Impact of Percoll purification on isolation of primary human hepatocytes – Supplementary information

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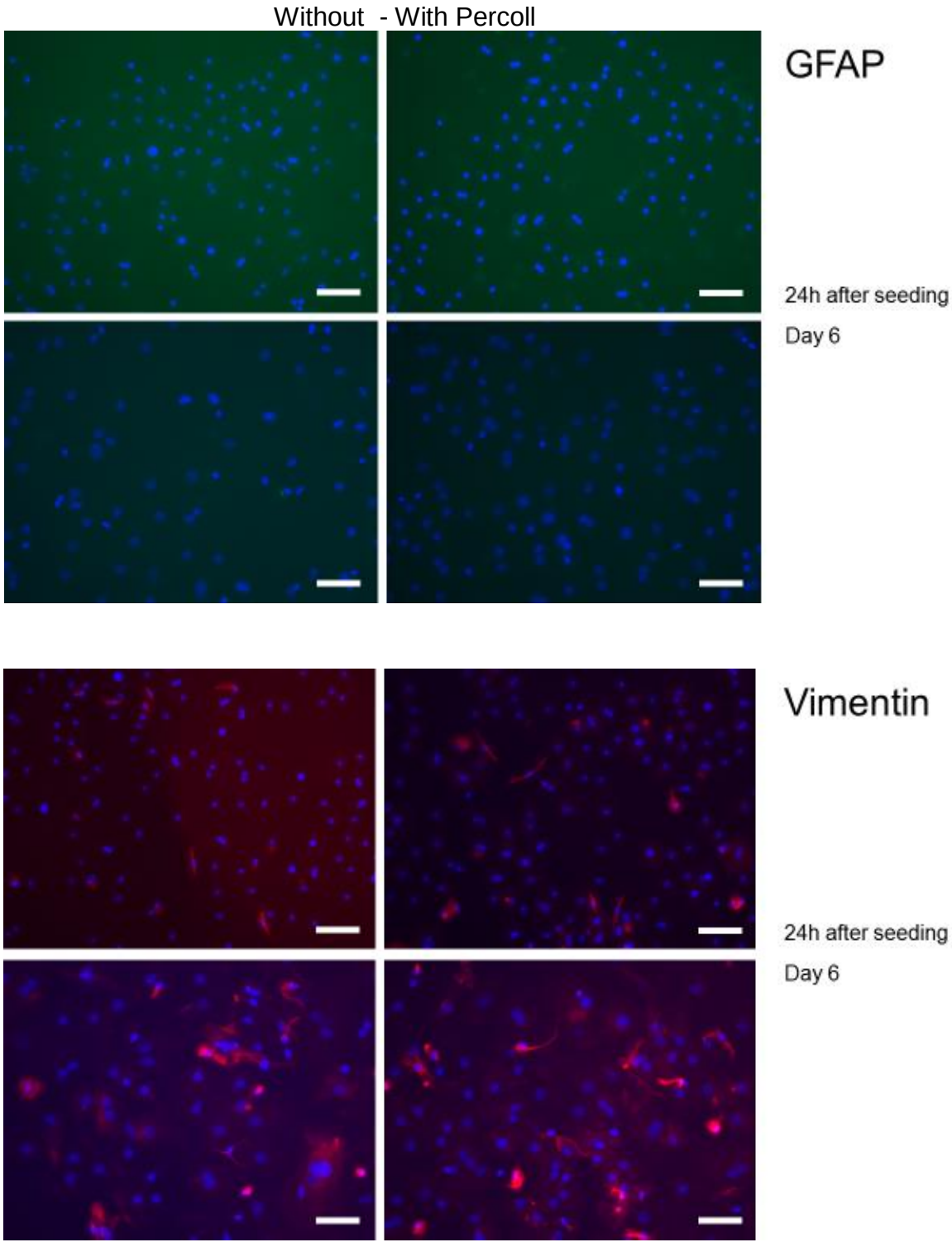
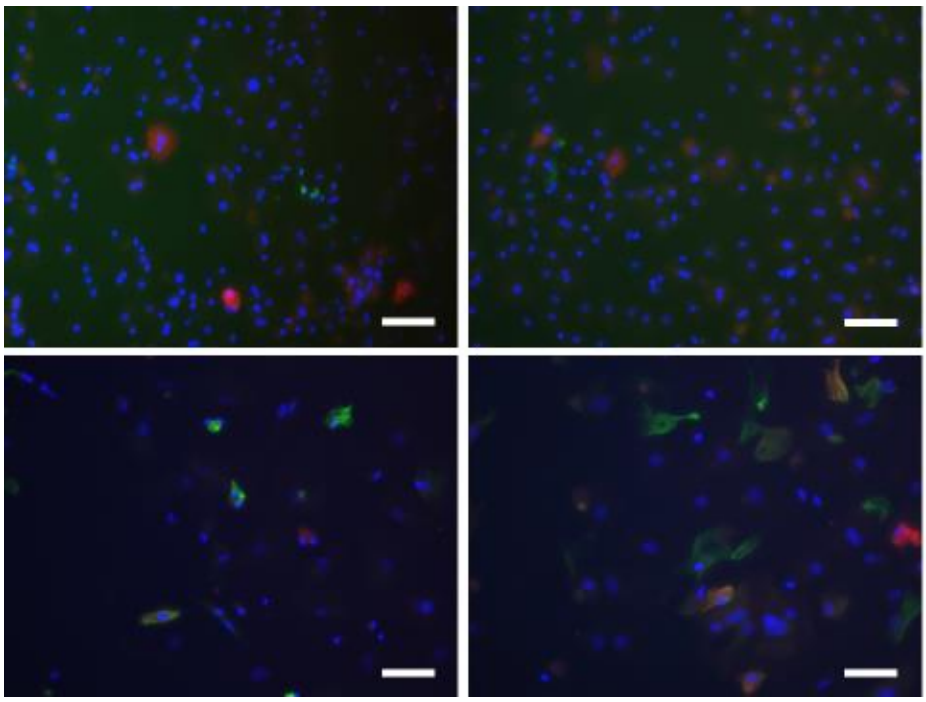
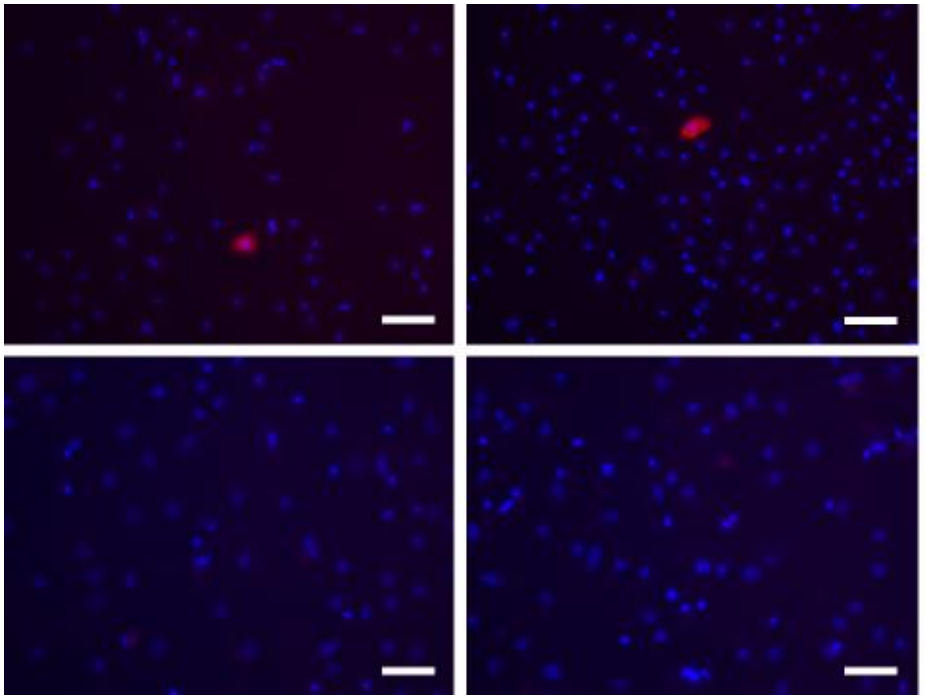
Supplementary information (1)

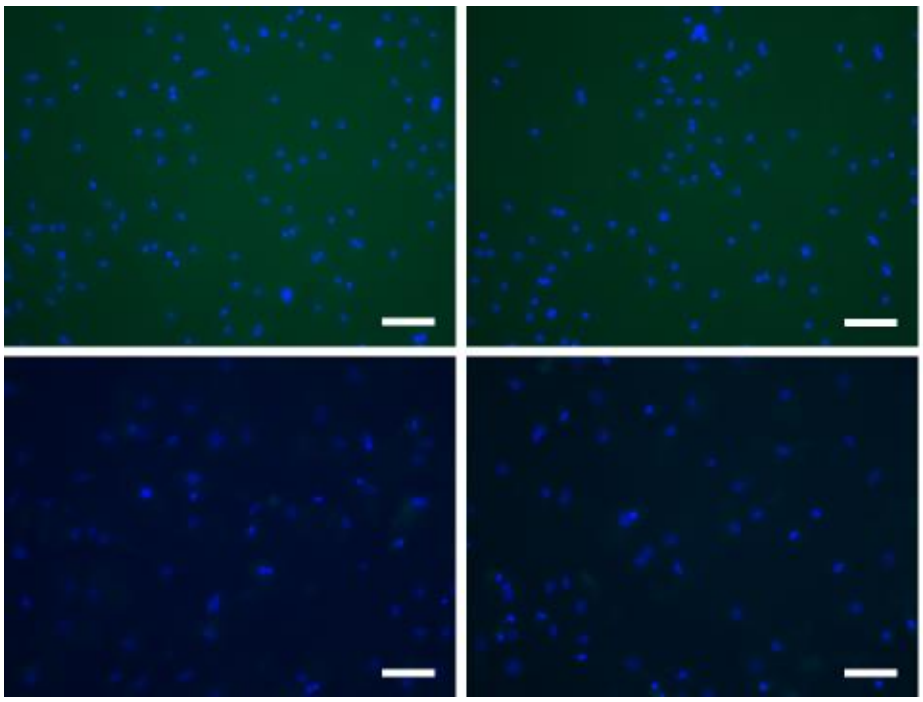
Immunofluorescence Staining of different liver cell types

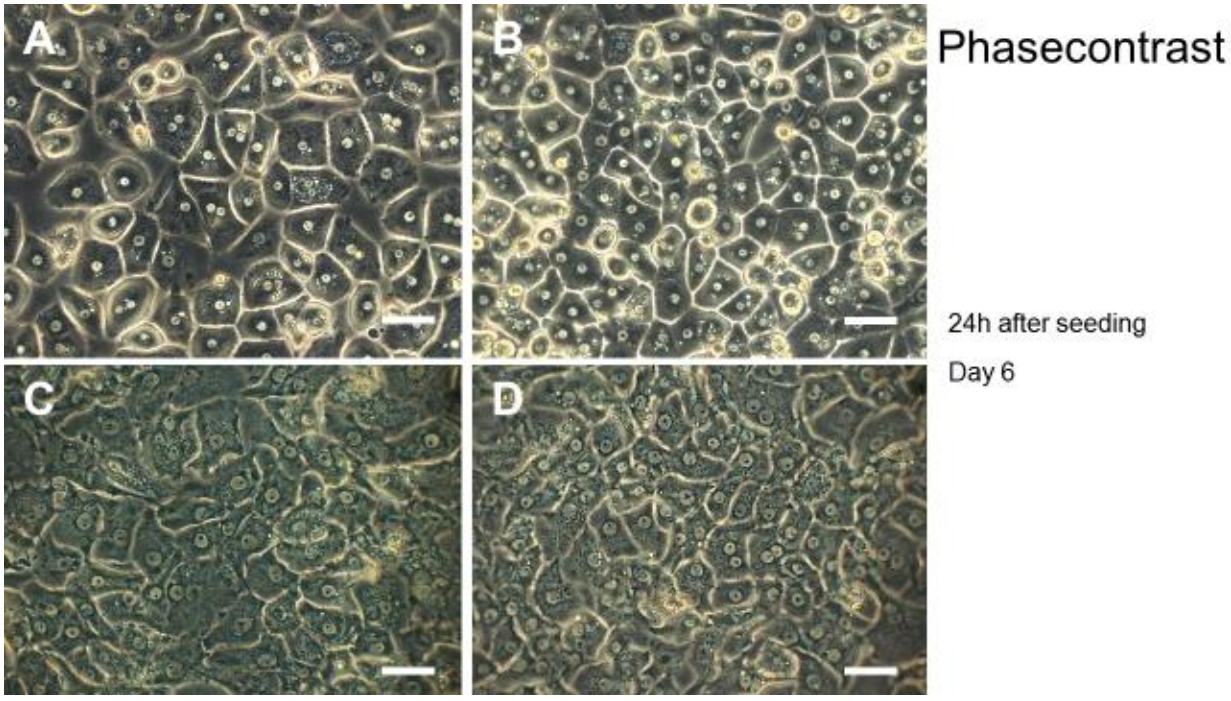
Fluorescence microscopy of hepatocyte cultures without (left column) of after (right column) purification with Percoll. Time points: 24 hours after seeding (first row for each staining respectively) and on Day 6 (second row for each staining respectively). A-D display KRT18-staining (hepatocytes), E-H display Pecam1-staining (endothelial cells). Scale bar: 50µm




KRT19/
KRT7
24h after seeding
Day 6


EpCam
24h after seeding
Day 6


CD68
24h after seeding
Day 6


A
B
C
D
Phasecontrast
24h after seeding
Day 6

Supplementary information (2) – Literature Research - Evaluation of the benefit of Percoll purification in primary human hepatocyte isolation

Search strategy and exclusion criteria

On May 28th, 2017, a systematic literature search was performed in PubMed (MEDLINE) using the following two search terms:

MeshTerm 1:

("Hepatocyte* Isolation" OR "primary hepatocyte*" OR "human hepatocyte*" OR ("Liver" AND "cell isolation")) AND ("plating efficiency" OR "density purification" OR Percoll OR Purification OR "Gradient Centrifugation" OR "density gradient" OR "culture purity")

MeshTerm 2:

"Hepatocyte* isolation"

All papers (n=352) where exported into Endnote X9 (Thomson Reuters, New York, US) and screened for duplicates (n=16). The resulting 326 papers were then screened by reading the title and abstract. After reading the abstracts, 170 papers were excluded because their abstract revealed that they fulfil exclusion criteria, including:

- Paper did not work with primary isolated hepatocytes but with cell lines like HuH7 or HepG2
- Paper focused on pharmaceutical drug-safety testing and gave no detailed information about the hepatocytes they used
- Paper used non-mammalian primary hepatocytes

After removal of papers which met the exclusion criteria, 156 papers were cross read. During this process publications were categorized for second exclusion:

Of all the publications that were identified to deal with primary hepatocytes and meet aforementioned criteria, 29 were not available with the literature licenses of Charité and 2 could not be retrieved in English or German. The remaining publications (n=125) were studied with special emphasis on the papers' methods part. 85 papers that gave a good explanation on the isolation were identified, while 22 were excluded because they did not state thoroughly enough, how they obtained hepatocytes and how they purified cell suspensions. Another 18 publications were excluded because the focus on another issue, so that hepatocyte isolation was just a tool to get their experimental materials.

Additionally, 16 papers were included after manual search. These papers were either suggested in references of other publications or recommended at the journals website.

Figure 1 illustrates the literature search strategy and workflow.

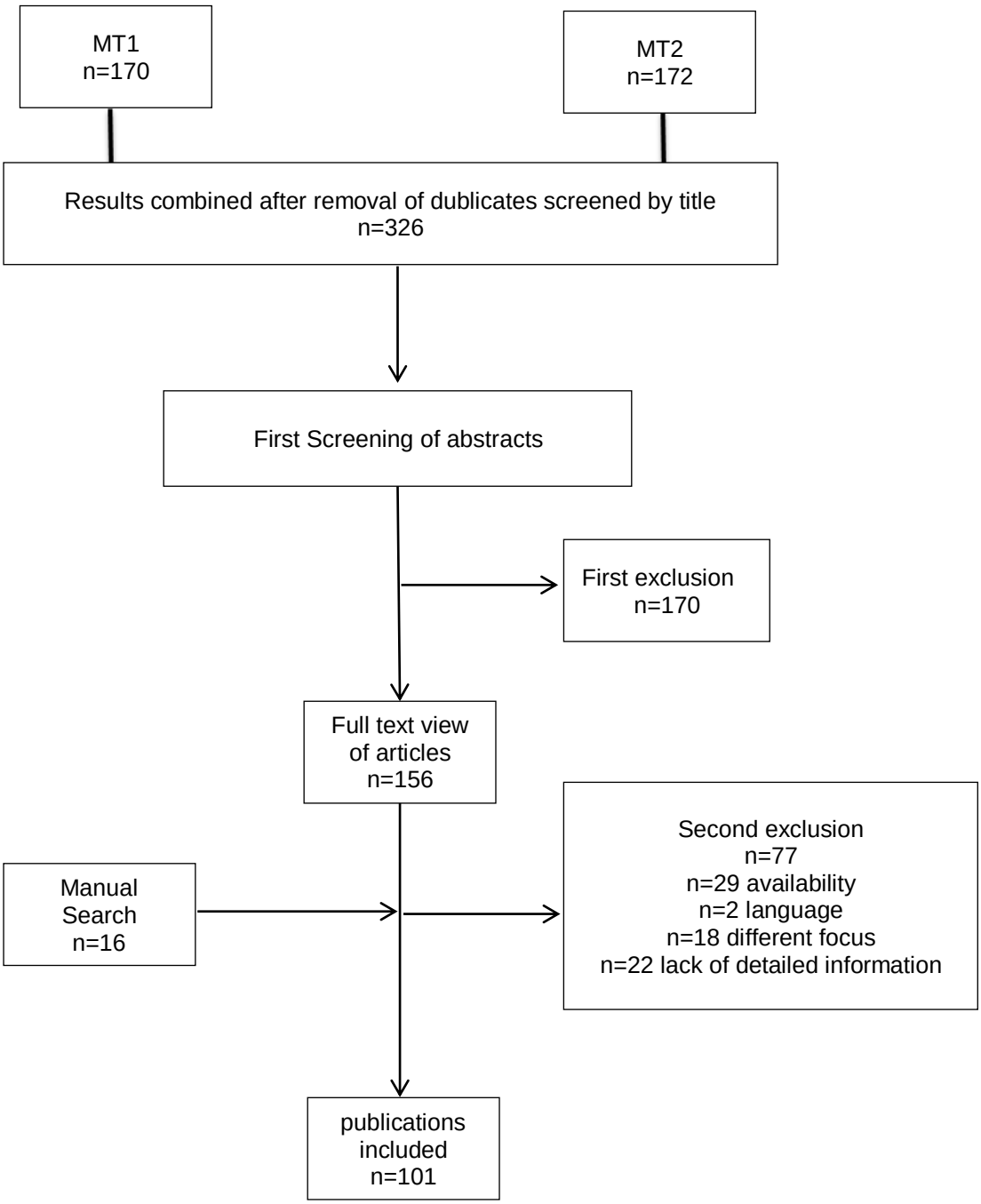


Figure 1: Flow diagram of search strategy

Quality assessment

Resulting papers' quality was assessed using a numeric scoring system. Since an established, published and peer-reviewed quality assessment lacks for evaluation of experimental studies, following questions were considered:

- Is the title adequate?
- Is the abstract adequate and does it accurately reflect the papers objectives?

- Is there an adequate description of statistic methods used and power to meet significance enough?
- Are there any errors in the paper concerning logic or facts?
- Is all the relevant information given to ensure reproducibility?

For each question, 1 point was accounted resulting in a score from 1 (poor quality) to 5 (excellent quality).

Results

101 publications were assessed for this review. Of these, 41 do not use density gradient centrifugation in their protocol, but end with the low speed centrifugation (mostly 50g for 5 minutes).

Figure 4 illustrates the publications reviewed and the different groups with references.

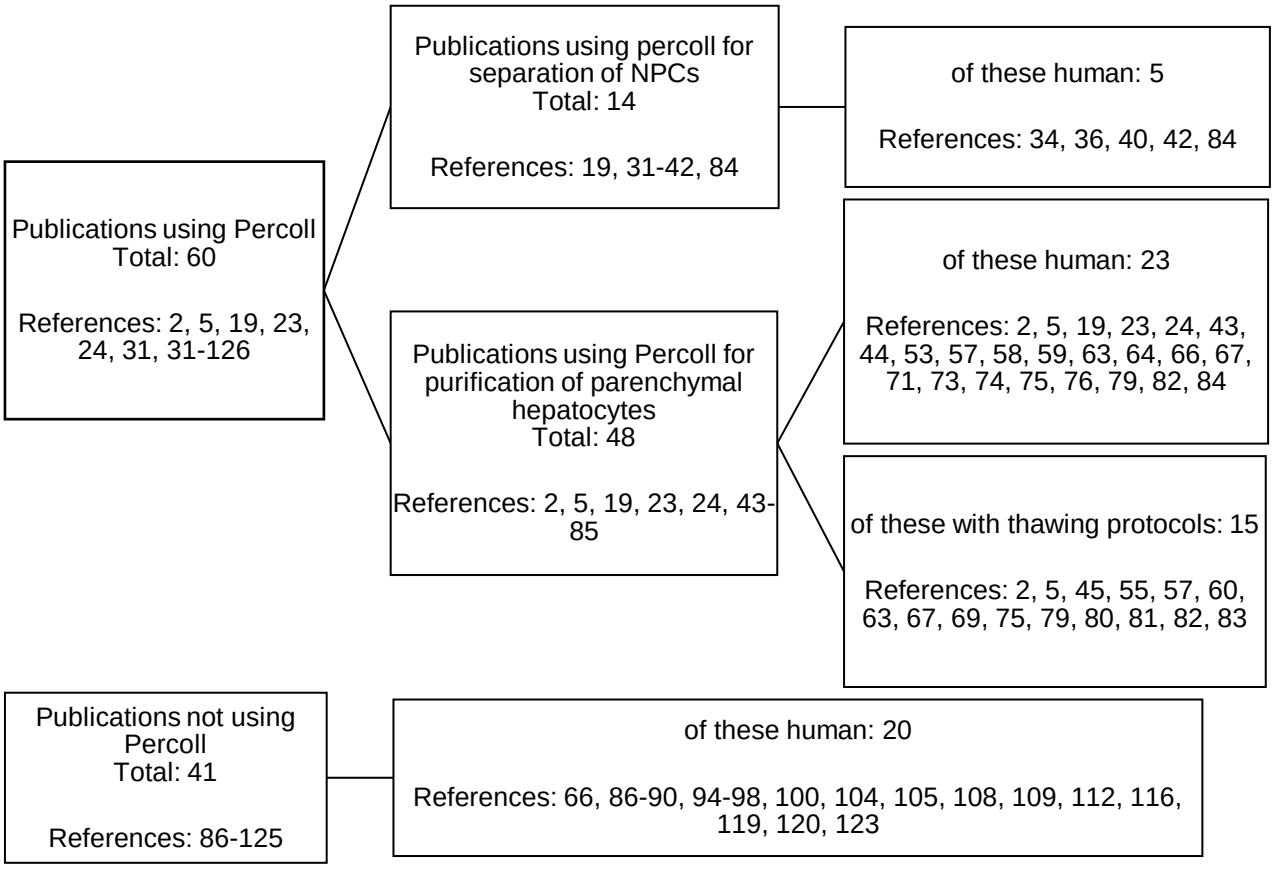


Figure 2. Overview of publications reviewed.

Percoll for purification of Non-Parenchymal cells

Of the 60 publications that mention purification with percoll density gradient in their methods part, 12 use this tool to isolate non-parenchymal cells like Kupffer cells (19, 31, 32, 34, 38, 39, 40), hepatic

progenitor cells (33, 42), oval cells (35) or stellate cells (31, 34, 36, 40); liver sinusoidal endothelial cells (19, 37). These authors use Percoll density gradient layers in different concentrations to separate different cell types of the liver from each other. However, the pellet resulting from the very first centrifugation without density gradient, in which the hepatocytes are concentrated, is not the focus of these works. The supernatant of this step gets centrifuged through several layers of differently concentrated percoll solution. Resulting cultures of NPCs are then cultivated and assessed regarding their function and yield. Detailed information about the pellet with the hepatocytes is missing.

Percoll for purification of parenchymal liver cells

Other 48 publications use percoll density gradient to further purify parenchymal hepatocyte suspensions. Set in relation to the ones not using percoll, 54% of screened publications focusing on hepatocytes use percoll to purify their culture. Of these 48 publications, 15 are using density gradient centrifugation just in conjunction with cryopreservation. Percoll is often used after thawing of freezed primary hepatocytes to split death cells and debris before subsequent use like culturing or transplantation.

Evaluation of purification of hepatocytes with Percoll

The literature search identified only 10 (19, 52, 53, 59, 63, 65, 73, 74, 84, 85) publications which systematically evaluate the benefit of Percoll on isolated hepatocytes, 5 of them (53, 59, 63, 73, 74) with human cells. Table 1 summarizes all information the publications give on their usage of percoll.

According to Schröder et al. in 1994 (74), who performed purification through a 50% concentrated Percoll-suspension for 10min at 50-250g, the reached viability was 94% and the cell culture reached to a high purity. Culture purity was assessed using immunohistological staining and revealed low contamination level of 1%, consisting of fibrocytes. However low yield after percoll purification is reported and in 7 out of 32 experiments, no cells could be recovered after the percoll step.

Olinga et al. (73) published a paper in 2000, in which they investigated, whether parameters of isolated hepatocytes can predict the graft function after transplanting the liver. They compared the yield and viability before and after purification through a Percoll density gradient (they don't state which concentration). It is reported that yield is significantly decreased (from 13.4 to 4.3 million cells per gram liver tissue) with an average percoll survival rate of 33%, but viability is strongly improved from 68.9 to 92%. They don't give information on quality and purity of the resulting cells though.

Laba et al. (2005, 59) centrifuged their cell suspensions through a 29-31%-concentrated Percoll layer before and after cryopreservation. Mean viability of crude cells before Percoll purification ranged to 72% and improved to 89% after Percoll. Taken freshly isolated crude cell suspension in comparison with cryopreserved, thawed and then Percoll-purified cells, they found a positive effect

on the plating efficiency after cryopreservation (35-40% improved to 65-85%) and lower levels of non-parenchymal cells when usage of Percoll. Therefore, markers where stained and assessed with immunohistochemistry and FACS: CD3, CD45, CD20, CD90, Ki76 where less positive in percoll-purified cultures, while expression of chemokines was similar in both groups. However, this study is focused on the comparison of fresh and cryopreserved and thawed hepatocytes, there is no metabolic test and limited due to the low number of experiments (n=5).

Yet, these authors do not reflect on the direct comparison on cells before and after Percoll purification regarding cell culture purity and plating efficiency, which are determining parameters for the hepatocytes' subsequent use.

Table 1. Overview of identified publications using percoll to purify parenchymal cells independently of cryopreservation (n=38):

Author	Percoll density gradient usage	species	Reference	Quality score
Bartlett, D. C., et al. (2014). Cytotherapy 16(6): 800-809.	Percoll was used, if initial viability was low and yield sufficiently high. No more detailed information.	human	43	4
Bhogal, R. H. et al. (2011). PloSOne Mar 29;6(3):e18222.	Used if initial viability was <50%. Effect: mean viability improved from 35% to 85%, while yield decreased from 550000 to 175000.	human	44	5
Clouet, A. S., et al. (1998). Analyst 123(12): 2489-2492.	used percoll centrifugation: 8min, 1400g. No more detailed information.	bovine	46	3
Falcieri, E., et al. (1990). Cytotechnology 4(3): 251-259.	After centrifugation through 50% percoll solution, complete purification and removal of debris is reported (less microvilli on cells in REM). No more detailed information.	rats	47	4
Fiegel, H. C., et al. (2000). Tissue Eng 6(6): 619-626.	used percoll 1,13g/ml (95%) 400g, 12min. No more detailed information.	rat	48	4
Foy, B. D., et al. (1994). Biotechnol Bioeng 43(7): 661-672.	used percoll 1.06 g/ml (45%), 50g, 5min, 90% viability. No more detailed information.	rat	49	5
Gerlach, J., et al. (1996). J Surg Res 62(1): 85-89.	used 1.065g/ml (50%) percoll "in selected isolations" viability improved up to 99%. No more detailed information.	pig	50	3
Gerlach, J., et al. (1993). Artif Organs 17(11): 950-953.	percoll improved viability up to a mean of 95%" – No more detailed information.	pig	51	4
Goncalves, L. A., et al. (2007). Malar J 6: 169.	used three different percoll concentrations: 1.12g/ml (99%), 1.08g/ml (60%),1.06g/ml (46%), 750g, 20min. Two sequential density gradient centrifugations. Purity was measured purity with facs: After each gradient centrifugation, there was less contamination with C45RB and CD95.	murine	52	5
Gramignoli, R., et al. (2014). Cell Transplant 23(12): 1545-1556.	Viability improved from 29% to 65% and from 75% to 90% respectively), plating efficiency (10-40% higher), ammonia metabolism and other metabolic activities improved. "Additional studies with cell separation techniques […] seem to be warranted."	human	53	5

Gumucio, J. J., et al. (1986). Hepatology 6(5): 932-944.	used percoll 63%, 40000g, 20min, 4°C; focused on the difference between anterograde and retrograde perfusion techniques.	rat	54	5
Horner, R., et al. (2016). Tissue Eng Part C Methods 22(9): 839-846.	used 25% concentrated percoll solution, 1474g, 20min; no comparison before and after percoll purification.	human	23	5
Johnston, D. E. and R. Jasuja (1994). Hepatology 20(2): 436-444.	Used 54%-percoll layer for purification of parenchymal hepatocytes. NPCs were sedimented between 25% and 50% layer of percoll. No more detailed information about the hepatocyte layer.	rat	56	5
Kawahara, T., et al. (2010). Liver Transpl 16(8): 974-982.	State usage of percoll but no more detailed information.	rat	58	4
Kluge, M., et al. (2016). Tissue Eng Part C Methods 22(1): 38-48.	used 25% concentrated percoll solution, 1474g, 20min; no comparison before and after	human	24	5
Laba, A., et al. (2005). Arch Immunol Ther Exp (Warsz) 53(5): 442-453.	29-31%, Viability improved from 72% to 89%, less contamination with NPCs, better plating efficiency (35-40% to 65-85%)	human	59	4
Odaira, M., et al. (2009). J Surg Res 152(2): 209-217.	Used percoll density gradient to enrich cell viability to at least 90% – no more detailed information.	human	61	5
Ohashi, K., et al. (2012). Cell Transplant 21(2-3): 429-436.	State usage of percoll but no more detailed information.	murine	62	5
Ostrowska, A., et al. (2000). Cell Tissue Bank 1(1): 55-68.	Used percoll at unknown concentration. Puritiy was assessed by CD45 staining, decreased from 8.8% to 0.7% after percoll. Increased viability from 85% to 93%	human	63	5
Puviani, A. C., et al. (1998). Comp Biochem Physiol A Mol Integr Physiol 121(2): 99-109.	State usage of percoll but no more detailed information.	human	64	3
Rajvanshi, P., et al. (1998). Exp Cell Res 244(2): 405-419.	Separated different subpopulations of hepatocytes by their ploidy state through percoll layers of different concentrations: 70%, 52%, 42%, 30%; 1000g, 30min - viablility of parenchymal cells is reported to rise, while yield decreases with increasing percoll concentration. Hepatocytes were split up regarding their state in the azinus before.	rat	65	4

Richert, L., et al. (2004). Liver Int 24(4): 371-378.	Multi-laboratory study. One used percoll, other not. No more detailed information.	human	66	5
Rivas, P. A., et al. (1993). Transplantation 55(2): 335-339.	State usage of percoll but no more detailed information.	rabbit	68	4
Shulman, M. and Y. Nahmias (2013). Methods Mol Biol 945: 287-302.	500g, 5min; but no more detailed information.	rat	70	5
Vondran, F. W., et al. (2008). Artif Organs 32(3): 205-213.	Use percoll: 25%, 1278g, 20min. Viability was risen from 70.3 to 82.5%, while yield was decreased from 18.7 to 13.2x10^6/g, percoll survival 68%	human	71	5
Wang, H., et al. (1998). J Biochem 124(5): 892-899.	Use percoll 45%, microscopically assessed purity was 99% and viablility was 98% measured by Trypan blue exclusion. No comparison before and after percoll.	murine	72	4
Smedsrød B, Pertoft H. (1985). J Leukoc Biol. 38(2):213-30	Used percoll for primary hepatocytes: 1.08% (60%), 50g, 2min; supernatant in different percoll-concentrations for NPCs. After 60% density centrifugation, PCs are stated to be nearly 100% viable and no detectable contaminating NPCs. 50-60% percoll survival.	rat	19	5
Olinga P, et al. (2000). Liver. 20(5):374-80.	Percoll concentration not stated. Focus more on the correlation of hepatocyte isolation with clinical features of the donor. Mean Percoll survival: 33%; mean yield was decreased from 13.4 to 4.3million cells/g tissue while viability was increased from 68.9 to 92% on average.	human	73	5
Schröder AJ, et al (1994). Zentralbl Chir., 119(2):127-38	50% percoll, 10min, 50-250g; Contamination level of NPCs =1% (fibrocytes); report low yield after percoll-purification (in 7 out of 32 experiments no outcome after percoll). Assessment of purity with immunohistochemical staining. No metabolical assay.	human	74	5
Godoy P et al. (2013). Arch Toxicol. 87(8):1315-530	Recommend 25%-concentrated percoll, 1200-1200g for 15-20min for the best separation between viable and dead cells. Recommend 48%-concentrated percoll, 28g for 5min to purify hepatocytes. No comparison.	Human/ rat	5	4
Li AP. (2007). Chem Biol Interact 168(1):16-29	State usage of percoll but no more detailed information about it.	human	75	4

Knobeloch D, et al. (2012). Methods Mol Biol. 806:99-120	Use percoll 25%, 1278g, 20min; no more detailed information about it.	human	76	4
Ehrhard S, et al. (2016). EXCLI J. 15:858-866.	Use percoll 1.124g/ml (92%), 1000g, no more detailed information.	bovine	77	4
Spotorno VG, et al. (2006). Cytotechnology. 51(2):51-6	Non-perfusion-technique. Compare before and after 60% percoll - metabolic activity higher – When Percoll purification was performed, only larger cells were selected with a lower yield. These cells were not as confluent but metabolically more active.	bovine	78	5
McGinnity DF, et al. (2004). Drug Metab Dispos. 32(11):1247-53.	25% Percoll purification increased dog hepatocyte viability from 52 to 78% and human hepatocyte viability from 66 to 88%. “Percoll concentrations above 25% led to an unacceptable loss of cells in both species.”	Human/ dog	79	5
Innes GK, et al. (1988). Cryobiology 25(1):23-30	Percoll density 1.07g/ml (55%) and 1.02g/ml (12.5%), 30000g, 90min, was performed and two separate populations of hepatocytes were obtained this way. “One denser than the other with significantly greater trypan blue dye-exclusion capabilities ($P < 0.05$).” Purified cells through the higher-concentrated Percoll layer performed better metabolically and had a better membrane integrity than the less dense cells.	rat	80	5
Tateno C, et al. (2000). Hepatology 31(1):65-74	Used Percoll concentrated 45%, 50g, 10min; separated different subpopulations of hepatocytes (“normal” vs “small hepatocytes”) and NPCs. Assessed effects of different co-cultures and growth potential and purity with FACS and immunohistochemistry. Focus is not on one population of parenchymal cells before and after density centrifugation.	rat	84	5
Kreamer BL, et al. (1986). 22(4):201-11.	Used two different density gradient protocols, one iso-density with 1.06g/ml (45%), 50g, 10min. Viability was improved from 87.1 to 98%. Rat hepatocytes purified this way performed better metabolically (cytochrome p-450, tyrosine aminotransferase, glutamic-oxaloacetic transaminase, hormone response). Percoll survival was 90%, cells sowed better plating efficiency, and a higher percentage of single cells, less debris and almost no NPCs. Also subsequent viability is reported to be better	rat	85	4

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