard curve method or a comparative C_T method. The real time PCR method was based on the 5' nuclease cleavage of a dual labeled oligo probe to report sequence specific primer amplification of the target sequence ("TaqMan™" assay). Expression of genes encoding cartilage link protein (HAPLN1) and microfibrillar associated protein 5 (MFAP5) were assayed using TaqMan™ Gene Expression Assays Hs00157103_m1 and Hs00185803_m1 (Applied Biosystems Inc.), respectively. The real time PCR was prepared with TaqMan™ Universal PCR Master Mix, no UNG (catalog number 4324018, Applied Biosystems Inc.), appropriate TaqMan™ Gene Expression Assay (Applied Biosystems) and sample cDNA were used according to the Universal PCR Mix protocol. The amplifications were run on an ABI 7500 Real-Time PCR system (Applied Biosystems Inc.) using the standard TaqMan™ cycling and data collection program for this configuration. Duplicate 25 µL reactions were run with up to 5 ng of input cDNA per well. A threshold of 0.1 units was used for all assays.

[0070] *Standard Curve Method*--The standard curve method was performed using the Eukaryotic 18S rRNA Endogenous Control assay (catalog number 4319413E, Applied Biosystems Inc.), in which 18S rRNA is used as an internal control to normalize RT-PCR results to account for input variation. For quantitation of the relative levels of expression of each gene, dilutions of the primary chondrocyte (PC) cDNA were run to generate a standard curve with the 7500 system software. The level of each test sample's expression was determined from the standard curve, and the resulting mRNA ratios to the primary chondrocyte control (PC) were divided by the sample's 18S rRNA ratio to PC to normalize for cDNA loading.

[0071] *Comparative C_T Method*--Comparative C_T analysis was performed to determine the relative gene expression ratios of HAPLN1 to MFAP5 in the various samples from the real time quantitative RT-PCR gene expression analysis raw data generated as described above. The comparative Ct method provides a

relative measure of the ratio of HAPLN1 to MFAP5 which allows for direct comparison between test samples without the need for standards, standard curve analysis, or actual calibrators. This method can be employed in the case of the HAPLN1 and MFAP5 assays used in this example because the following four conditions were met: 1) the assay performance was consistent from run to run; 2) equivalent amounts of RNA were run in HAPLN1, MFAP5, and endogenous control assays; 3) the C_T value for the endogenous control gene, 18S rRNA, was always lower than either the HAPLN1 C_T or MFAP5 C_T when equivalent amounts of RNA were run in each assay, and thus 18S C_T was always quantifiable when either HAPLN1 or MFAP5 were quantifiable; and 4) the method used an arbitrarily selected theoretical calibrator defined as a theoretical sample containing the ratio of HAPLN1 / MFAP5 which yielded a HAPLN1 C_T value equal to the MFAP5 C_T value when the other three conditions listed above were met. The derivation of the equation used for this comparative C_T method is as follows. Where the amount of target gene in a sample, normalized to an endogenous control gene and relative to a calibrator is given by:

$$(1+E)^{(-\Delta\Delta C_{T,\text{target gene}})}$$

wherein

E = amplification efficiency

wherein

$$\Delta\Delta C_{T,\text{target gene}} = \text{sample } \Delta C_{T,\text{target gene}} - \text{calibrator } \Delta C_{T,\text{target gene}}$$

and wherein

$$\Delta C_{T,\text{target gene}} = C_{T,\text{target gene}} - C_{T,\text{endogenous control gene}}$$

(See Liu, W. and Saint, D.A., Analytical Biochemistry, 302: 52-59 (2002); Livak, K.J, ABI Prism 7700 Sequence Detection System, User Bulletin 2, ABI publication 4303859, 1997). Then the ratio of HAPLN1 to MFAP5 can be described as:

$$(1+E)^{(-\Delta\Delta C_{T,HAPLN1})} / (1+E)^{(-\Delta\Delta C_{T,MFAP5})}$$

which equals:

$$(1+E)^{(-\{[\text{sample } C_{T,HAPLN1} - \text{sample } C_{T,\text{ endogenous control gene}}] - [\text{calibrator } C_{T,HAPLN1} - \text{calibrator } C_{T,\text{ endogenous control gene}}]\})} / (1+E)^{(-\{[\text{sample } C_{T,MFAP5} - \text{sample } C_{T,\text{ endogenous control gene}}] - [\text{calibrator } C_{T,MFAP5} - \text{calibrator } C_{T,\text{ endogenous control gene}}]\})}$$

If the same amount of sample is run in each assay, then the C_T of the sample endogenous control gene can be represented by the term *x*. If the same amount of calibrator is run in each assay, then the C_T of the calibrator endogenous control gene can be represented by the term *y*. Substituting these terms, the equation derives to:

$$(1+E)^{(-\{[\text{sample } C_{T,HAPLN1} - x] - [\text{calibrator } C_{T,HAPLN1} - y]\})} / (1+E)^{(-\{[\text{sample } C_{T,MFAP5} - x] - [\text{calibrator } C_{T,MFAP5} - y]\})}$$

which equals:

$$(1+E)^{([x - \text{sample } C_{T,HAPLN1}] - [y - \text{calibrator } C_{T,HAPLN1}])} / (1+E)^{([x - \text{sample } C_{T,MFAP5}] - [y - \text{calibrator } C_{T,MFAP5}])}$$

If the calibrator is defined as a theoretical sample containing the ratio of HAPLN1 / MFAP5 which yields a $C_{T,HAPLN1}$ value equal to the $C_{T,MFAP5}$ value when equivalent amounts of calibrator are run in each assay, then the term *z* can be substituted for the calibrator $C_{T,HAPLN1}$ and the calibrator $C_{T,MFAP5}$. The equation then derives to:

$$(1+E)^{([x - \text{sample } C_{T,HAPLN1}] - [y - z])} / (1+E)^{([x - \text{sample } C_{T,MFAP5}] - [y - z])}$$

which equals:

$$(1+E)^{([x - \text{sample } C_{T,HAPLN1}] - [y - z] - [x - \text{sample } C_{T,MFAP5}] + [y - z])}$$

which equals:

$$(1+E)^{([x - \text{sample } C_{T,HAPLN1}] - [x - \text{sample } C_{T,MFAP5}])}$$

which equals:

$$(1+E)^{(\text{sample } C_{T,MFAP5} - \text{sample } C_{T,HAPLN1})}$$

And if $E = 1$ (100% efficiency), then the relative ratio of HAPLN1 to MFAP5 equals:

$$2^{(\text{ sample } C_{T,MFAP5} - \text{sample } C_{T,HAPLN1})}$$

The above equations derive to a final formula leaving only two variables, the sample HAPLN1 C_T and sample MFAP5 C_T, as unknowns. This formula applies when samples are assayed under the conditions described above, and the theoretical calibrator employed is set as described above.

[0072] Figure 2 depicts HAPLN1 expression levels in several cell strains as determined by a standard curve method of RT-PCR. Figure 3 depicts expression levels of MFAP5 in the same cell samples as shown in Figure 2, as determined by a standard curve method of RT-PCR. HAPLN1 was expressed at higher levels in the chondrocyte cell cultures than in the synoviocyte and fibroblast

cell cultures. MFAP5 was expressed at higher levels in synoviocyte and fibroblast cell cultures than in the chondrocyte cell cultures.

[0073] Figure 4 depicts the ratios of HAPLN1 and MFAP5 expression levels from Figures 2 and 3. The ratio in primary chondrocytes (PC) was scaled to 1; other ratios were scaled accordingly.

[0074] Figure 5 depicts the ratios of HAPLN1 and MFAP5 expression levels in the same strains as shown in Figure 2, however, the expression levels were determined by a comparative C_T method of RT-PCR. The results of the C_T method were similar to the results obtained by the standard curve method.

Example 2: Expression of HAPLN1 and MFAP5 in Additional Strains of Chondrocytes, Synoviocytes and Fibroblasts

[0075] Expression levels of HAPLN1 and MFAP5 were determined in additional cell cultures to confirm fidelity of the method for differentiating between chondrocyte and synoviocyte cultures. The cultures used in this Example are listed in Table 3.

Table 3: Cell cultures used in RT-PCR Analysis (Example 2)

Cell Culture	Cell Type	Type of Cell Culture
PC	Chondrocyte	Primary culture
C3	Chondrocyte	Second passage
C4	Chondrocyte	Second passage
C5	Chondrocyte	Second passage
C6	Chondrocyte	Second passage
C7	Chondrocyte	Second passage
S3	Synoviocyte	Second passage
S4	Synoviocyte	Second passage
S5	Synoviocyte	Second passage
S6	Synoviocyte	Second passage
S7	Synoviocyte	Second passage

[0076] *Cell isolation and culture*--Human chondrocyte cell cultures C3, C4, C5, C6, and C7 were isolated and cultured using the method for producing Carticel® autologous chondrocytes as described in Example 1. Human synoviocyte cultures (synovium derived cell cultures, also known as synovial fibroblasts) were either isolated at Genzyme or obtained from Cell Applications Inc. (San Diego, CA). Strains S4, S6, and S7 were isolated at Genzyme using various procedures. S4 was isolated by subjecting minced synovium tissue to digestion in collagenase solution for 3.5 hours at 37°C, followed by a second digestion in trypsin solution for 1 hour at 37°C. Strain S6 was isolated by subjecting minced synovium tissue to digestion in a solution containing collagenase and DNase for 2 hours at 37°C. Strain S7 was isolated by subjecting minced synovium to the method for producing Carticel® autologous chondrocytes. After isolation, the synovium derived cells were plated in tissue culture flasks with EGHXX medium and cultured using the method for producing Carticel® autologous chondrocytes as described in Example 1. Strains S3 and S5 were obtained from Cell Applications Inc. as cryopreserved first passage cells. After thawing, the cells from strains S3 and S5 were plated in tissue culture flasks with EGHXX medium and cultured using the method for producing Carticel® autologous chondrocytes as described in Example 1.

[0077] *RNA isolation and cDNA preparation*--RNA preparations for chondrocyte cell cultures C3, C4, C5, C6, C7 and synoviocyte cultures S3, S4, S5, S6, S7 were performed as described in Example 1. The RNA from these samples was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer's instructions. The PC cDNA from Example 1 was used in this Example. The cDNA was stored at -20°C or -80°C until analysis.

[0078] *Gene expression analysis*--Gene expression analysis was performed using RT-PCR as described in Example 1.

[0079] Figure 6 depicts HAPLN1 expression levels in a number of additional chondrocyte and synoviocyte strains. The expression levels were determined by a standard curve method of RT-PCR and normalized to 18S ribosomal RNA.

[0080] Figure 7 depicts MFAP5 expression levels in the same strains as shown in Figure 6. The expression levels were determined by a standard curve method of RT-PCR and normalized to 18S ribosomal RNA.

[0081] Figure 8 depicts the ratios of HAPLN1 and MFAP5 expression levels from Figures 6 and 7. The ratio in primary chondrocytes (PC) was scaled to 1; other ratios were scaled accordingly.

[0082] Figure 9 depicts the ratios of HAPLN1 and MFAP5 expression levels in the same strains as shown in Figure 6. The expression levels were determined by a comparative C_T method of RT-PCR, and the ratios were calculated as $2^{(C_{T,MFAP5} - C_{T,HAPLN1})}$.

[0083] The RT-PCR results in the additional cell strains were consistent with the results obtained in Example 1.

Example 3: Expression of HAPLN1 and MFAP5 in Chondrocytes, Synoviocytes and Fibroblasts Using Custom-designed Primers and Probes

[0084] Testing of various chondrocyte, synoviocyte, and dermal fibroblast cultures was performed with primers and probes of known oligonucleotide sequences.

[0085] *Cell isolation and culture*-- The cell strains used in this Example are listed in Tables 4 and 5 below. Human chondrocyte cell cultures C1, C2, C3, C4, C5, C6, C7, C8, C26, C28, C30, and C34 were isolated and cultured using the method for producing Carticel® autologous chondrocytes as described in Example 1. Human chondrocyte cell cultures C21, C22, C23, C24, C25, C27, C29, C31, C32, and C33 were isolated (using the Protease method) and cultured as described in Example 1. Cell isolation and culture methods for human synoviocyte cultures S1, S2, S3, S4, S5, S6, and S7 were described in Examples 1 and 2. Synoviocyte culture S9 was isolated by subjecting minced synovium tissue to digestion in a solution containing collagenase and DNase for 2 hours at 37°C. Synoviocyte culture S10 was isolated by subjecting minced synovium tissue to digestion in collagenase solution for 3.5 hours at 37°C, followed by a second digestion in trypsin solution for 1 hour at 37°C. Synoviocyte strains S11, S12,

S13, S14, S15, S16, S17, and S18 were obtained from Cell Applications Inc. as cryopreserved first passage cells. Dermal fibroblast strains F1, F2, F3, F4, F5, F6, F8, F9, F10, and F11 were obtained from Cell Applications Inc. as cryopreserved primary cultured cells. All cell cultures were cultured using the method for producing Carticel® autologous chondrocytes as described in Example 1.

Table 4: First Set of Cell Cultures used in RT-PCR Analysis (Example 3)

Cell Culture	Cell Type	Type of Cell Culture
C1	Chondrocyte	Second passage
C2	Chondrocyte	Second passage
C3	Chondrocyte	Second passage
C4	Chondrocyte	Second passage
C5	Chondrocyte	Second passage
C6	Chondrocyte	Second passage
C7	Chondrocyte	Second passage
C8	Chondrocyte	Second passage
S1	Synoviocyte	Second passage
S2	Synoviocyte	Second passage
S3	Synoviocyte	Second passage
S4	Synoviocyte	Second passage
S5	Synoviocyte	Second passage
S6	Synoviocyte	Second passage
S7	Synoviocyte	Second passage
S9	Synoviocyte	First passage
S10	Synoviocyte	Third passage
S11	Synoviocyte	Third passage
S12	Synoviocyte	Third passage
S13	Synoviocyte	Third passage
F1	Dermal fibroblast	Second passage
F2	Dermal fibroblast	Second passage
F3	Dermal fibroblast	Second passage
F4	Dermal fibroblast	Second passage
F5	Dermal fibroblast	Second passage
F6	Dermal fibroblast	Second passage

Table 5: Second Set of Cell Cultures used in RT-PCR Analysis (Example 3)

Cell Culture	Cell Type	Type of Cell Culture
C21	Chondrocyte	Second Passage
C22	Chondrocyte	Second Passage
C23	Chondrocyte	Second Passage
C24	Chondrocyte	Second Passage
C25	Chondrocyte	Second Passage
C26	Chondrocyte	Second Passage
C27	Chondrocyte	Second Passage
C28	Chondrocyte	Second Passage
C29	Chondrocyte	Second Passage
C30	Chondrocyte	Second Passage
C31	Chondrocyte	Second Passage
C32	Chondrocyte	Second Passage
C33	Chondrocyte	Second Passage
C34	Chondrocyte	Second Passage
S14	Synoviocyte	Third Passage
S15	Synoviocyte	Third Passage
S16	Synoviocyte	Third Passage
S17	Synoviocyte	Second Passage
S18	Synoviocyte	Second Passage
F8	Dermal fibroblast	Second Passage
F9	Dermal fibroblast	Second Passage
F10	Dermal fibroblast	Second Passage
F11	Dermal fibroblast	Second Passage

[0086] *RNA isolation and cDNA preparation*--RNA preparations from chondrocyte strains C1, C2, C3, C4, C5, C6, C7, synoviocyte strains S1, S2, S3, S4, S5, S6, S7, and dermal fibroblast strains F1, and F2 were described in Examples 1 and 2. For preparation of RNA from chondrocyte strain C8, synoviocyte strains S9, S10, S11, S12, S13, S14, dermal fibroblast strains F3, F4, F5, and F6, and all strains listed in Table 5, the RNeasy™ Mini Kit (Qiagen, Valencia, CA) RNA isolation method was used. For the RNeasy™ isolation, 360 µL of lysis solution was added to cell pellets containing up to one million cells.

The samples were immediately vortexed at full speed for 30 seconds, then placed at 37°C for 5 minutes. After incubation, the samples were shaken by hand for 10 seconds, followed by another 30 second vortex at full speed. The contents of each tube were collected, and the lysate was run through a Qiashredder™ column (Qiagen). Three hundred and fifty µL of the Qiashredded lysate was used in the RNeasy™ procedure following the manufacturer's protocol for the isolation of RNA from animal cells. The columns were eluted with a single elution consisting of 30 µL of water. The RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer's instructions. The resulting cDNA was stored at -20°C or -80°C until analysis.

[0087] *Gene expression analysis*--RT-PCR assays were performed with custom-designed primers and probes specific for regions of HAPLN1 and MFAP5 mRNAs. The sequence information for the custom primers and probes is shown in Table 6. Abbreviations: 6FAM = 6-Carboxyfluorescein, VIC™ is a trademark of Applied Biosystems Inc. and is a fluorophore, MGBNFQ = minor groove binder non-fluorescent quencher. Primers were obtained from Invitrogen Corp. (Carlsbad, CA). Probes were obtained from Applied Biosystems Inc. For HAPLN1, the target of the forward primer is nucleotides 543 to 570 of the HAPLN1 sequence deposited under GenBank Accession No. NM_001884.2 (SEQ ID NO:2), the target of the reverse primer is nucleotides 603 to 622, and the target of the probe is nucleotides 584 through 601 of the same sequence. For MFAP5, the target of the forward primer is nucleotides 301 through 322 of the MFAP5 sequence deposited under Genbank Accession No. NM_003480.2 (SEQ ID NO:1); the target of the reverse primer is nucleotides 353 through 372, and the target of the probe is nucleotides 334 through 350 of the same sequence. Real time PCR was performed with TaqMan™ Fast Universal PCR Master Mix, no UNG (catalog number 4352042, Applied Biosystems Inc.), 900 nM primers, 250 nM probes, and up to 5 ng of sample cDNA, according to the TaqMan™ Fast Universal PCR Mix protocol. The reaction volume was 13 µL and the amplifications were run on an ABI 7500 Real-Time PCR system (Applied Biosystems Inc.) using the default Fast TaqMan™ cycling and data collection program for this configuration. A threshold of 0.1 units was used for all assays.

The expression levels were determined by a comparative C_T method of RT-PCR described in Example 1.

Table 6: Custom Primer and Probe Sequences

Marker	Forward primer	Reverse Primer	Probe
HAPLN1	5' TGAAGGATTAGAAG ATGATACTGTTGTG 3' (SEQ ID NO:16)	5' GCCCCAGTCG TGGAAAGTAA 3' (SEQ ID NO:17)	5' VIC / TACAAGGTGTGGTATTCC / MGBNFQ 3' (SEQ ID NO:18)
MFAP5	5' CGAGGAGACGA TGTGACTCAAG 3' (SEQ ID NO:19)	5' AGCGGGATCA TTCACCAGAT 3' (SEQ ID NO:20)	5' 6FAM / ACATTCACAGAAGATCC / MGBNFQ 3' (SEQ ID NO:21)

[0088] Figure 10A depicts the ratios of HAPLN1 and MFAP5 expression levels in the same strains as shown in Figures 2 and 6, as well as additional chondrocyte, synoviocyte, and dermal fibroblast strains from Table 4. Figure 10B depicts the ratios of HAPLN1 and MFAP5 expression levels in strains from Table 5. The results obtained with the custom-designed primers and probes were similar to the results described in Examples 1 and 2.

Example 4: Comparison of HAPLN1 and MFAP5 Expression Levels in Chondrocyte, Synoviocyte and Fibroblast Cultures in Monolayers and Collagen Scaffolds

[0089] Expression levels of HAPLN1 and MFAP5 were compared in various types of cultures in monolayers and collagen scaffolds.

[0090] *Cell isolation and culture*--Chondrocyte cultures C9, C10, C11, C12, C13, C14, C15, C16, C17, and C18 were isolated using the Protease method as described in Example 1, and cultured as described in Example 1. Synoviocyte culture S7 was isolated and cultured as described in Examples 1 and 2. Dermal fibroblast cultures F2 and F7 were obtained from Cell Applications Inc. as cryopreserved primary cultured cells and cultured as described in Example 1. Upon completion of second passage culture (third passage for culture S7), a sample was taken for RNA isolation (the "Day 0" or monolayer sample), and then

the cells were resuspended in EGHXX medium and seeded onto a 20 cm^2 MAIX™ scaffold (ACI- MAIX™ collagen membrane, CE, Matricel GmbH, D-52134 Herzogenrath, Germany). The cells were allowed to attach for 1 hour at 37°C, then the scaffold was fed additional EGHXX and cultured for 4 days. Scaffold cultures containing synoviocytes and dermal fibroblasts were also prepared in the same manner. After 4 days of scaffold culture, the cultures were sampled using an 8 mm biopsy punch (the "Day 4" or scaffold sample), and RNA isolation was performed.

[0091] *RNA Isolation and cDNA preparation*--RNA was isolated using the RNeasy™ Mini Kit (Qiagen, Valencia, CA). For the RNeasy™ isolation, 360 µL of lysis solution was added to MACI® implant samples (up to two 8 mm MACI® implant punches per preparation). The samples were immediately vortexed at full speed for 30 seconds, then placed at 37°C for 5 minutes. After incubation, the samples were shaken by hand for 10 seconds to unfold the membrane followed by another 30 second vortex at full speed. The contents of each tube were collected, and the lysate was run through a Qiashredder column (Qiagen). Three hundred and fifty µL of the Qiashredded lysate was used in the RNeasy™ procedure following the manufacturer's protocol for the isolation of RNA from animal cells. The columns were eluted with a single elution consisting of 30 µL of water. Preparation of cDNA from the sample RNA was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer's instructions. The cDNA was stored at -20°C or -80°C.

[0092] Table 7 lists the cell cultures used and configurations used in this Example.

Table 7: Cell cultures used in RT-PCR Analysis (Example 4)

Culture Code	Cell Type	Culture Type	Configuration
C9 day 0	Chondrocyte	Second passage	5 X 10^5 cell pellet
C10 day 0	Chondrocyte	Second passage	5 X 10^5 cell pellet
C11 day 0	Chondrocyte	Second passage	5 X 10^5 cell pellet
C12 day 0	Chondrocyte	Second passage	5 X 10^5 cell pellet
C13 day 0	Chondrocyte	Second passage	5 X 10^5 cell pellet
C14 day 0	Chondrocyte	Second passage	5 X 10^5 cell pellet
C15 day 0	Chondrocyte	Second passage	5 X 10^5 cell pellet
C16 day 0	Chondrocyte	Second passage	5 X 10^5 cell pellet
C17 day 0	Chondrocyte	Second passage	5 X 10^5 cell pellet
C18 day 0	Chondrocyte	Second passage	5 X 10^5 cell pellet
S7 day 0	Synoviocyte	Third passage	5 X 10^5 cell pellet
F7 day 0	Dermal fibroblast	Second passage	5 X 10^5 cell pellet
F2 day 0	Dermal fibroblast	Second passage	5 X 10^5 cell pellet
C9 day 4	Chondrocyte	MACI® implant	8 mm punch
C10 day 4	Chondrocyte	MACI® implant	8 mm punch
C11 day 4	Chondrocyte	MACI® implant	8 mm punch
C12 day 4	Chondrocyte	MACI® implant	8 mm punch
C13 day 4	Chondrocyte	MACI® implant	8 mm punch
C14 day 4	Chondrocyte	MACI® implant	8 mm punch
C15 day 4	Chondrocyte	MACI® implant	8 mm punch
C16 day 4	Chondrocyte	MACI® implant	8 mm punch
C17 day 4	Chondrocyte	MACI® implant	8 mm punch
C18 day 4	Chondrocyte	MACI® implant	8 mm punch
S7 day 4	Synoviocyte	MACI® implant	8 mm punch
F7 day 4	Dermal fibroblast	MACI® implant	8 mm punch
F2 day 4	Dermal fibroblast	MACI® implant	8 mm punch

[0093] *Gene expression analysis*--Gene expression analysis of the monolayer and MACI® implant cDNA was performed in the manner outlined above in Example 1.

[0094] Figure 11 shows a comparison between expression level ratios for HAPLN1 and MFAP5 in monolayer and collagen-scaffold cultures. HAPLN1 and MFAP5 expression levels were determined by a standard curve method of RT-PCR. Expression levels were normalized to 18S ribosomal RNA. The ratio in monolayer culture of primary chondrocytes (PC) was scaled to 1; other ratios were scaled accordingly.

[0095] Figure 12 shows a comparison between expression level ratios for HAPLN1 and MFAP5 in the monolayer and collagen-scaffold cultures using the same strains as shown in Figure 11. The expression levels of HAPLN1 and MFAP5 were determined by a comparative C_T method of RT-PCR, and the ratios were calculated as $2^{(C_{T,MFAP5} - C_{T,HAPLN1})}$.

[0096] The results obtained in scaffold cultures were similar to those obtained in monolayer cultures.

Example 5: Expression of HAPLN1 and MFAP5 As A Function of The Passage Number

[0097] The ratio of HAPLN1 to MFAP5 at various culture levels was investigated.

[0098] *Cell isolation and culture*--Chondrocyte cultures C19, C20, C31, C32, and C33 were isolated using the Protease method as described in Example 1, and cultured as described in Example 1. Synoviocyte cultures S6 and S7 were isolated and cultured as described in Examples 1 and 2. Synoviocyte culture S8 was isolated by subjecting minced synovium tissue to digestion in a solution containing collagenase and DNase for 2 hours at 37°C. Cell culture of S8 was performed as described in Example 1. For chondrocyte cultures C19, C20, C31, C32, and C33, samples of cartilage derived cells (labeled "0" in Figures 14A and 14B), primary cultured cells (labeled "1" in Figures 14A and 14B), first passage cells (labeled "2" in Figures 14A and 14B), and second passage cells (labeled "3" in Figures 14A and 14B) were taken. For synoviocyte culture S7, samples of

primary cultured (labeled "1" in Figure 13), first passage (labeled "2" in Figure 13), second passage (labeled "3" in Figure 13), third passage (labeled "4" in Figure 13), and fourth passage (labeled "5" in Figure 13) cells were taken. For synoviocyte cultures S6 and S8, samples of first passage (labeled "2" on Figure 13), second passage (labeled "3" on Figure 13), third passage (labeled "4" on Figure 13), and fourth passage (labeled "5" on Figure 13) cells were taken.

[0099] *RNA isolation and cDNA preparation*--RNA and cDNA were prepared using the RNeasy™ Mini Kit (Qiagen) and the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc.) as described in Example 3.

[00100] *Gene expression analysis*--Gene expression analysis of the synoviocyte samples was performed as described in Example 1. Gene expression analysis of the chondrocyte samples was performed as described in Example 3.

[0100] Figures 13, 14A, and 14B depict the change in the expression level ratios for HAPLN1 and MFAP5 as a function of the passage number. The expression levels were determined by a comparative C_T method of RT-PCR. The HAPLN1:MFAP5 ratios were calculated as $2^{(C_{T,MFAP5} - C_{T,HAPLN1})}$.

[0101] The ratios of HAPLN1 to MFAP5 were consistently low at all culture levels for the synoviocyte samples relative to the chondrocyte samples. The ratios of HAPLN1 to MFAP5 were consistently high at all culture levels for the chondrocyte samples relative to the synoviocyte samples.

Example 6: Expression of HAPLN1 and MFAP5 in Mixed Cell Cultures

[0102] Gene expression analysis was applied to mixed cultures of chondrocyte and synoviocyte cells to evaluate sensitivity level of the method in mixed cultures. Cell cultures of human chondrocytes and human synoviocytes were used to prepare mixtures of the two cell types at the following proportions:

1) 0% chondrocytes / 100% synoviocytes;
2) 25% chondrocytes / 75% synoviocytes;
3) 50% chondrocytes / 50% synoviocytes;
4) 75% chondrocytes / 25% synoviocytes; and
5) 100% chondrocytes / 0% synoviocytes.

[0103] *Cell isolation and culture*--Chondrocyte strains C5, C6, and C8 were isolated and cultured as described in Examples 1 and 2. Synoviocyte cultures S6, S7, and S9 were isolated and cultured as described in Examples 1, 2, and 3. For Mixing Experiment 1, second passage cultures of chondrocyte strain 6 (C6) and synoviocyte strain 6 (S6) were used. For Mixing Experiment 2, first passage cultures of chondrocyte strain 8 (C8) and synoviocyte strain 7 (S7) were used. For Mixing Experiment 3, first passage cultures of chondrocyte strain 5 (C5) and synoviocyte strain 9 (S9) were used.

[0104] *RNA isolation and cDNA preparation*--RNA and cDNA were prepared using the RNeasy™ Mini Kit (Qiagen) and the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc.) as described in Example 3.

[0105] *Gene expression analysis*--Gene expression analysis was performed as described in Example 3.

[0106] The expression levels were determined by a comparative C_T method of RT-PCR. The HAPLN1:MFAP5 ratios were calculated as $2^{(C_{T,MFAP5} - C_{T,HAPLN1})}$. The results of the mixing experiments are provided in Figure 15, which shows the ratios of expression levels of HAPLN1 and MFAP5 in samples consisting of mixed populations of chondrocytes and synoviocytes. In the cultures that contained 75% or less chondrocytes and 25% or more synoviocytes, the HAPLN1:MFAP5 expression level ratios were about 1 or lower. Higher ratios corresponded to higher proportions of chondrocytes in the tested samples. The ability of the assay to discern mixtures of cell cultures showed that, on average, samples composed of at least 67% chondrocytes with the balance consisting of synovial fibroblasts to produce a positive $C_{T,MFAP5} - C_{T,HAPLN1}$. Contamination by other cell types, such as dermal fibroblasts, can also be detected with this assay.

Example 7: Analysis of Relationship Between Assay Response and Molecular Ratio of Markers

[0107] Synthetic RNA transcripts of HAPLN1 and MFAP5 were employed to determine the relationship between the assay response and the molecular ratio of the markers in test samples. First, primary PCR (Platinum PCR Supermix, Invitrogen catalog number 11306-016) was performed on human cDNA using the primers listed in Table 8. For HAPLN1, these primers amplified nucleotides from positions 256 to 1171 of the HAPLN1 gene (Accession No. NM_001884.2). For MFAP5, these primers amplified nucleotides from positions 32 to 728 of the MFAP5 gene (Accession No. NM_003480.2). PCR products were analyzed on 1.5% tris-acetate EDTA (TAE) agarose gels using a 100 base pair molecular size ladder (100 bp PCR Molecular Ruler, BioRad catalog number 170-8206) for reference and SYBR Green I (Invitrogen catalog number S-7563) gel staining. The resulting primary amplicons were gel purified with 4% native tris-borate EDTA (TBE) polyacrylamide (PAGE) gels and used as a template for secondary PCR with the primers listed in Table 8. Gel-purified primary amplicon template for the secondary MFAP5 amplification was loaded at 0.56 ng per 200 µL of secondary reaction. Gel-purified primary amplicon template for the secondary HAPLN1 amplification was loaded at 2.5 ng per 900 µL of secondary reaction. Secondary amplicons were purified from native TBE PAGE gels as described and used as templates for *in vitro* transcription with an Ambion Megascript T7 Kit. Up to 1.3 µg of secondary amplicon template was used per 20 µL transcription reaction. The resulting transcripts were gel purified with 6% TBE-Urea (TBU) polyacrylamide gels, resuspended in 0.1 mM EDTA, and quantified spectrophotometrically with readings performed in duplicate using a conversion factor of 1_{A260} unit corresponding to a concentration of 40 ng/µL RNA. The gel purified transcripts were analyzed on 6% TBU gels to assess purity. After determination of purity by PAGE analysis and quantitation by spectrophotometry, the number of transcript copies per µL was determined using the conversion factors listed in Table 9. These conversion factors assume an average base molecular weight of 343 Daltons. Avagadro's constant was considered to be 6.02×10^{23}/mole. It was also assumed that the first base transcribed was the +1 G from the T7 promoter,

followed by the target sequence. The transcripts were diluted in yeast RNA carrier buffer (20 ng/µL solution of Yeast RNA (Ambion catalog number AM7120G) in nuclease free water) at concentrations ranging from 10^3 to 10^8 copies/µL. The dilutions were then tested using the RT-PCR procedure given in Example 3 of the patent application, except that 2 µL of cDNA was used per 13 µL PCR reaction. The ratio of HAPLN1:MFAP5 was then calculated for each dilution using the comparative Ct method as described in Example 4 of the patent application.

[0108] The assay response using the copy number standards was compared to the known molecular ratio. These results are shown in Table 10. With the relationship between the assay response and the molecular ratio determined, the exact molecular ratios at various assay responses were calculated. These results, shown in Table 11, indicate that when the comparative Ct determined acceptance boundary equals 1 (for example, where HAPLN1:MFAP5 = 1), this corresponds to an exact molecular ratio of HAPLN1:MFAP5 of 2.212.

Table 8: Primers Used for Amplification of Copy Number Standards

Marker	**Forward Primer (Primary PCR)**	**Forward Primer (Secondary PCR)**	**Reverse Primer (Primary and Secondary PCR)**
HAPLN1	5' GCCAAGGTGTTTT CACACAG 3' (SEQ ID NO:22)	5' TAATACGACTCACTATAGGGGC CAAGGTGTTTTCACACAG 3' (SEQ ID NO:23)	5' CTCTGAAGCAGTA GACACCA 3' (SEQ ID NO:24)
MFAP5	5' CCTAGCCTGGCTT TCTTGCTC 3' (SEQ ID NO:25)	5' TAATACGACTCACTATAGGGCCT AGCCTGGCTTTCTTGCTC 3' (SEQ ID NO:26)	5' CCATTGGGTCTCT GCAAATCC 3' (SEQ ID NO:27)

Table 9: Expected Transcript Sizes and Conversion Factors

Transcript	Expected transcript size (bases)	Molecular weight (Daltons)	Copies/ng
HAPLN1	917	3.145 X 10^5 g/mole	1.914 X 10^9/ng
MFAP5	698	2.394 X 10^5 g/mole	2.515 X 10^9/ng

Table 10: Assay Response Versus Molecular Ratio of Markers

Copies of HAPLN1	Copies of MFAP5	Exact molecular ratio of HAPLN1:MFAP5	Assay response, HAPLN1:MFAP5
1 X 10^8	1 X 10^8	1	0.470
1 X 10^7	1 X 10^7	1	0.448
1 X 10^6	1 X 10^6	1	0.423
1 X 10^5	1 X 10^5	1	0.452
1 X 10^4	1 X 10^4	1	0.443
1 X 10^3	1 X 10^3	1	0.478
			Average = 0.452

Table 11: Exact Molecular Ratio of Markers at Various Assay Responses

Assay Response, HAPLN1:MFAP5	Exact molecular ratio of HAPLN1:MFAP5
500	1106
100	221.2
5	11.06
1	2.212
0.2	0.4425
0.01	0.02212
0.002	0.004425

Example 8: Absolute Quantitation Analysis of Chondrocyte and Fibroblast Markers

[0109] Gene expression analysis using an absolute quantitation method was performed. Table 12 lists the cell cultures used in this Example. The various cultures were isolated and cultured as discussed in the Examples above. RNA from the cell cultures was isolated using the RNeasy Kit as discussed in Example 3. RT-PCR was performed on the cell cultures as described in Example 3, except that 2µl of cDNA was used per 13 µL PCR reaction. *In vitro* transcribed RNA standards for HAPLN1 and MFAP5 (prepared as described in Example 7) were diluted to yield cDNA with final concentrations of 5×10^2, 5×10^3, and 5×10^4 copies per uL. Standard curves were generated by graphing the Ct results from the standards on the y-axis, versus the logarithm of the number of copies per reaction (10^3, 10^4, and 10^5) on the x-axis. A linear trendline was fitted to the data, and the quantities of HAPLN1 and MFAP5 mRNA copies present in each test sample were determined mathematically. This method of quantitation has been previously described, e.g., in Real-Time PCR Systems: Applied Biosystems 7900HT Fast Real-Time PCR System and 7300/7500 Real-Time PCR Systems, Chemistry Guide, Applied Biosystems, 2005, Part No. 4348358 Rev. E. The molar ratio of HAPLN1:MFAP5 was then calculated for each sample. Figure 16 depicts molar ratios of HAPLN1:MFAP5 in various cell cultures. These results indicate that the molar ratio of HAPLN1:MFAP5 in chondrocytes is high relative to synoviocytes and dermal fibroblasts.

Table 12: Cell cultures used in Absolute Quantitation Analysis (Example 8)

Cell Culture	Cell Type	Type of Cell Culture
C26	Chondrocyte	Second Passage
C27	Chondrocyte	Second Passage
C28	Chondrocyte	Second Passage
S14	Synoviocyte	Third Passage
S15	Synoviocyte	Third Passage
S16	Synoviocyte	Third Passage
F1	Dermal Fibroblast	Second Passage
F2	Dermal Fibroblast	Second Passage

* * *

CLAIMS

1. A method of evaluating the composition of a cell culture, said method comprising:

a) obtaining a plurality of cells from a cell culture;

b) determining an average expression level of microfibrillar associated protein 5 (MFAP5) in the plurality of cells, said average expression level of MFAP5 being such that its normalized expression levels are lower in chondrocytes than in fibroblasts or synoviocytes; and

c) determining the composition of the culture based on the average expression level of MFAP5.

2. The method of claim 1, wherein the cell culture comprises chondrocytes.

3. The method of claim 1, wherein an average expression level of MFAP5 below a predetermined threshold indicates that the cell culture comprises chondrocytes.

4. The method of claim 1, wherein a normalized expression level of MFAP5 is lower in passaged chondrocytes than in dermal fibroblasts or synoviocytes.

5. The method of claim 1, further comprising determining an average expression level of a chondrocyte marker in the plurality of cells; and, in step c), determining the composition of the culture based on the average expression level of the chondrocyte marker and the average expression level of MFAP5.

6. The method of claim 5, wherein the MFAP5 and the chondrocyte marker are such that the ratio of their expression levels (chondrocyte marker to MFAP5) in chondrocytes is equal to or greater than 5 times the ratio in dermal fibroblasts or synoviocytes.

7. The method of claim 5, wherein the chondrocyte marker is HAPLN1 , MGP, EDIL3, WISP3, AGC1, COMP, COL2A1, COL9A1, COL11A1, LECT1, S100B, CRTAC1, SOX9, or NEBL.

8. The method of claim 5, wherein the chondrocyte marker is HAPLN1.

9. The method of claim 5, wherein the expression levels of MFAP5 and chondrocyte marker are determined at the RNA level.

10. The method of claim 9, wherein the expression levels are determined using PCR.

11. The method of claim 10, wherein the expression levels are determined using a comparative C_T PCR method.

12. The method of claim 5, wherein an expression level ratio of the chondrocyte marker to MFAP5 of greater than 0.25, as determined using a comparative C_T PCR method, indicates that the cell culture contains chondrocytes.

13. The method of claim 12, wherein said ratio indicates that the cell culture contains at least 50% chondrocytes.

14. The method of claim 5, wherein a molar ratio of the chondrocyte marker to MFAP5 of greater than 0.55 indicates that the cell culture contains chondrocytes.

15. The method of claim 14, wherein said molar ratio indicates that the cell culture contains at least 50% chondrocytes.

16. The method of claim 1, wherein the cell culture comprises cells obtained from a cartilage biopsy.

17. The method of claim 16, wherein the cartilage biopsy is taken from a knee joint.

18. Use of the cell culture as defined in claim 16 for autologous chondrocyte implantation.

19. A method of evaluating the phenotype of a cell, the method comprising:

a) determining the expression level of MFAP5 in the cell; and
b) determining the phenotype of the cell based on the expression level of MFAP5.

20. The method of claim 19, further comprising determining the expression level of a chondrocyte marker in the cell; and, in step b), determining the phenotype of the cell based on the expression levels of the chondrocyte marker and MFAP5.

21. The method of claim 20, wherein the chondrocyte marker is HAPLN1 , MGP, EDIL3, WISP3, AGC1, COMP, COL2A1, COL9A1, COL11A1, LECT1, S100B, CRTAC1, SOX9, or NEBL.

22. The method of claim 20, wherein the chondrocyte marker is HAPLN1.

23. The method of claim 20, wherein the expression levels of MFAP5 and the chondrocyte marker are determined at the RNA level.

24. An *in vitro* method of evaluating the composition of a cell culture, said method comprising:

a) isolating cells from a cartilage biopsy from a mammal;
b) culturing cells isolated in step a) in a cell culture;
c) obtaining a sample of the cell culture;
d) determining the expression levels of MFAP5 and HAPLN1 in one or more cells from the sample; and
e) determining the composition of the culture based on the expression levels of MFAP5 and HAPLN1.

25. The method of claim 24, wherein the expression levels of MFAP5 and HAPLN1 are determined at the RNA level.

26. The method of claim 25, wherein the expression levels are determined using PCR.

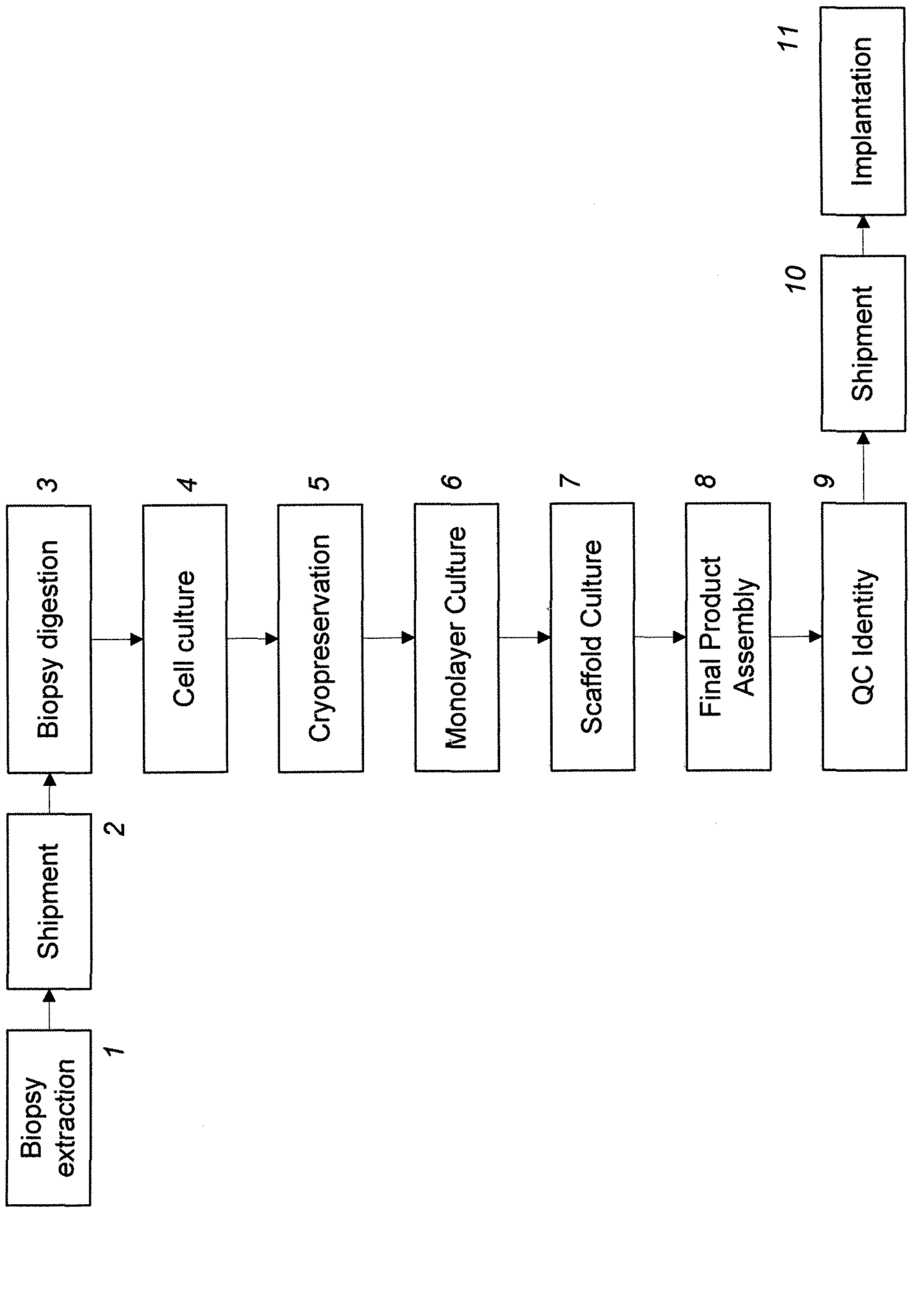
27. The method of claim 26, wherein the expression levels are determined using a comparative C_T PCR method.

28. The method of claim 24, wherein an expression level ratio of HAPLN1 to MFAP5 greater than 0.25, as determined using a comparative C_T PCR method, indicates that the cell culture contains chondrocytes.

29. The method of claim 28, wherein said ratio indicates that the cell culture contains at least 50% chondrocytes.

30. The method of claim 24, wherein a molar ratio of HAPLN1 to MFAP5 greater than 0.55 indicates that the cell culture contains chondrocytes.

31. The method of claim 30, wherein said molar ratio indicates that the cell culture contains at least 50% chondrocytes.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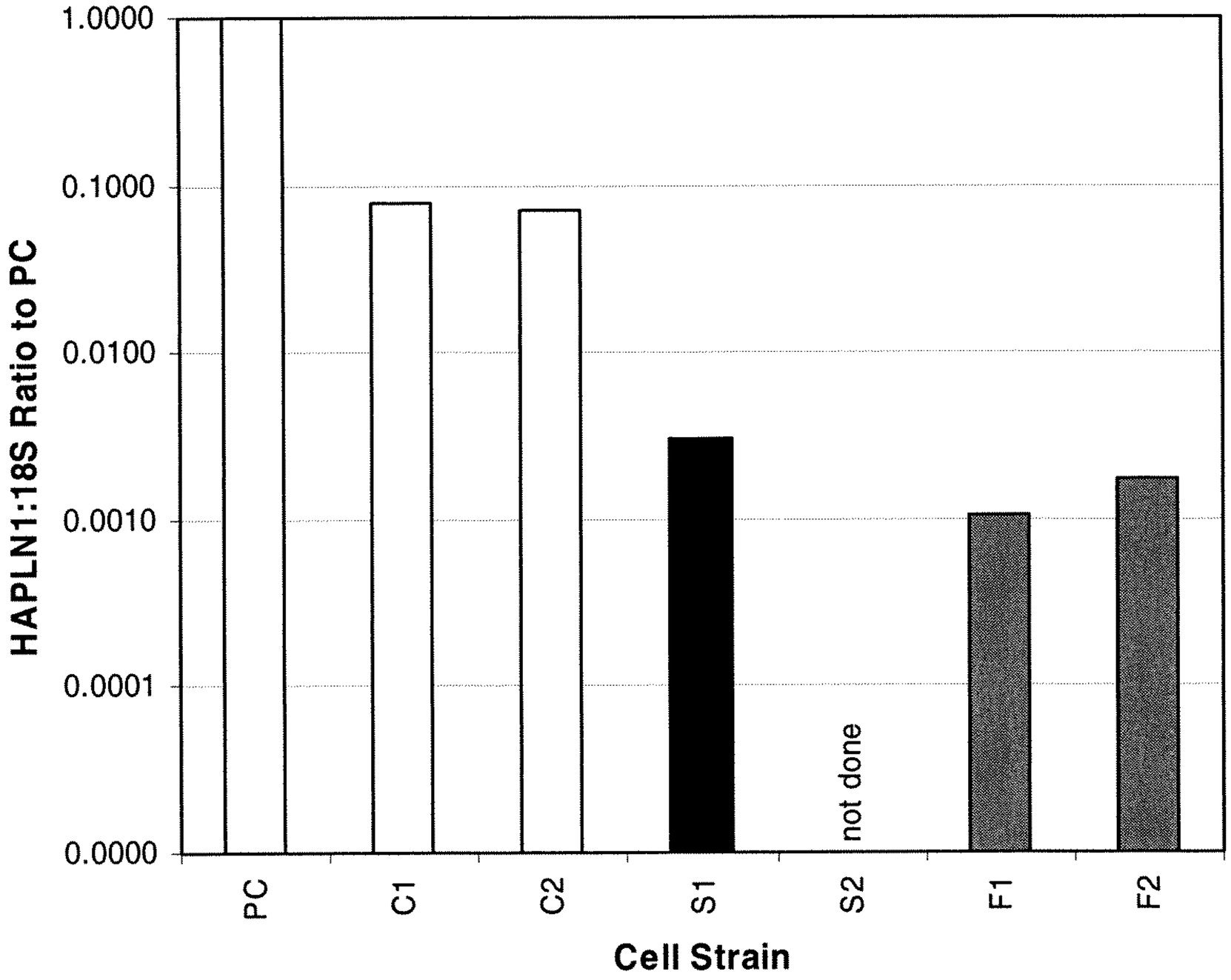
9
Shipment
10
Implantation
11

Fig. 1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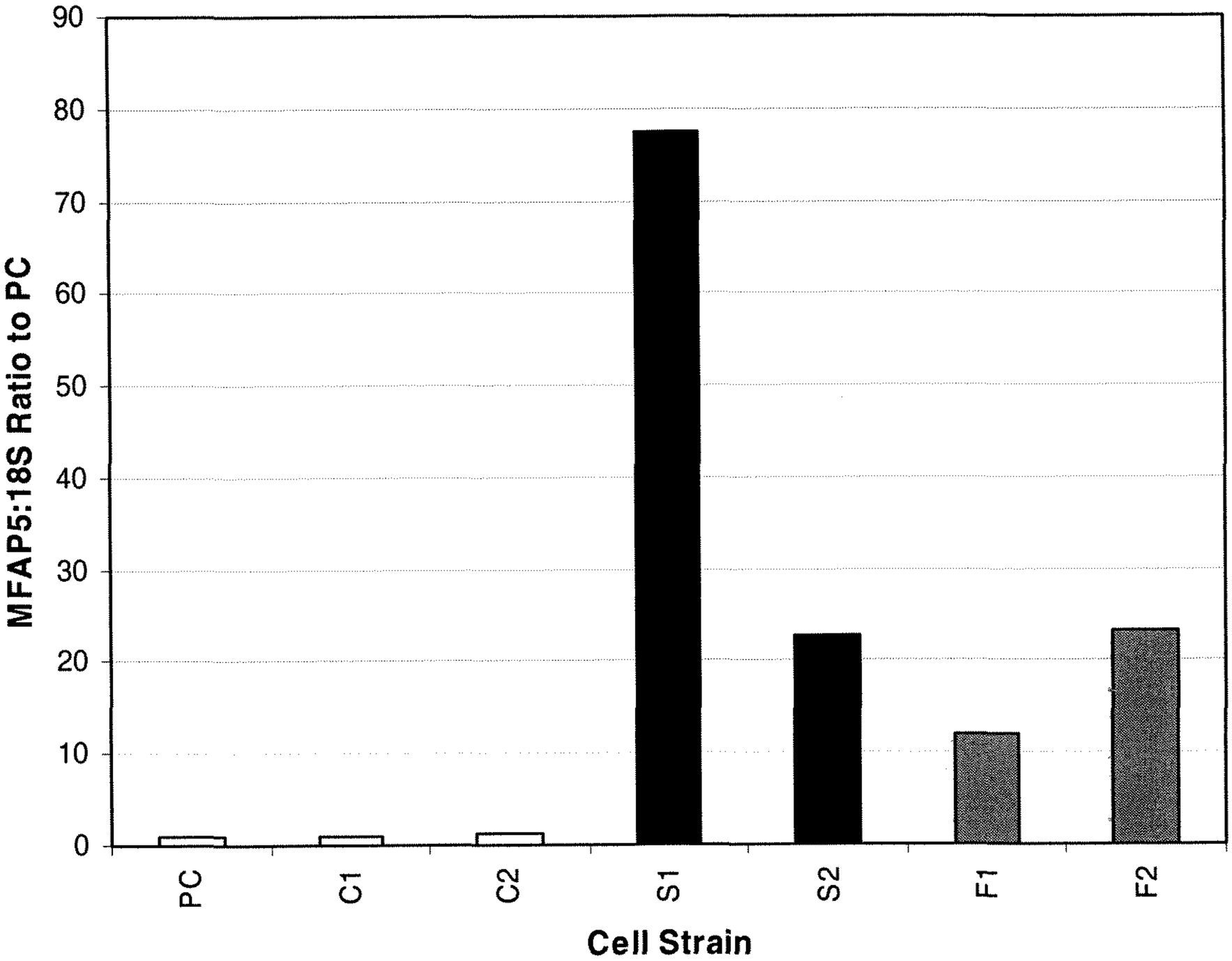
Cell Strain

Fig. 2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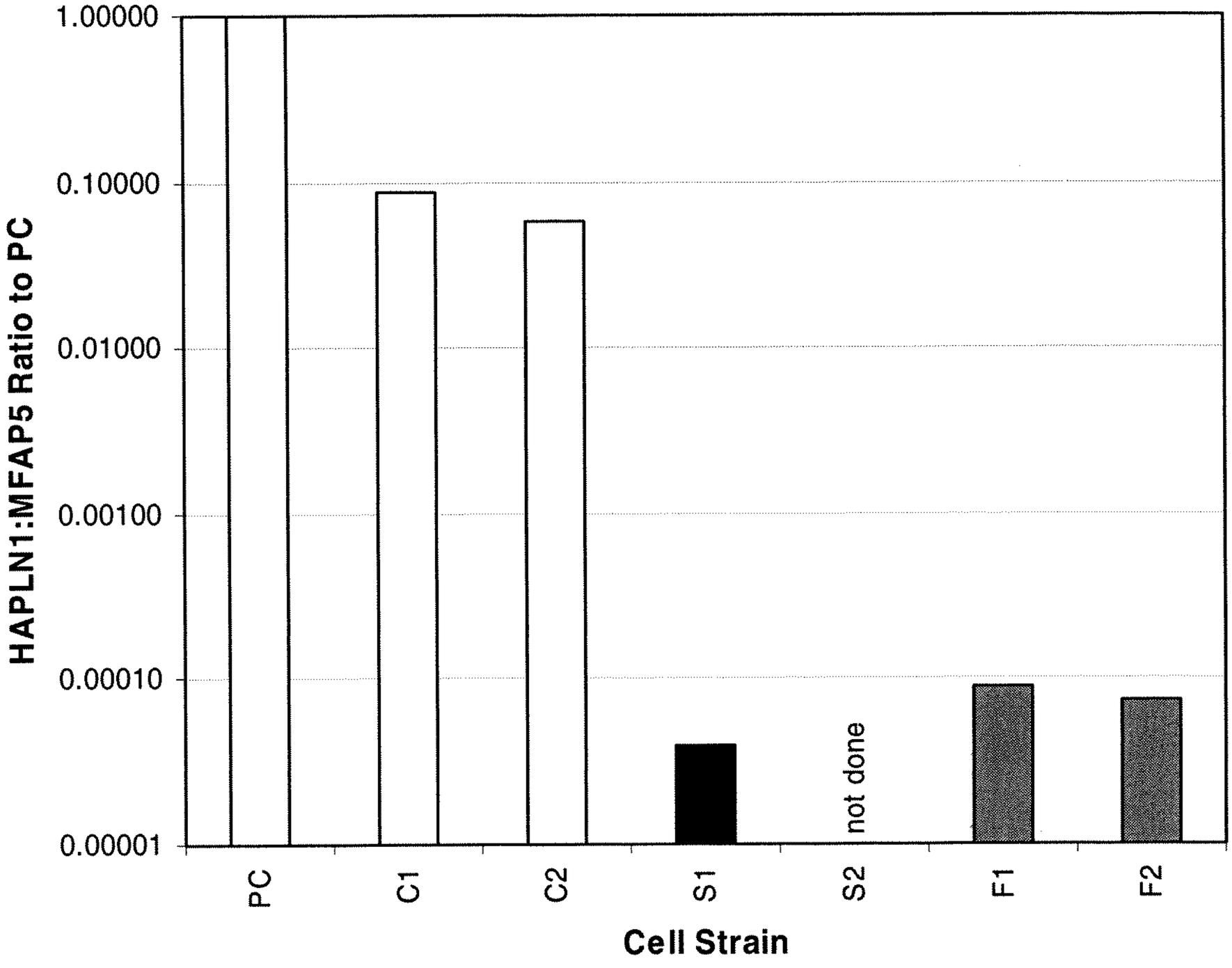
Cell Strain

Fig. 3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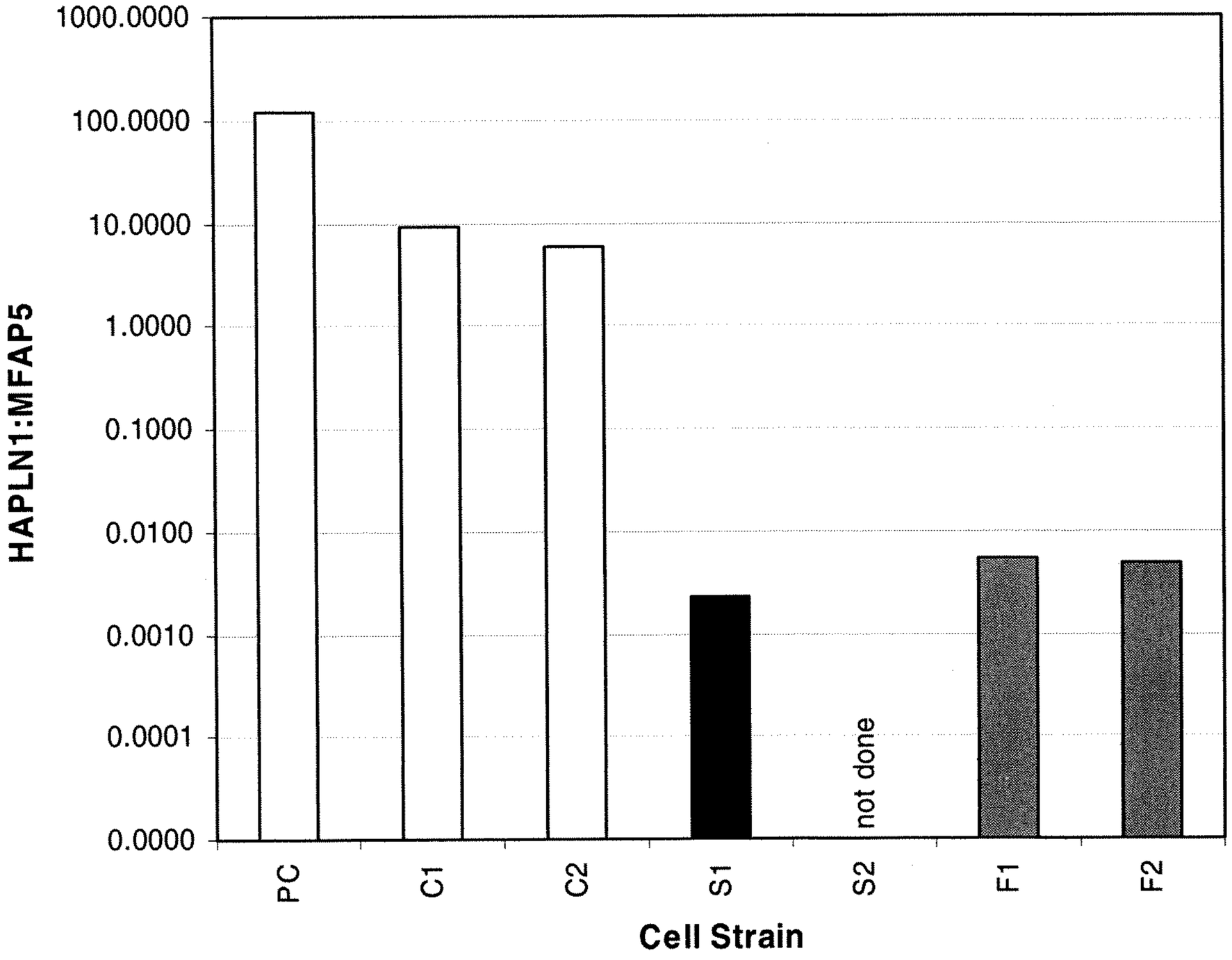
Cell Strain

Fig. 4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Cell Strain

Fig. 5

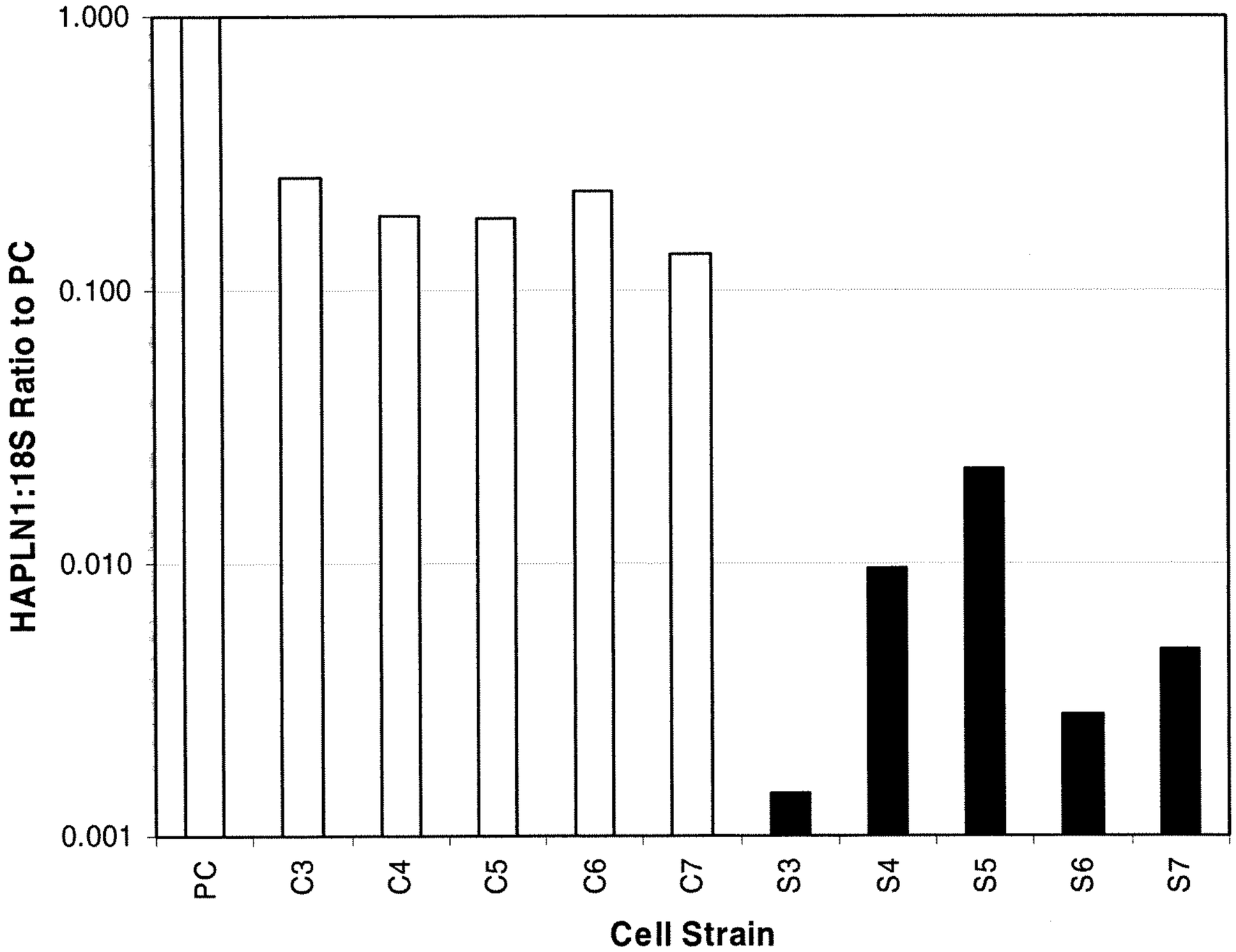

1.000
0.100
0.010
0.001
HAPLN1:18S Ratio to PC
PC
C3
C4
C5
C6
C7
S3
S4
S5
S6
S7
Cell Strain

Fig. 6

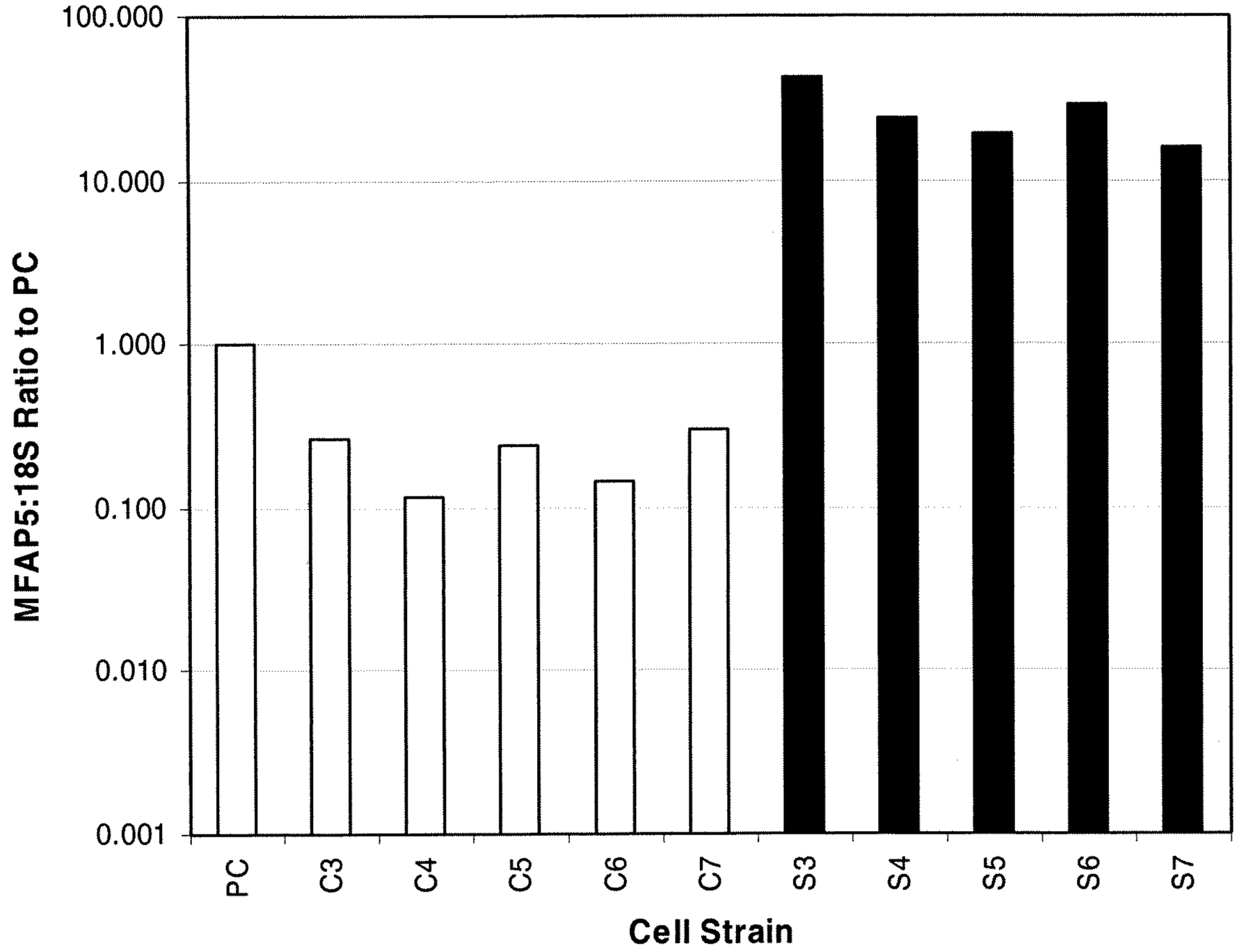

100.000
10.000
1.000
0.100
0.010
0.001
MFAP5:18S Ratio to PC
PC
C3
C4
C5
C6
C7
S3
S4
S5
S6
S7
Cell Strain

Fig. 7

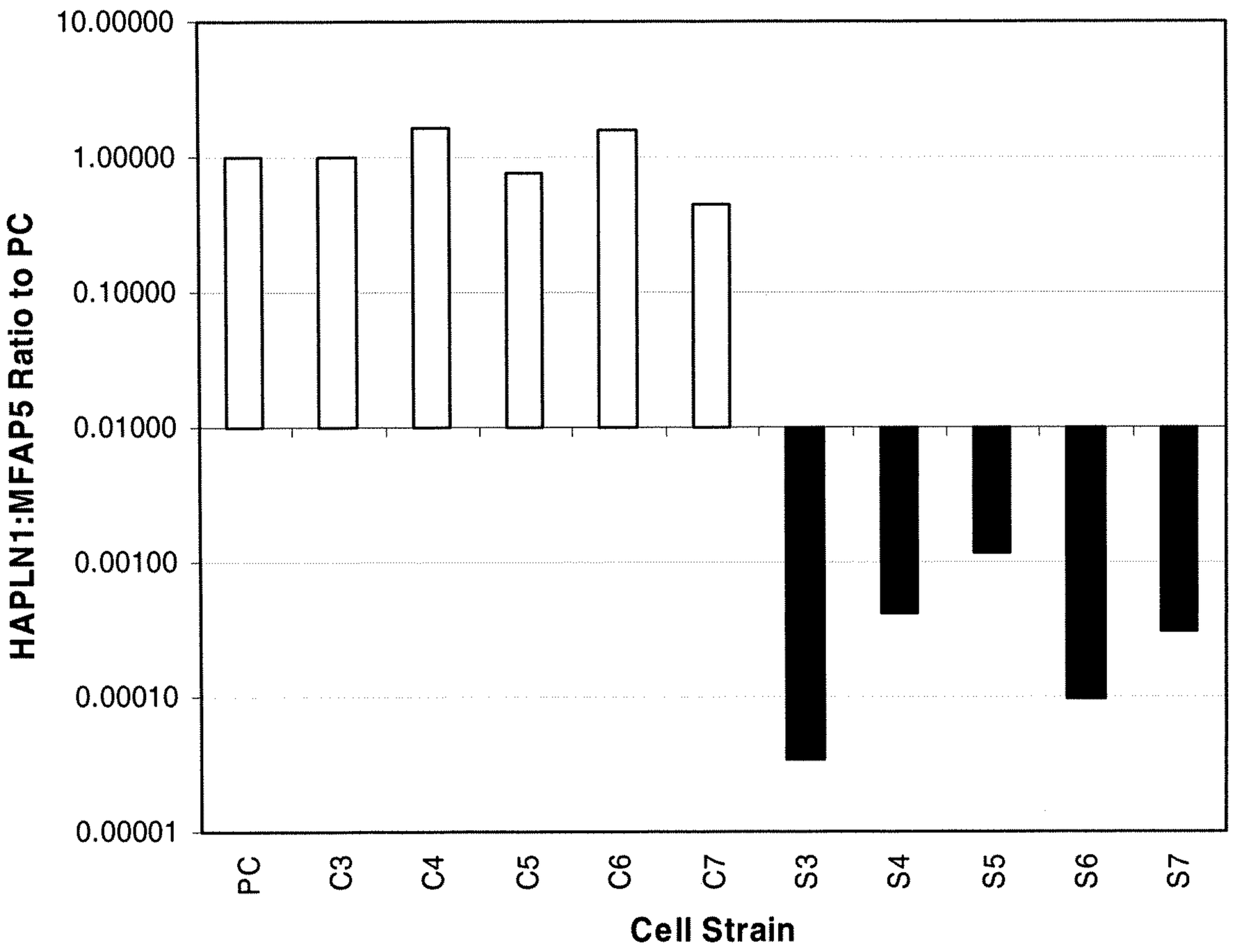

10.00000
1.00000
0.10000
0.01000
0.00100
0.00010
0.00001
HAPLN1:MFAP5 Ratio to PC
PC
C3
C4
C5
C6
C7
S3
S4
S5
S6
S7
Cell Strain

Fig. 8

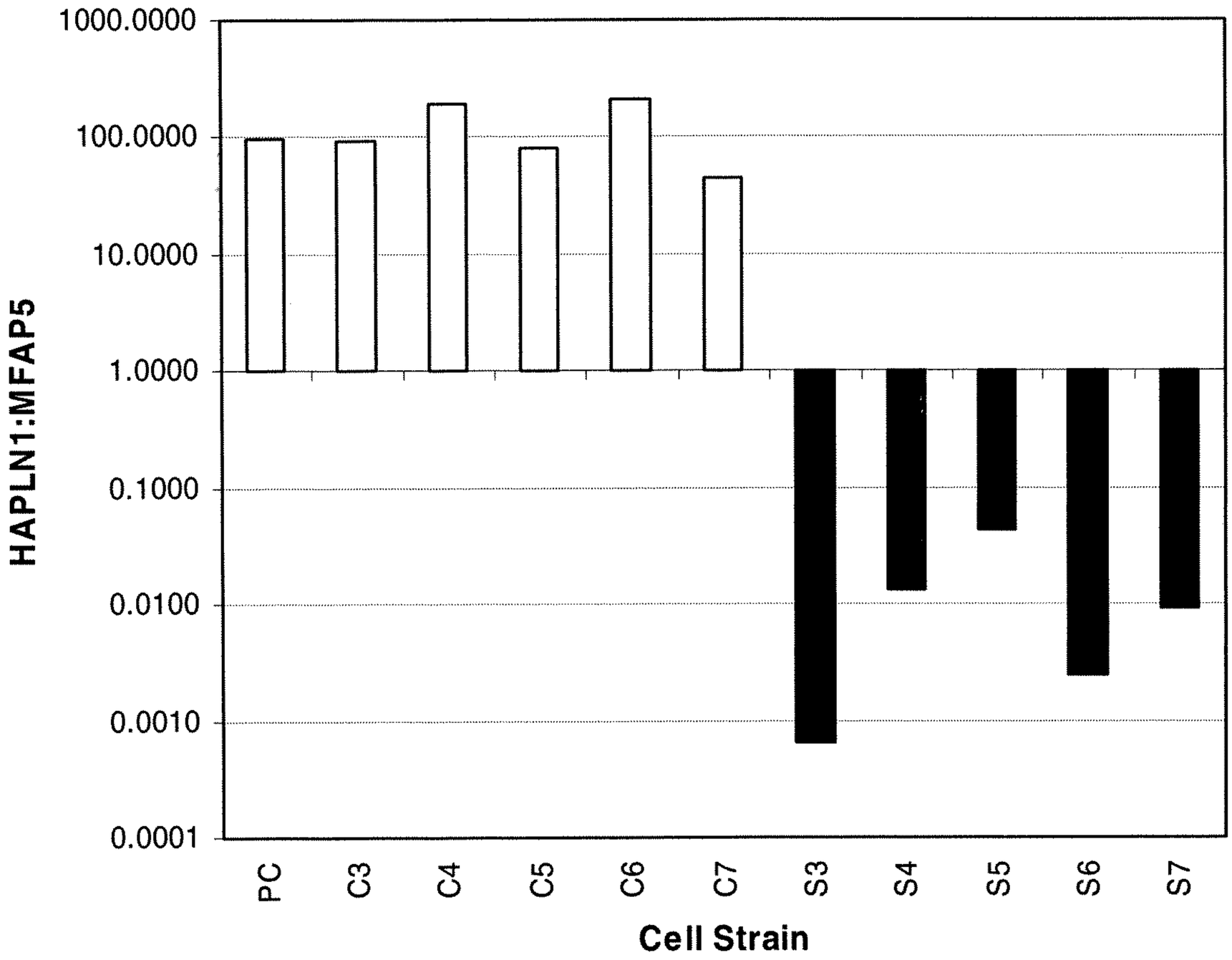

1000.0000
100.0000
10.0000
1.0000
0.1000
0.0100
0.0010
0.0001
HAPLN1:MFAP5
PC
C3
C4
C5
C6
C7
S3
S4
S5
S6
S7
Cell Strain

Fig. 9

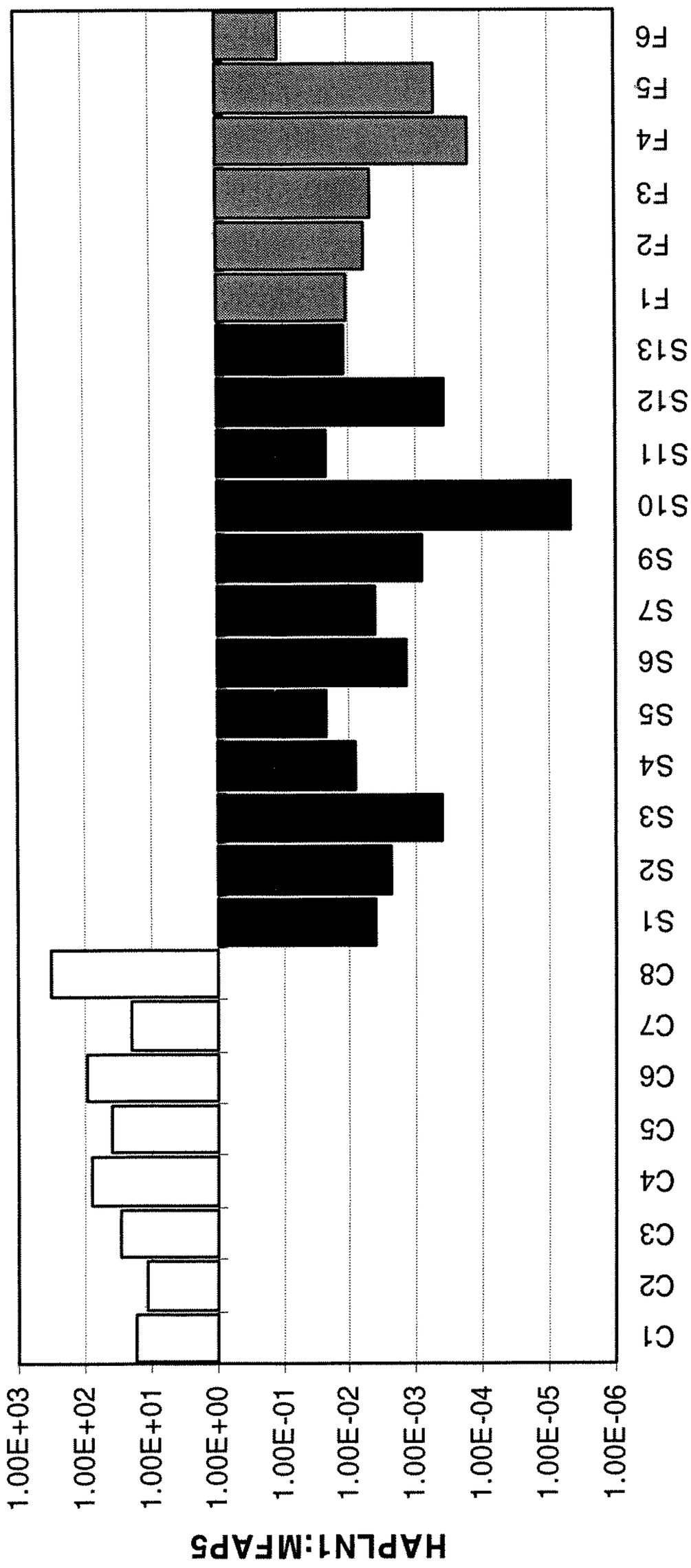

F6
F5
F4
F3
F2
F1
S13
S12
S11
S10
S9
S7
S6
S5
S4
S3
S2
S1
C8
C7
C6
C5
C4
C3
C2
C1
Cell Strain
1.00E+03
1.00E+02
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
1.00E-05
1.00E-06
HAPLN1:MFAP5

Fig. 10A

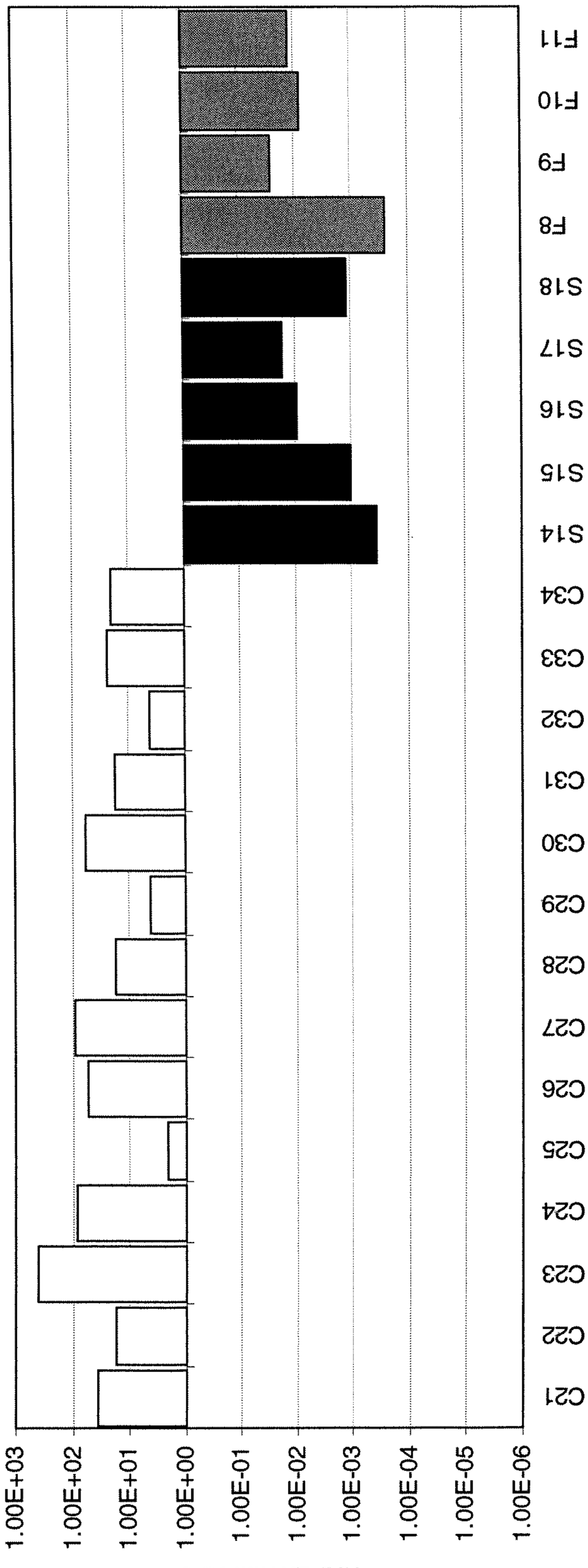

F11
F10
F9
F8
S18
S17
S16
S15
S14
C34
C33
C32
C31
C30
C29
C28
C27
C26
C25
C24
C23
C22
C21
Cell Strain
1.00E+03
1.00E+02
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
1.00E-05
1.00E-06
HAPLN1:MFAP5

Fig. 10B

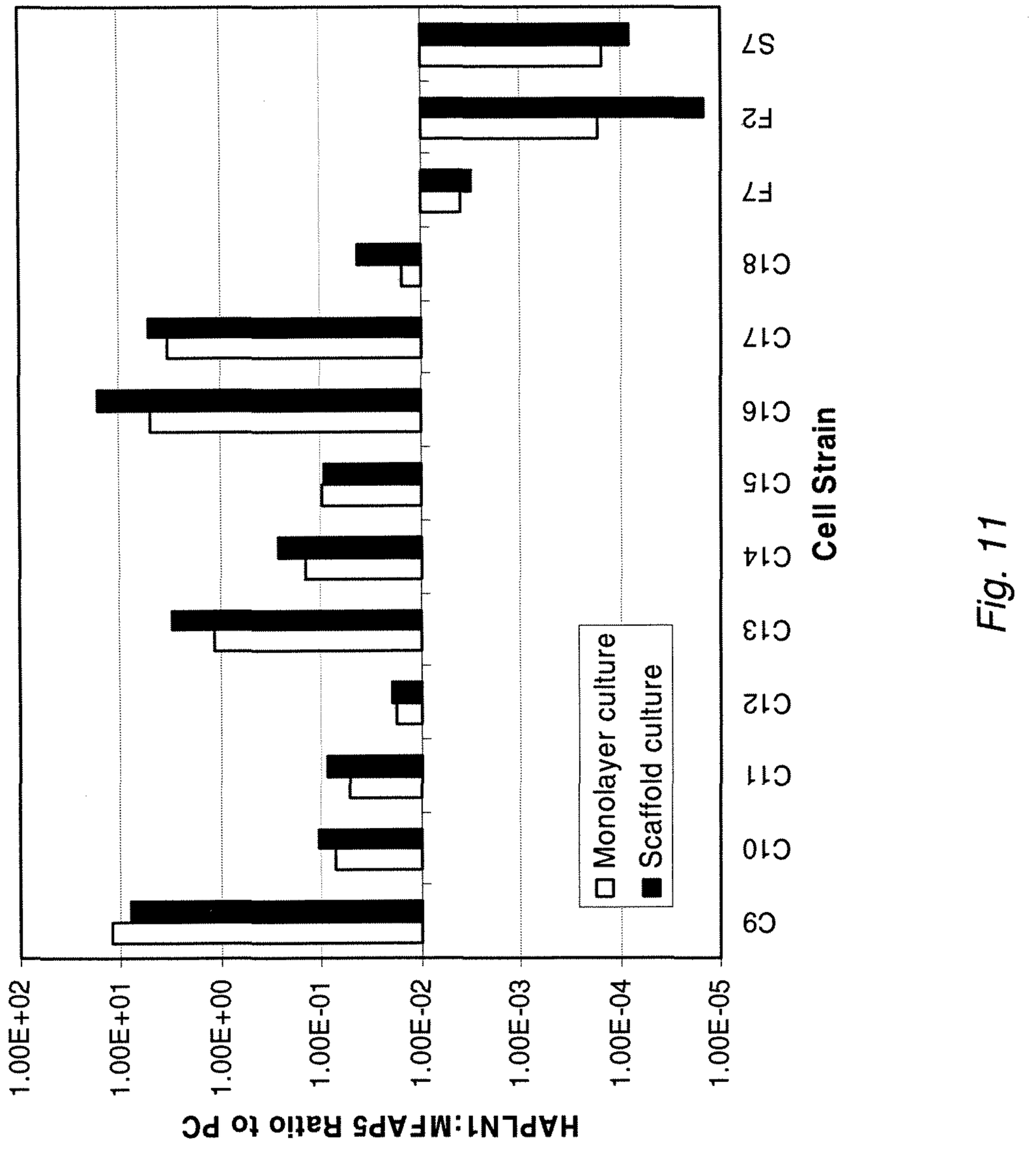

HAPLN1:MFAP5 Ratio to PC
1.00E+02
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
1.00E-05
Monolayer culture
Scaffold culture
C9
C10
C11
C12
C13
C14
C15
C16
C17
C18
F7
F2
S7
Cell Strain

Fig. 11

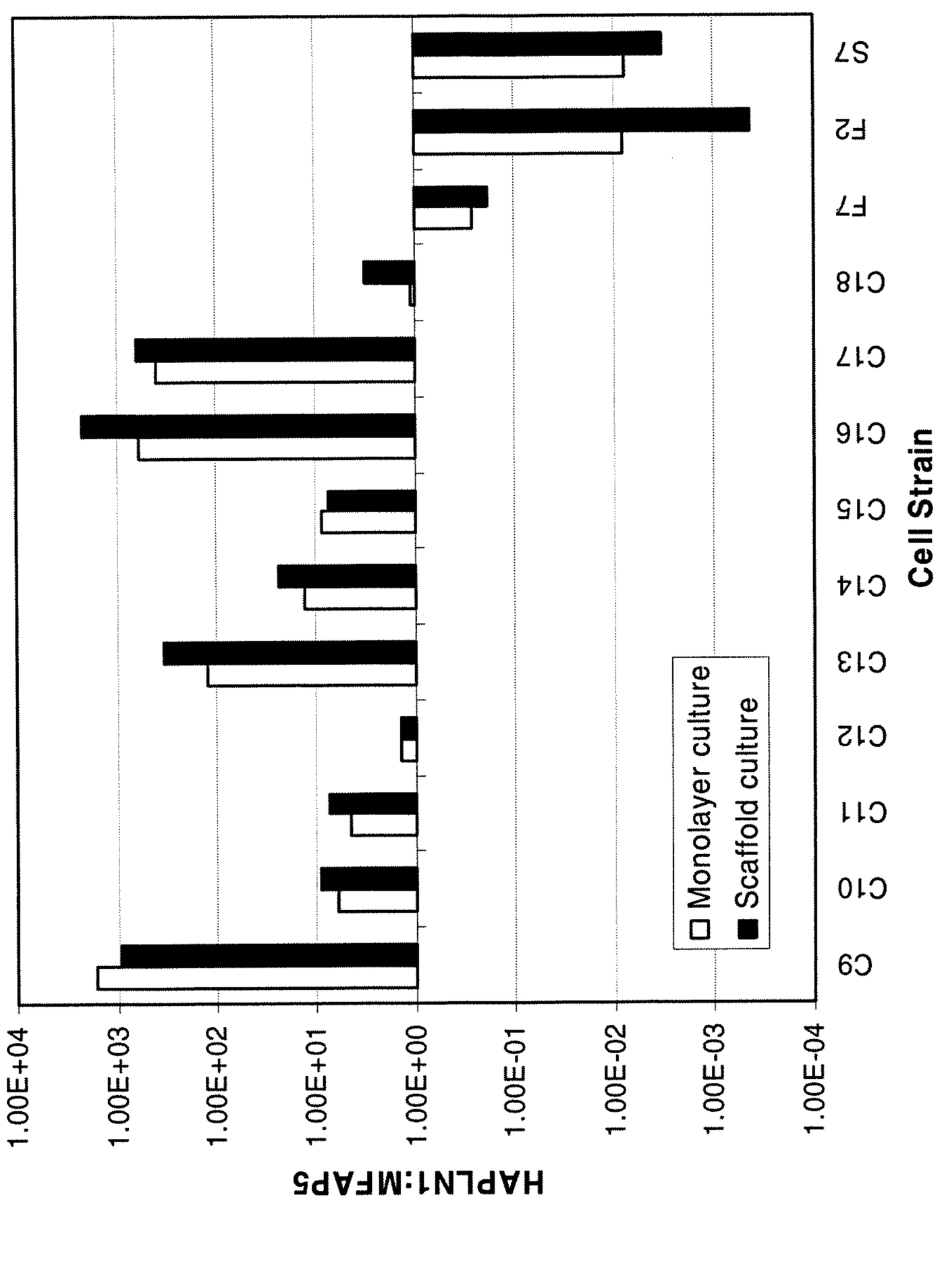

HAPLN1:MFAP5
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
C9
C10
C11
C12
C13
C14
C15
C16
C17
C18
F7
F2
S7
Cell Strain
Monolayer culture
Scaffold culture

Fig. 12

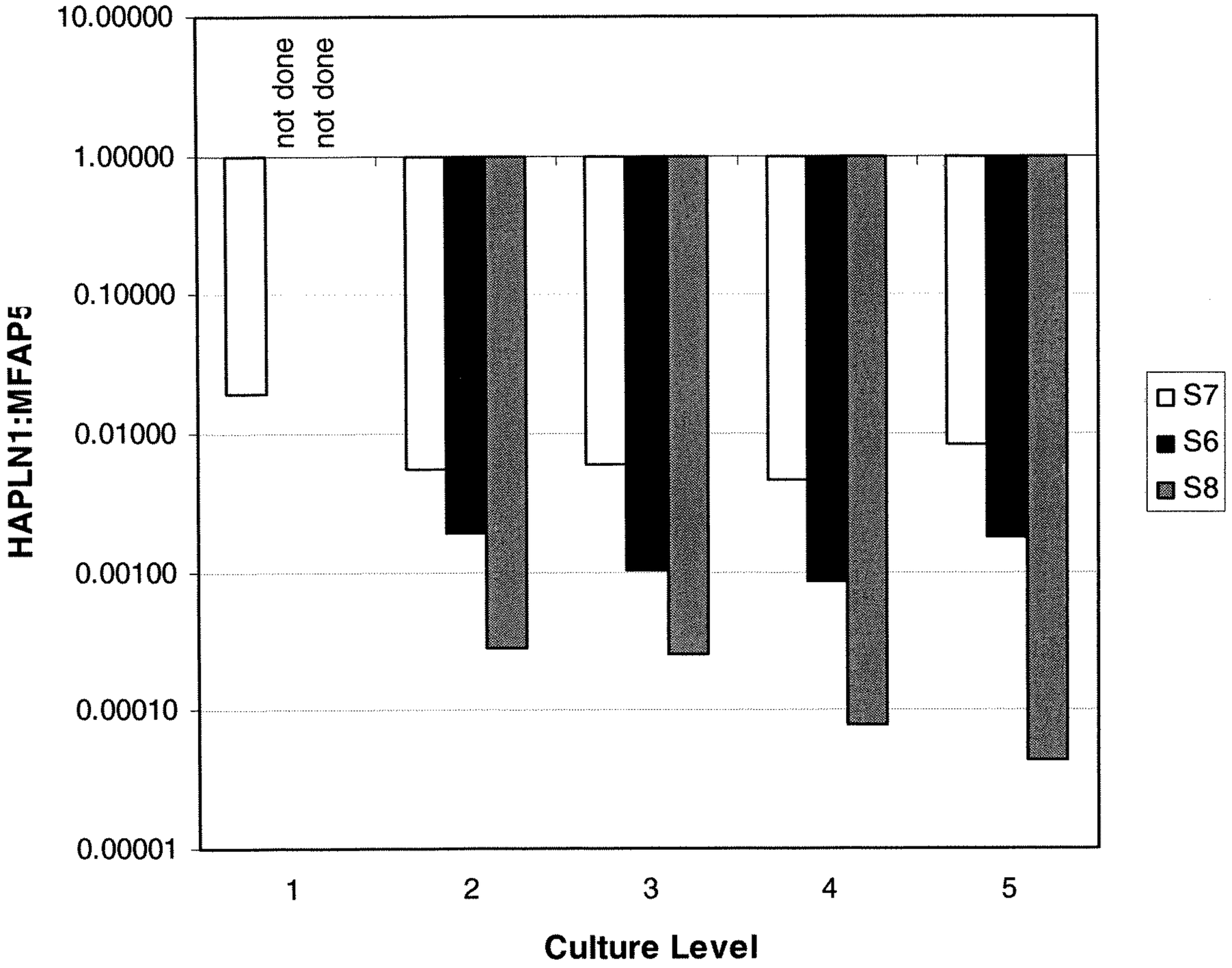

10.00000
1.00000
0.10000
0.01000
0.00100
0.00010
0.00001
not done
not done
HAPLN1:MFAP5
S7
S6
S8
1
2
3
4
5
Culture Level

Fig. 13

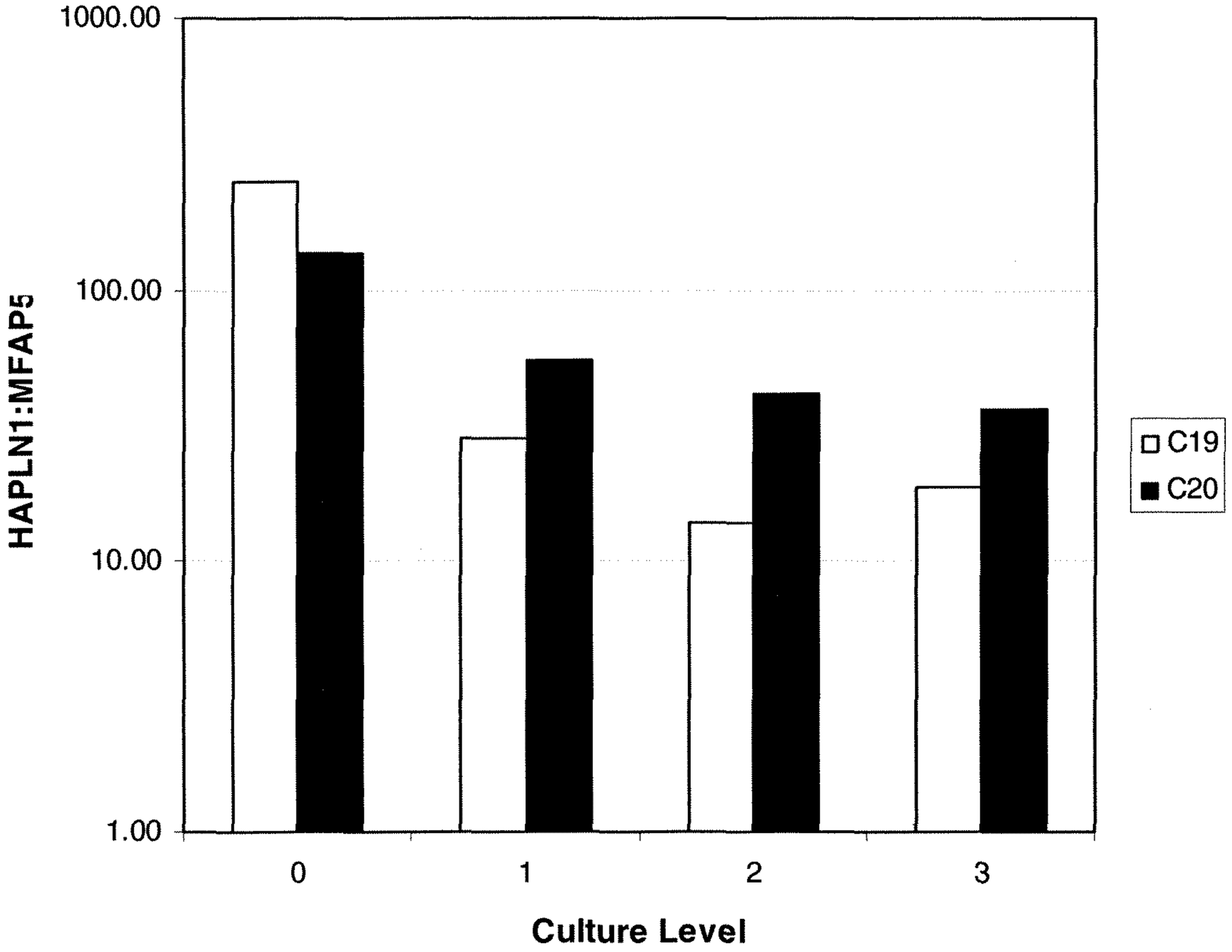

1000.00
100.00
10.00
1.00
HAPLN1:MFAP5
0
1
2
3
Culture Level
C19
C20

Fig. 14A

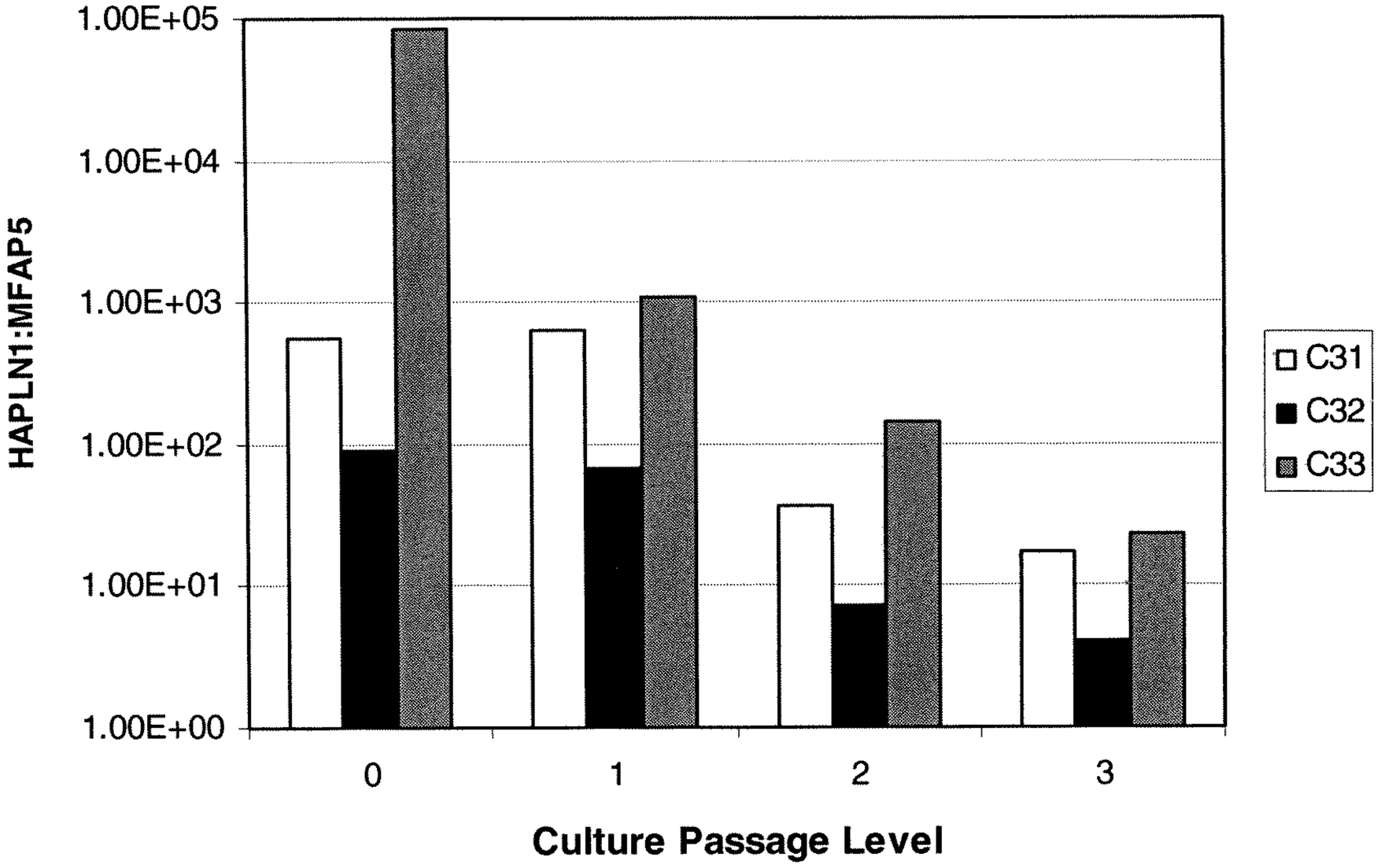

1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
HAPLN1:MFAP5
0
1
2
3
C31
C32
C33
Culture Passage Level

Fig. 14B

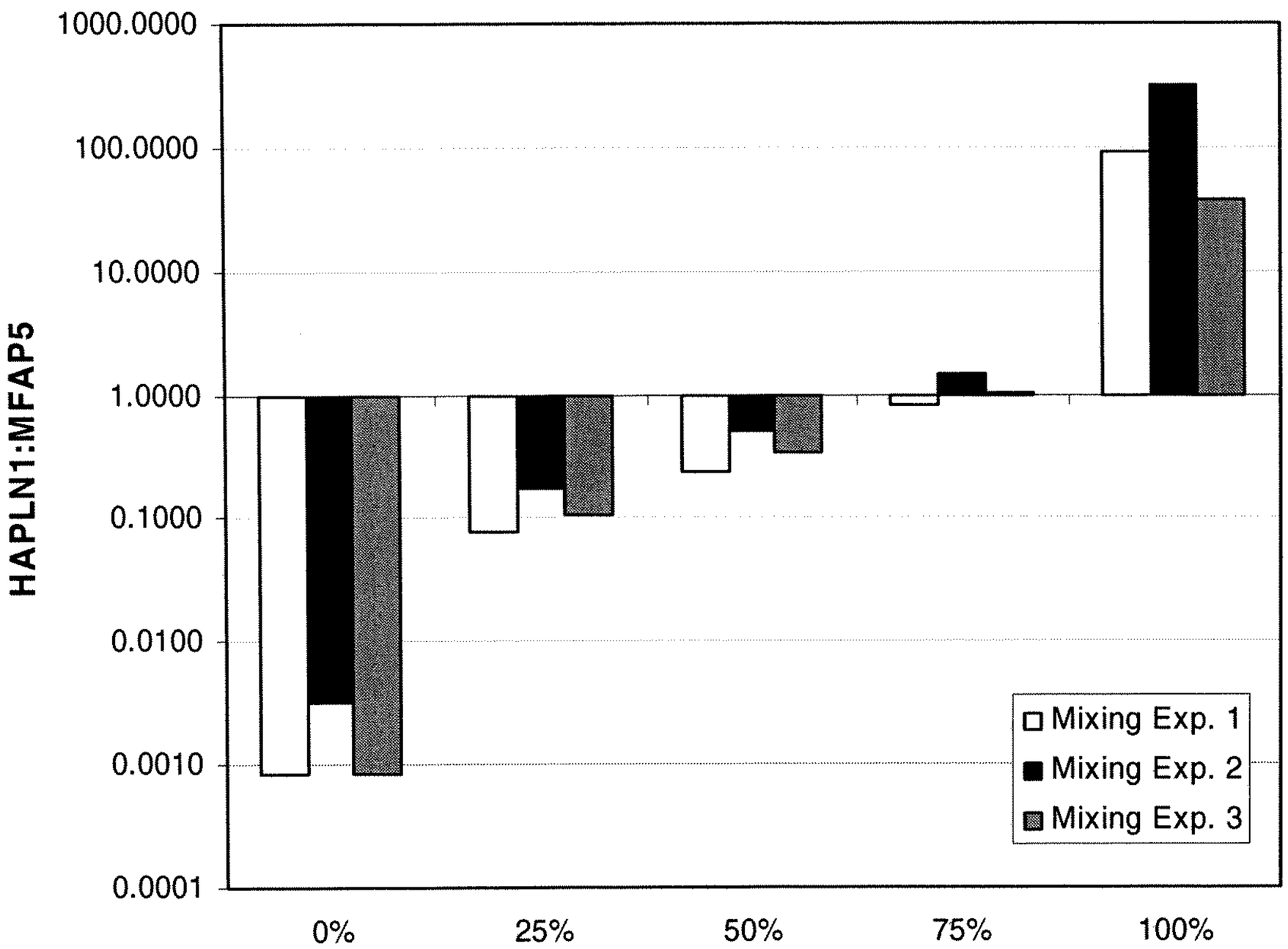

1000.0000
100.0000
10.0000
1.0000
0.1000
0.0100
0.0010
0.0001
HAPLN1:MFAP5
0%
25%
50%
75%
100%
Mixing Exp. 1
Mixing Exp. 2
Mixing Exp. 3
Percent chondrocytes with the balance synovial fibroblasts

Fig. 15

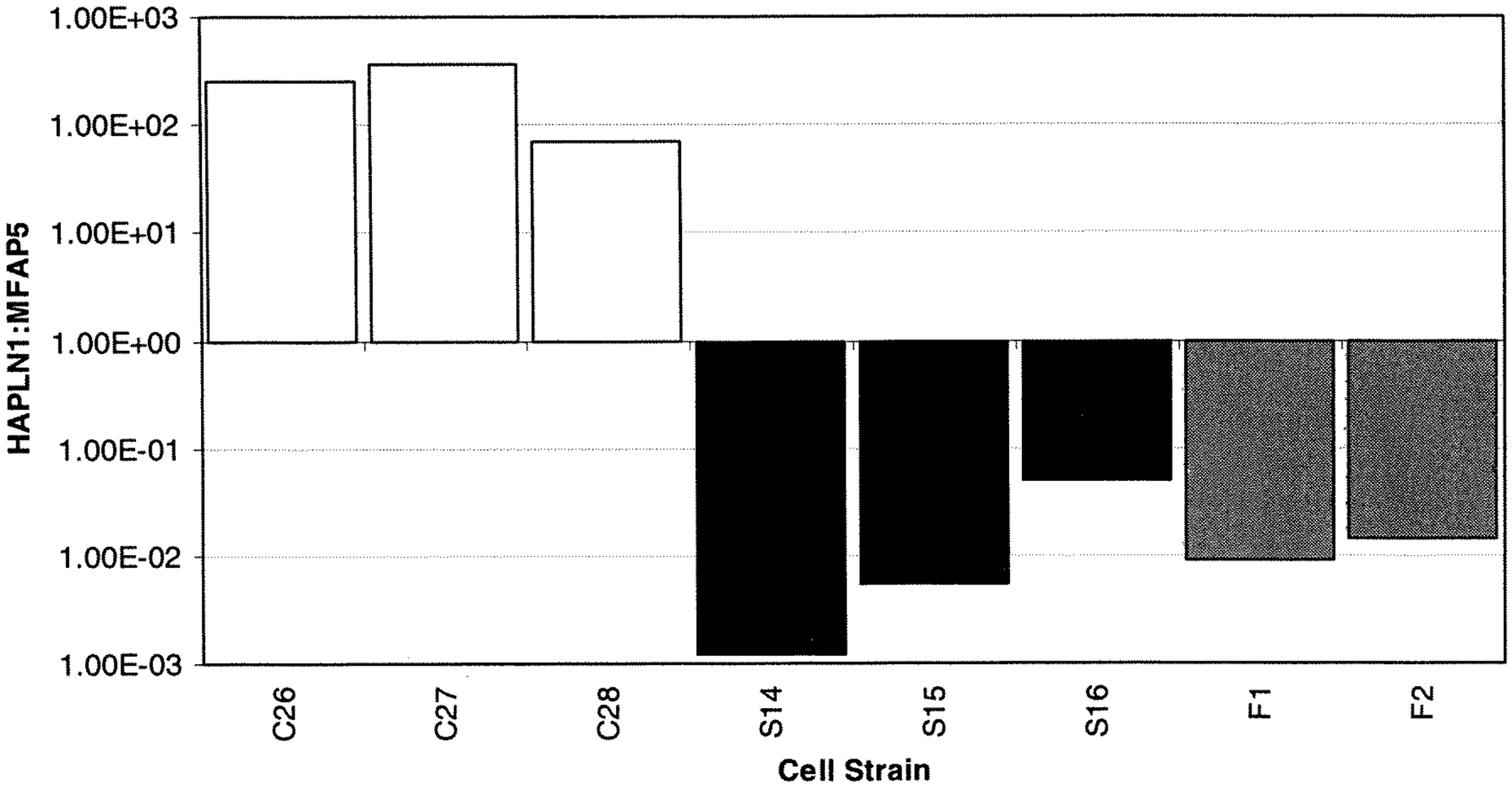
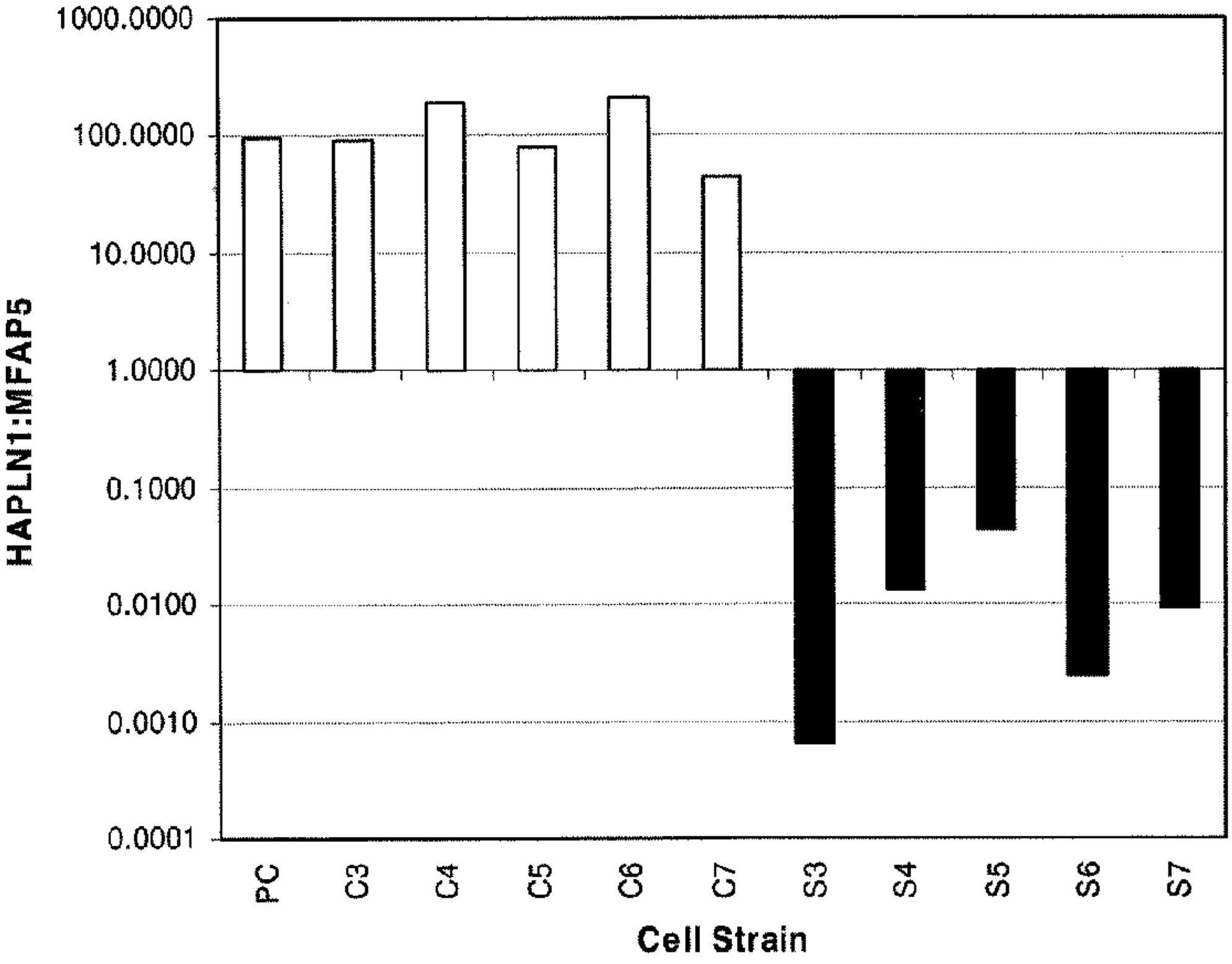

1.00E+03
1.00E+02
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1.00E-03
HAPLN1:MFAP5
C26
C27
C28
S14
S15
S16
F1
F2
Cell Strain

Fig. 16


1000.0000
100.0000
10.0000
1.0000
0.1000
0.0100
0.0010
0.0001
HAPLN1:MFAP5
PC
C3
C4
C5
C6
C7
S3
S4
S5
S6
S7
Cell Strain