1 **DNA methylation and gene expression changes derived from assisted reproductive**
2 **technologies can be decreased by reproductive fluids**

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4 Sebastian Canovas [1,2], Elena Ivanova [3], Raquel Romar [1,2], Soledad García-Martínez [1,2], Cristina
5 Soriano-Úbeda [1,2], Francisco A. García-Vázquez [1,2], Heba Saadeh [3,4,Ç], Simon Andrews [4], Gavin
6 Kelsey [3,5] and Pilar Coy [1,2]*

7 **Affiliations:**

8 [1]Physiology of Reproduction Group, Departamento de Fisiología, Facultad de Veterinaria,
9 Universidad de Murcia, Campus Mare Nostrum. Murcia-30100. Spain

10 [2]Instituto Murciano de Investigación Biosanitaria. IMIB-Arrixaca-UMU. Murcia-30120, Spain.

11 [3]Epigenetics Programme, The Babraham Institute, Cambridge, CB22 3AT, United Kingdom.

12 [4]Bioinformatics Group, The Babraham Institute, Cambridge, CB22 3AT, United Kingdom.

13 [5]Centre for Trophoblast Research, University of Cambridge, Cambridge, CB2 3EG, United
14 Kingdom.

15 [Ç]Computer Science Department, KASIT, The University of Jordan, Jordan.

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17 *To whom correspondence should be addressed: pcoy@um.es

18 **Running title:** Reproductive fluids help the correct establishment of DNA methylation

19 **Keywords:** *in vitro* fertilization, blastocyst, ART, epigenetics, pig, reproductive fluids,
20 imprinting, oviductal fluid

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24

25 **Abstract**

26 The number of children born since the origin of Assisted Reproductive Technologies (ART)
27 exceeds 5 million. The majority seem healthy, but a higher frequency of defects has been
28 reported among ART-conceived infants, suggesting an epigenetic cost. We report the first whole-
29 genome DNA methylation datasets from single pig blastocysts showing differences between *in*
30 *vivo* and *in vitro* produced embryos. Blastocysts were produced *in vitro* either without (C-IVF)
31 or in the presence of natural reproductive fluids (Natur-IVF). Natur-IVF embryos were of higher
32 quality than C-IVF in terms of cell number and hatching ability to. RNA-Seq and DNA
33 methylation analyses showed that Natur-IVF embryos have expression and methylation patterns
34 closer to *in vivo* blastocysts. Genes involved in reprogramming, imprinting and development
35 were affected by culture, with fewer aberrations in Natur-IVF embryos. Methylation analysis
36 detected methylated changes in C-IVF, but not in Natur-IVF, at genes whose methylation could
37 be critical, such as IGF2R and NNAT.

38 **Introduction**
39
40 "Most fertility researchers are trying to improve [ART] success as measured by a single, clear
41 standard: the birth of an apparently healthy baby. Only a few are trying to discern whether IVF
42 leaves a subtle legacy in children. What will happen to these kids when they are middle-
43 aged?"[1]. In humans, according to a study by the World Health Organisation (WHO) in 190
44 countries, infertility affects 20% of couples and it was estimated that at least 40.5 million women
45 were seeking infertility medical care in 2007 [2]. Assisted Reproductive Technologies (ART)
46 provide a helpful alternative for a high proportion of infertility cases and the number of children
47 born to date using these methods exceeds 5 million [3]. Although the majority of them seem
48 healthy, studies have reported higher rates of preterm births [4], non-chromosomal birth defects

49 and adverse perinatal effects in ART pregnancies [5], with long-term effects being under study in
50 humans[6]. Epidemiological data suggest that perturbed epigenetic gene regulation by the
51 application of ART could be a contributory factor in these adverse outcomes [5, 7], although
52 such alterations could also be considered as consequences of parental characteristics, gamete
53 quality or other non-epigenetic technique-derived effects [8]. To clarify the impact of each of
54 these factors the use of an animal model that avoids, as much as possible, the effect of parental
55 circumstances and the use of protocols minimizing the technique-derived effects would help to
56 attain the goal of offering safer ART for patients.
57
58 For modelling ART-related disorders in human, swine could be a good candidate for several
59 reasons: their genetic, anatomical and physiological similarities with human [9], their size and
60 length of gestation, and the availability of individuals genetically selected by their excellent
61 reproductive performance in artificial insemination centres. Importantly, this last trait could be
62 useful to remove the paternal factor (low quality male gametes) from studies as a possible reason
63 for any epigenetic alterations found. However, most protocols for processing boar spermatozoa
64 for *in vitro* fertilization (IVF) include their selection by density gradient centrifugations and just
65 a few used the swim-up procedure to isolate highly motile spermatozoa which is the routine
66 selection in human infertility clinics. Since it was observed that spermatozoa selected by swim-
67 up show higher rates of normal morphology and motility, and decreased DNA fragmentation and
68 methylation levels [10], it would be necessary to adapt the sperm selection protocols in pig
69 before using them to model ART-derived epigenetic alterations.
70 In both mouse and human accumulating evidence indicates that the embryo is sensitive to its
71 very early environment, and that culture media used in ART (as factors involved in technique-

72 derived effects) may have long-lasting consequences [11, 12]. Several imprinting disorders and
73 abnormal phenotypes have been linked to ART, but of special significance is the relationship
74 between the presence of serum in culture media and the incidence of Large Offspring syndrome
75 (LOS) in ruminants [13], which includes diverse pathologic alterations and shows phenotypic
76 and epigenetic similarities with the imprinted disorder Beckwith-Wiedemann syndrome (BWS)
77 in humans [14]. Since it was proposed that serum in the culture medium could be a crucial factor
78 in LOS incidence, the tendency in the procedures for both human and livestock was to move
79 towards the use of chemically defined media, limiting the presence of proteins in the culture
80 medium to serum albumin. Although practical, this approach may have unpredictable
81 consequences, because it ignores the fact that the reproductive fluids have a different
82 composition to serum and are extremely rich in proteins other than serum albumin (more than
83 150 have been described in the oviductal fluid [15]). If these proteins are physiologically present,
84 they must play a variety of roles supporting the normal development of the embryo, roles that
85 serum albumin alone cannot properly provide and serum cannot fully mimic. In addition,
86 although ART in species such as cattle and sheep usually results in foetal overgrowth [16, 17],
87 opposing phenotypes such as low birth weights (excluding BWS) are often seen in humans [18]
88 and pigs [19]. A study showing the relationship between child birth weight and the protein
89 source in embryo culture media [20] reinforces the hypothesis that the protein composition of the
90 culture media plays a role in the correct regulation of epigenetic marks in the growing embryo. A
91 similar conclusion can be reached from a clinical trial showing that protein enrichment of media
92 compared with addition of serum albumin alone improved the blastocyst implantation rate and
93 may increase human births by more than 8% [21]. Therefore, as with breast milk, which is so
94 complex and so rich in bioactive factors that cannot be easily replaced with any artificial

95 composition [22], the idea that reproductive secretions could be necessary in the culture media
96 should not be underrated. At least, it should be explored under experimental conditions to unveil
97 the relevance of these secretions.
98
99 DNA and RNA sequencing have become affordable cutting-edge technologies that could help to
100 understand the mechanisms underlying abnormalities observed in ART-derived offspring.
101 However, so far, single blastocyst whole-genome DNA methylation profiles comparing *in vivo*
102 and *in vitro* produced embryos have not been published for any mammalian species and we
103 therefore aim to produce these in this study.
104
105 We report here that modified swim-up protocols for the selection of spermatozoa in pigs and the
106 use of reproductive secretions as additives in the culture media significantly increase the yield
107 and quality of the blastocysts produced from a morphological, epigenetic and gene expression
108 point of view. Using genome-wide analyses of gene expression by RNA-Seq and DNA
109 methylation by Bisulfite-Seq in single blastocysts we provide datasets of pig blastocysts
110 produced *in vitro* with and without reproductive secretions as additives in the culture medium
111 and show that the former are more similar to the *in vivo* specimens than the later. This suggests
112 an alternative approach for conceiving healthier ART-derived children.
113

114 Results

115 *Swim-up method improves the yield of pig IVF*

116 In order to select spermatozoa before IVF, a swim-up protocol was set up and compared with a
117 conventional selection system by density gradient centrifugations. To do this it was necessary to

118 design a suitable washing and sperm selection medium imitating, as far as possible, *in vivo*
119 conditions (NaturARTs® PIG sperm washing medium and NaturARTs® PIG sperm swim-up
120 medium, EmbryoCloud, Murcia, Spain). The swim-up medium was supplemented either with
121 bovine serum albumin (BSA) (Swim-up BSA group) or porcine oviductal fluid (POF, Swim-up
122 fluid group) collected at the late follicular (LF) phase of the estrous cycle (NaturARTs® POF-
123 LF, EmbryoCloud, Murcia, Spain) (Figure 1). All the fluids used in this study were directly
124 aspirated from the lumen of ovarian follicles, oviducts or uterus and processed according to the
125 information described in the material and methods section, at http://embryocloud.com, and in
126 previous references [23].

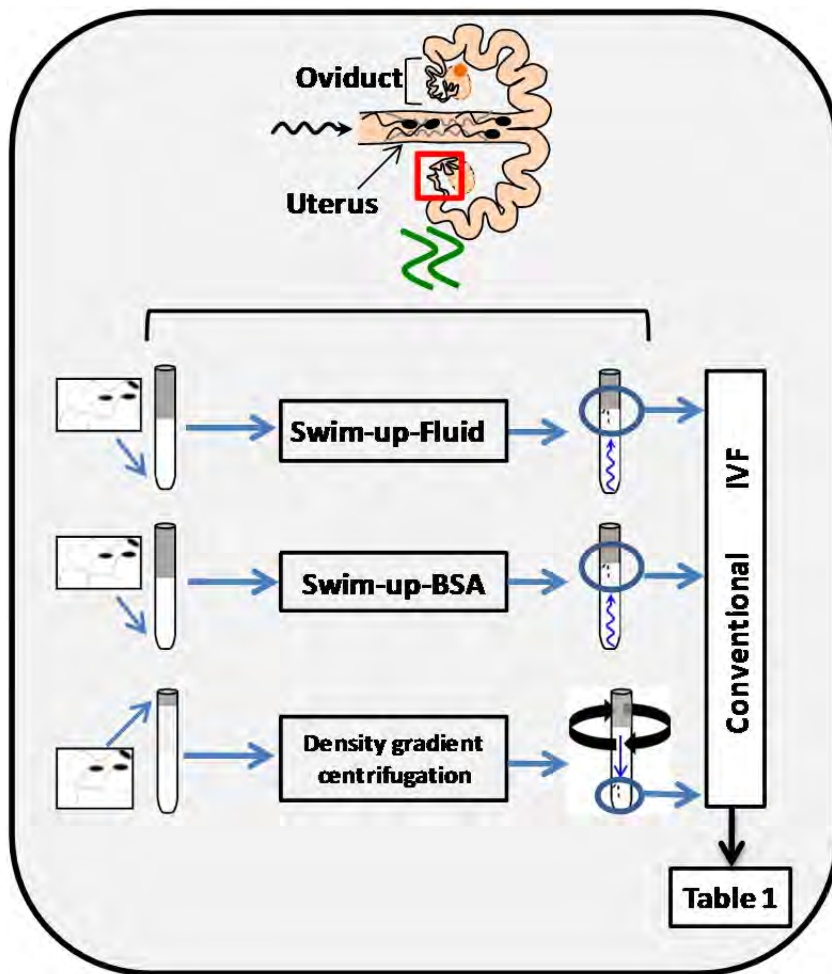

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Figure 1. Schematic representation of 3 different sperm processing protocols used for *in vitro* fertilization. Swim-up-BSA: NaturARTs® PIG medium + BSA; Swim-up-Fluid: NaturARTs® PIG medium + POF-LF*. Density gradient centrifugation: centrifugation through a discontinuous Percoll®: gradient (45 and 90% v/v). *POF-LF: porcine oviductal fluid collected at the late follicular phase of the estrous cycle. Red box represents the portion of the reproductive tract whose conditions we tried to resemble *in vitro*. IVF results after using these 3 different sperm processing protocols are included in Table 1.

Polyspermy after IVF is a major issue in the pig [24]. With these new protocols we obtained significantly higher rates of monospermy than with conventional ones (49.6±4.5 *vs*. 17.4±4.1, Table 1) and the final percentage of putative zygotes (evaluated at 24 hours post insemination, hpi) was significantly higher (35.2±0.2 *vs*. 14.6±0.1, Table 1). Moreover, the addition of POF-LF to the Swim-up media instead of BSA increased the final yield of the system (35.2±0.2 *vs*. 29.7±0.2, Table 1).

Reproductive fluids added to the culture media increase blastocyst quality

In a second experiment, and using the Swim-up protocol for sperm selection, a new IVF/Embryo culture (EC) system (Natur-IVF) was developed, which included preincubation of oocytes in oviductal fluid (NaturARTs® PIG OF-LF) and the presence of reproductive fluids as additives in the IVF and EC media (0-8 h: NaturARTs® POF-LF; 8-48 h: oviductal fluid from the early luteal–EL- phase of the estrous cycle, NaturARTs® POF-EL; 48-180 h: uterine fluid -UF-from this same phase, NaturARTs® PUF-EL) (Figure 2). Corresponding controls with BSA instead of OF/UF for each step (referred as C-IVF group) were analysed (Figure 2). Evaluation at 24 hpi revealed higher penetration rate (66.6±0.1 *vs*. 43.7±0.1, $P<0.05$) and similar monospermy rate (78.6±0.1 *vs*. 72.7±0.1, $P<0.05$) for the Natur-IVF and C-IVF groups, respectively. Regarding embryo development, more than 40% of cleaved embryos reached the blastocyst stage in both

groups (Table 2A). However, the Natur-IVF blastocysts showed a significant increase in their mean number of cells (81.8±7.2, Table 2A) compared to the C-IVF ones (49.9±3.7) and this number was similar to that observed in the *in vivo* samples (*In-vivo* group, 87.0±7.2). Moreover, at day 7.5, embryos reaching the hatching or hatched stages were observed only in the Natur-IVF group (Table 2B). Taken together, these data indicate a higher quality, in terms of cell number and ability to hatch, in the ART-derived blastocysts when reproductive fluids were added to the culture medium.

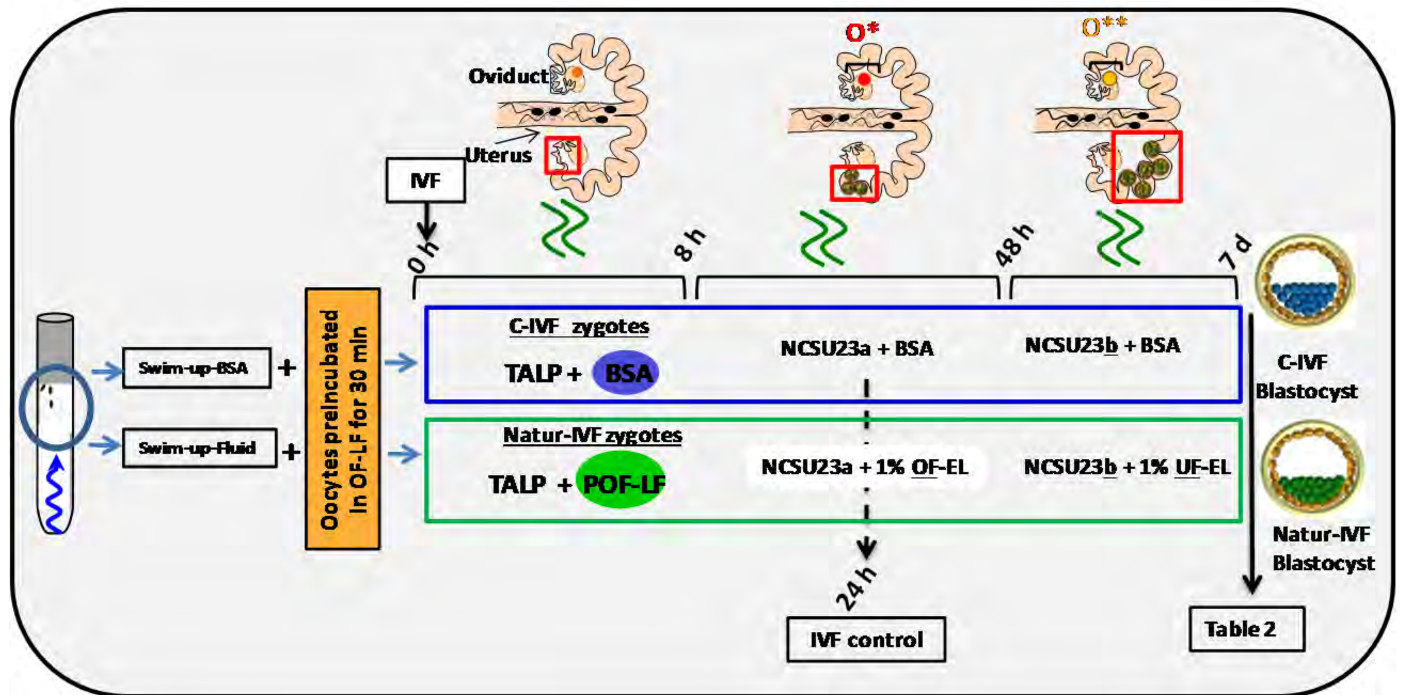
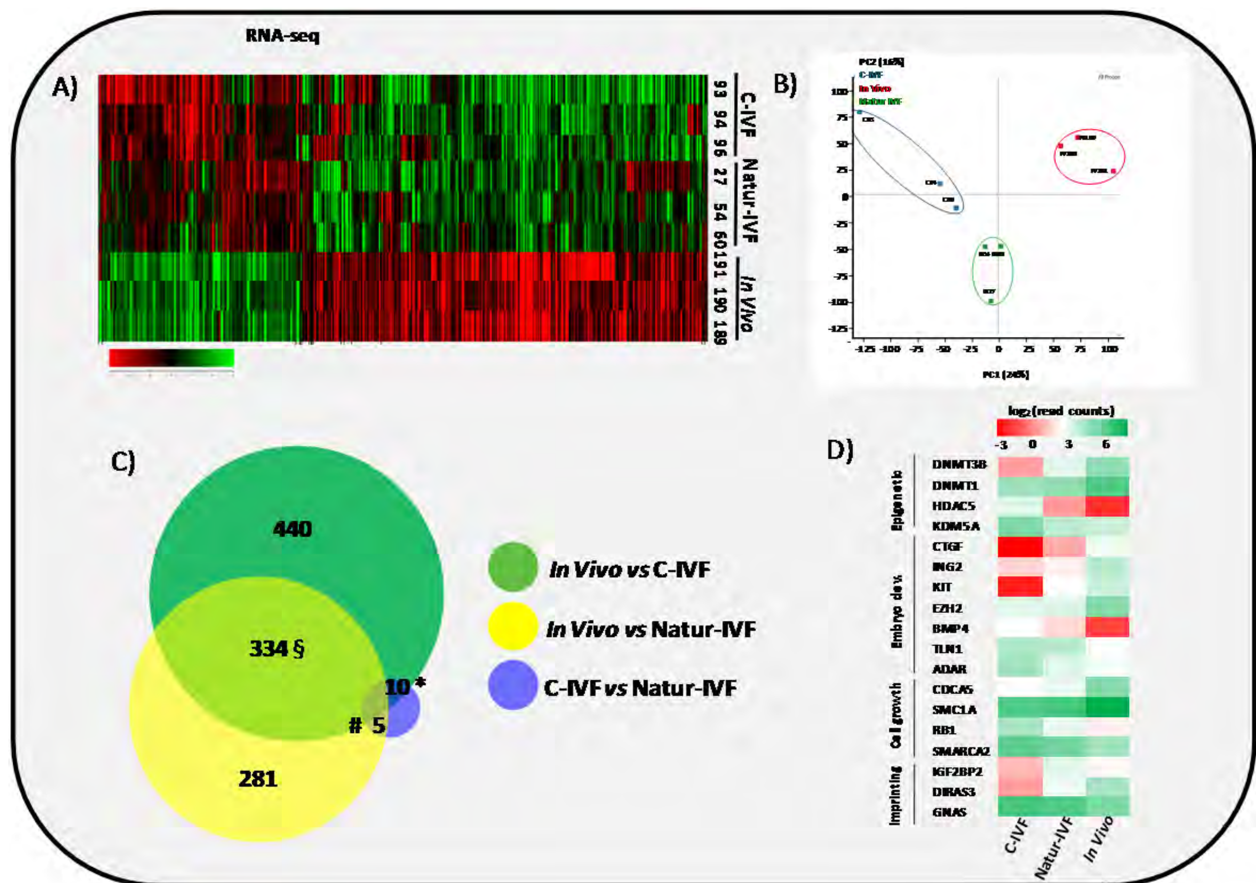


Figure 2. Schematic representation of the different steps of the new IVF/EC system. Swim-up-BSA or Swim-up-Fluid protocols were used for IVF. Previously, oocytes were preincubated in OF-LF for 30 min. Then, each group of putative zygotes were incubated in different media (0-8 h, 8-48h and 48h-7days) as indicated in the diagram. O*: ovary with hemorrhagic corpus luteum; O**: early corpus luteum; OF-LF: oviductal fluid-late follicular phase of the estrous cycle; OF-EL: oviductal fluid-early luteal phase of the estrous cycle; UF-EL: uterine fluid-early luteal phase of the estrous cycle. Swim-up-BSA: NaturARTs® PIG medium + BSA; Swim-up-Fluid: NaturARTs® PIG medium + POF-LF. TALP: culture medium used for IVF. NCSU23: culture medium used for embryo development *in vitro* supplemented with sodium lactate, pyruvate and

non-essential amino acids (NCSU23a) or with glucose and essential and non-essential amino acids (NCSU23b).

The blastocyst transcriptome can be modulated in vitro by reproductive fluids

In vitro culture systems significantly alter embryonic gene expression as previously observed in pooled pig blastocysts [26]. Here, the transcriptomes from 3 individual day 7.5 blastocysts produced by C-IVF or Natur-IVF were compared with their *in vivo* counterparts (Figure 3A-B). RNA libraries showed acceptable quality in all 9 blastocysts. Mean number of raw reads was 14.24±2.23 (±SD) millions, and transcripts from 13,309 to 14,512 different genes (from a total of 20,789 annotated pig mRNAs) were detected in each individual. Principal Component Analysis (PCA) showed that, despite expected individual variability, the 3 embryos from each group clustered together (Figure 3B), with the C-IVF embryos showing higher variability, which could represent high embryo plasticity in response to suboptimal culture conditions. Therefore, after combining the triplicates, data from both *in vitro* groups showed high correlation (Pearson correlation coefficient [r] = 0.964), but Natur-IVF was closer to the *In-vivo* group ([r] = 0.95) than C-IVF ([r] = 0.938). RNA-Seq data analysis (DESeq2 P<0.05 after multiple testing correction) identified 787 differentially expressed genes (DEG) between the C-IVF and *In-vivo*, and 621 DEGs between Natur-IVF and *In-vivo* (Source data 1, including also all the expression values for all the genes). Of the genes that were significantly different (adjusted P-value <0.05, Fold Change>1.5) in the pair-wise comparisons, there was a higher number of up-regulated (534/787 -68%- in C-IVF embryos and 431/621 -69%- in Natur-IVF) than down-regulated (253 and 190, respectively) (Figure 3C, source data 1).



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196 **Figure 3.** Gene expressed analysis in blastocysts obtained *in vivo*, by the Natur-IVF system or by
197 C-IVF system. **A)** Heatmap of global gene expression (with log2 fold change >1.5 and adjusted
198 B-H P-value< 0.05). Numbers denote ID of a specific embryo. **B)** Principal Component Analysis
199 (PCA) of the RNA-Seq samples: *In-vivo* embryos (IV, red), Natur-IVF (N, green) and C-IVF (C,
200 blue). Numbers denote ID of specific embryos. **C)** Venn diagram with DEGs (Source data 1). ***,**
201 **#, §** denotes DEGs exclusive for C-IVF, Natur-IVF and *In-vivo*, respectively (Source data 2). **D)**
202 Heat map of gene expression of key genes associated with embryo development/differentiation,
203 epigenetic reprogramming, cell cycle/cell growth, gene expression and imprinting.

204

205 Top Canonical Pathways, Physiological Systems and Molecular and Cellular Functions related to
206 DEGs were identified (summarized in Supplementary file 1) using the Ingenuity Pathway
207 Analysis (IPA) software. Globally, down-regulated genes in C-IVF and in Natur-IVF were
208 linked to similar Top-cellular functions (Supplementary file1). Equally, top Canonical Pathways
209 affected by up-regulated genes were similar for both groups. In contrast, two pathways were

210 identified in down-regulated DEGs in C-IVF embryos, but not in Natur-IVF DEGs
211 (Supplementary file1). Increased pathways in Natur-IVF and C-IVF included cholesterol,
212 mevalonate, serine and glycine biosynthesis and p53 signaling. Decreased pathways (protein
213 ubiquitination and 14-3-3 mediated signaling) were detected only in C-IVF. Similarly,
214 Physiological Systems and Functions over-represented by up-regulated or down-regulated DEGs
215 were different between C-IVF or Natur-IVF. These results show that, in spite of similarity, there
216 were differences that could influence specific pathways and affect key molecular and cellular
217 functions in the embryos from each group.

218

219 *Natur-IVF blastocysts show fewer aberrantly expressed genes than C-IVF blastocysts*

220 Natur-IVF and C-IVF blastocysts shared 334 genes that were aberrantly expressed in both
221 groups *vs. In-vivo* (Exclusive DEGs, Figure 3C- source data 2). However, there were 440 genes
222 (from the 784 DEGs in C-IVF) that showed aberrant expression only in C-IVF *vs. In-vivo* (DEGs
223 only in C-IVF, Figure 3C), while 40% fewer genes (n=281 from the 620 DEGs in Natur-IVF)
224 showed aberrant expression only in the Natur-IVF group *vs. In-vivo* (DEGs only in Natur-IVF,
225 Figure 3C). Importantly, several genes related to epigenetic reprogramming (down: *DNMT3B*,
226 *DNMT1*; up: *HDAC5, KDM5A*), embryo development (down: *CTGF*, *ING2*, *KIT*, *EZH2*; up:
227 *BMP4*, *TLN1*, *ADAR*), cell growth (down: *CDCA5*, *SMC1A*; up: *RB1*, *SMARCA2*), or imprinting
228 (up: *IGF2BP2, GNAS*; down: *DIRAS3*) were amongst the C-IVF-specific DEGs (Figure 3D).

229

230 Direct comparison between Natur-IVF *vs. In-vivo* and C-IVF *vs. In-vivo* DEGs revealed that only
231 29 genes reached significant expression differences between the two *in vitro* groups after
232 DESeq2 analysis (Source data 1). Interestingly, of these 29 DEGs, 13 were similarly expressed

233 in Natur-IVF and *In-vivo,* and only 7 showed similar expression between C-IVF and *In-vivo*
234 groups (Figure 3C, Source data 2). Although the number of these genes was low, they could be
235 critical because among the 13 genes exclusively different in the C-IVF blastocysts (Source data
236 2), those down-regulated (n= 6) were *KIT*, *MPPA6*, *MTA3*, *KIF4A*, *UBR2* and *ISOC1* (Log Fold
237 Change from -5.9 to -54.18). For all six genes data were available for the corresponding knock-
238 out mice or knock-down studies, which showed phenotypes of altered/abnormal growth/size,
239 reproduction/fertility, mortality/aging, hematopoietic system, homeostasis/metabolism and other
240 abnormalities (Supplementary file 2).

241 These data suggest that *in vitro* culture significantly alters embryonic gene expression to a lesser
242 extent than previously proposed [26], and a better modulation of the blastocyst transcriptome
243 was achieved by mimicking physiological conditions of fertilization and early embryo
244 development by the addition of reproductive fluids (Natur-IVF).

245

246 *Genome-wide DNA methylation of the pig blastocyst is affected by the in vitro culture system*

247 In this study, for the first time, whole-genome DNA methylation profiles on individual porcine
248 blastocysts were generated by a low-cell adaptation of the post-bisulphite adaptor-tagging
249 (PBAT) method [27, 28]. Three blastocysts from each group were analysed. The number of
250 unique alignments in the samples ranged from 13,150,508 to 42,208,651 and the coverage of
251 CpGs (≥1 read) from 52% to 59.2%. The global methylation percentages of CpGs were
252 15.02±3.3, 11.09±2.6 and 12.33±3.6 for the C-IVF, Natur-IVF and *In-vivo* groups, respectively.
253 The distribution of methylation levels in windows of 150 CpGs across the genome and a general
254 view of the methylation profiles of the 9 individual blastocysts are shown in Figure 4A-B. The
255 generally low level of methylation suggests that the genome has experienced substantial loss of

256 methylation from the gametes, analogous to that observed in other mammals [29, 30]. The
257 landscape of methylated cytosines suggests some structure across the genome, with regions with
258 more methylation consistent between the individual blastocysts (Figure 4B). What contributes to
259 this structure, e.g., the regions of relatively higher methylation, is not immediately obvious, as
260 methylation was similar in different genomic contexts with no marked enrichment in repetitive
261 elements, for example (Table 3). Regarding the different classes of blastocysts, methylation over
262 specific genomic features followed the same tendency as the global differences, with higher
263 values for C-IVF (Table 3).

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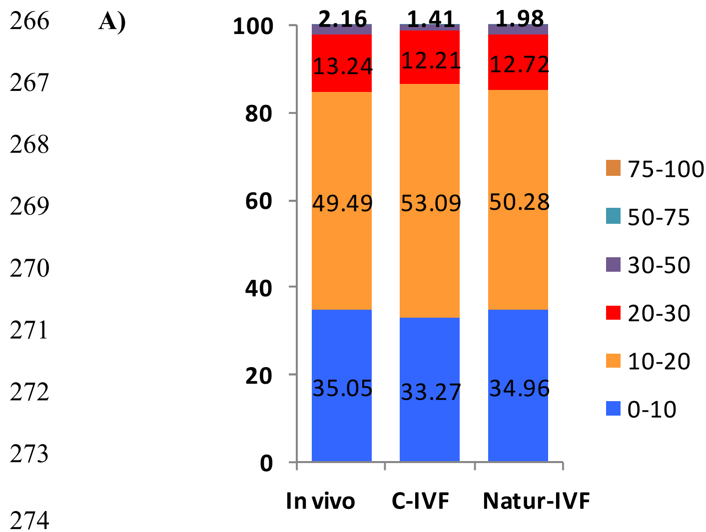
266 A)



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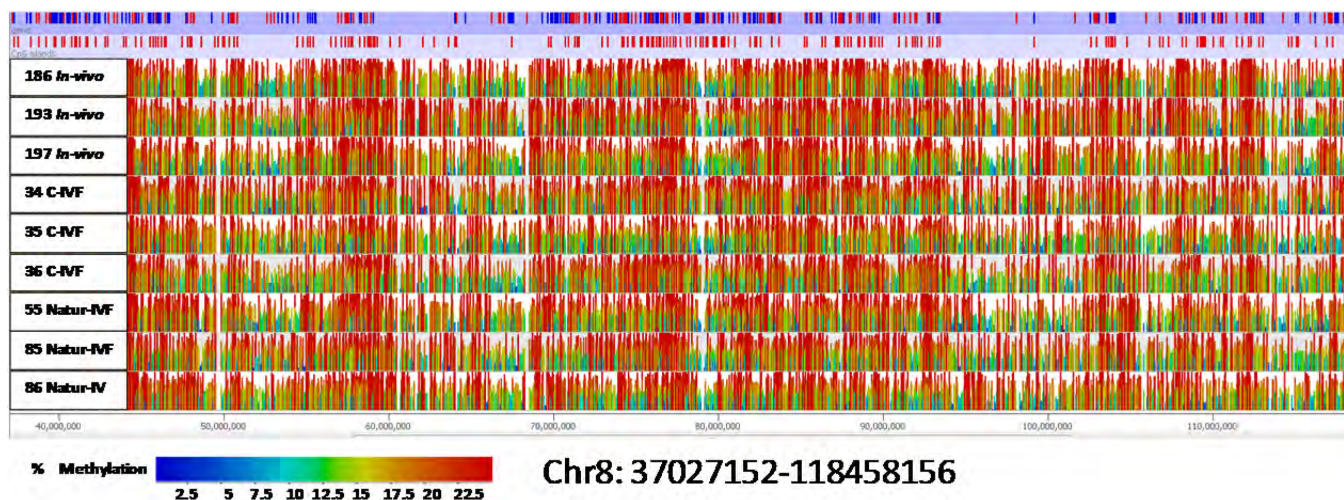
275 B)



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277 **Figure 4. A)** Distribution of methylation percentages across tiles of 150 CpGs on the pig genome
278 for three groups of blastocysts (*In-vivo*, C-IVF and Natur-IVF). **B)** Random browser shot as
279 example of methylation landscape of the 9 individual blastocysts analysed (Chr8:37027152-
280 118458156). The two first rows in the picture represent the genes and CpG islands annotated
281 (Ensembl, RRID:SCR_006773 *Sus scrofa* 10.2) in the pig genome, respectively. Colour scale
282 represents methylation levels from red (highest methylation, up to 25%) to blue (lowest
283 methylation-0%).

284
285

286 PCA revealed a good level of clustering for *In-vivo* and Natur-IVF embryos but not for C-IVF
287 embryos (Figure 5A). In particular, embryos C34 and C36 were far from the other 7 embryos
288 analyzed.

289 The low level of global methylation suggested that few differentially methylated regions (DMRs)
290 could be found. For this reason, and to obtain an unbiased measure of differences in genome
291 methylation, a fixed size of 150 CpGs was used for analysis, as this was found to give a modal
292 tile size of around 3kb with about 150 reads per tile for most individuals. To make the data
293 comparable to enable the detection of DMRs, separately from the global changes, the tiles
294 informative in all samples (258,885) were extracted and quantile normalised. To identify DMRs,
295 the comparison was filtered to require a consistent ≥5% absolute methylation change between all
296 replicates of the first and second condition, followed by a T-test (B-H adjusted $P<0.05$).
297 Differences between the groups were observed with fewer than 4,000 DMRs for each pair-wise
298 comparison (Source data 3). Globally, fewer DMRs showed higher methylation in *In-vivo vs.*
299 Natur-IVF (n=1,660) than in *In- vivo vs.* C-IVF (n=2,244) (Figure 5B).

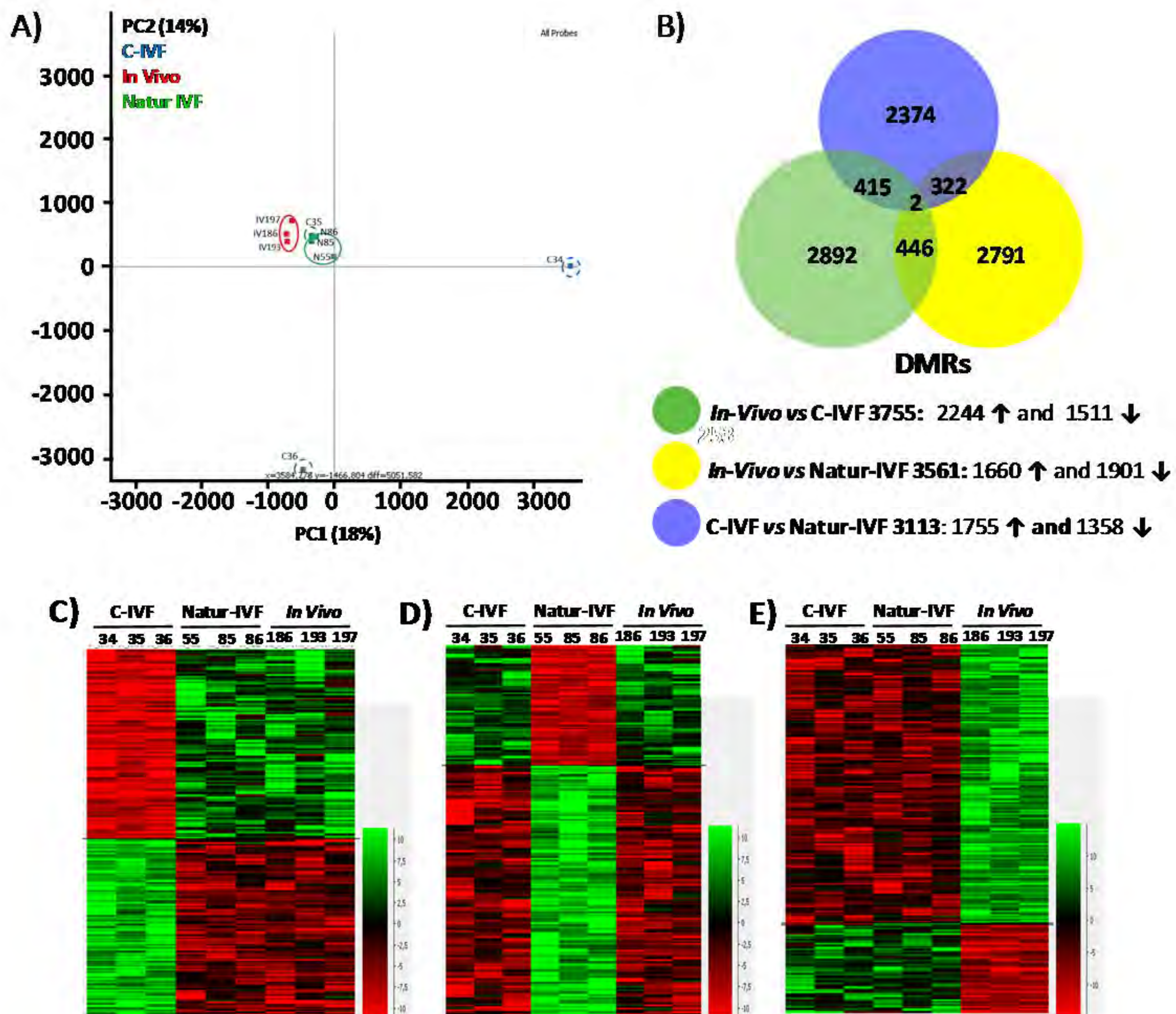

Figure 5. DNA-Methylation analysis in blastocysts obtained *in vivo*, by the Natur-IVF system or by C-IVF system. **A)** Principal Component Analysis (PCA) of the DNA methylation samples: *In-vivo* embryos (red), Natur-IVF (green) and C-IVF (blue). Numbers denote ID of specific embryo. **B)** Venn diagram of DMRs by pair-wise comparison (adjusted-P<0.05). Number of DMRs with higher (↑) or lower (↓) methylation in each pair-wise comparison are indicated (Source data 3). **C)** Heatmap of the 417 DMRs between the C-IVF group and the other two groups (In-vivo and Natur-IVF). **D)** Heatmap of the 324 DMRs between Natur-IVF group and the other two groups (In-vivo and C-IVF). **E)** Heatmap of the 448 DMRs between the In-vivo group and the other two groups (Natur-IVF and C-IVF). For C, D and E (Source data 4): Relative methylation measure as the difference in percent of methylation from the median methylation across all samples.

To better characterize the changes in methylation exclusively affecting one of the groups (P<0.05 for both comparisons), the corresponding subsets of DMRs ("exclusive" DMRs for each group)

were obtained by combining the previous lists (Figure 5B, C, D and E; Source data 4), and the enrichment in specific features in those DMRs was evaluated (Supplementary file 3). For the three subsets of DMRs there was a lower proportion of promoters compared to the global average ($P<0.001$). A lower proportion of LINE1s ($P< 0.05$) was also found for the C-IVF group, while the Natur-IVF blastocyst group showed a higher proportion of DMRs in transcription units (defined over the annotated genes from 500 bp downstream of the annotated TSS, $P<0.05$). Both C-IVF and Natur-IVF DMRs were less enriched in intergenic regions ($P<0.001$) and at LTRs ($P< 0.05$) than *In-vivo* blastocysts. These departures from the methylation state might reflect global differences in the DNA methylation and/or demethylation capacity of the different groups at a developmental time when DNA methylation is rather dynamic.

Exclusive DMRs for each group were linked to Canonical Pathways ($P<0.01$) and Diseases and Bio Functions (adjusted P-value<0.05; Figure 6) by IPA software. Representative genes for specific DMRs in each group are listed in Supplementary file 4. A DMR overlapping *IGF2R,* a gene directly related with the LOS in ruminants and mouse, was found in the subset of exclusive C-IVF DMRs (Source data 4). The methylation percentages for this region (Chr1: 9,199,522-9,201,143) were 12.45%, 28.3% and 35.5% for C-IVF, Natur-IVF and *In-vivo*, respectively (Figure 7A). In addition, a CpG island (oe=0.89, Chr1:9,200,658-9,202,276) that overlapped the DMR showed significant differences in methylation ($P<0.05$): 14.1%, 27.8% and 29.4% for C-IVF, Natur-IVF and *In-vivo* groups, respectively (Figure 7B), although we should be cautious about their significance since the CpG island distribution in the pig genome is very different to the human or mouse genome.

338 Top Diseases and Bio Functions linked by IPA to DMRs exclusive for each group with low or
339 high methylation are represented in Figure 6. Top Molecular and Cellular Functions and
340 representative genes related to DMRs with higher or lower methylation in each group (C-IVF,
341 Natur-IVF and *In-vivo*) are listed in Supplementary File 4.
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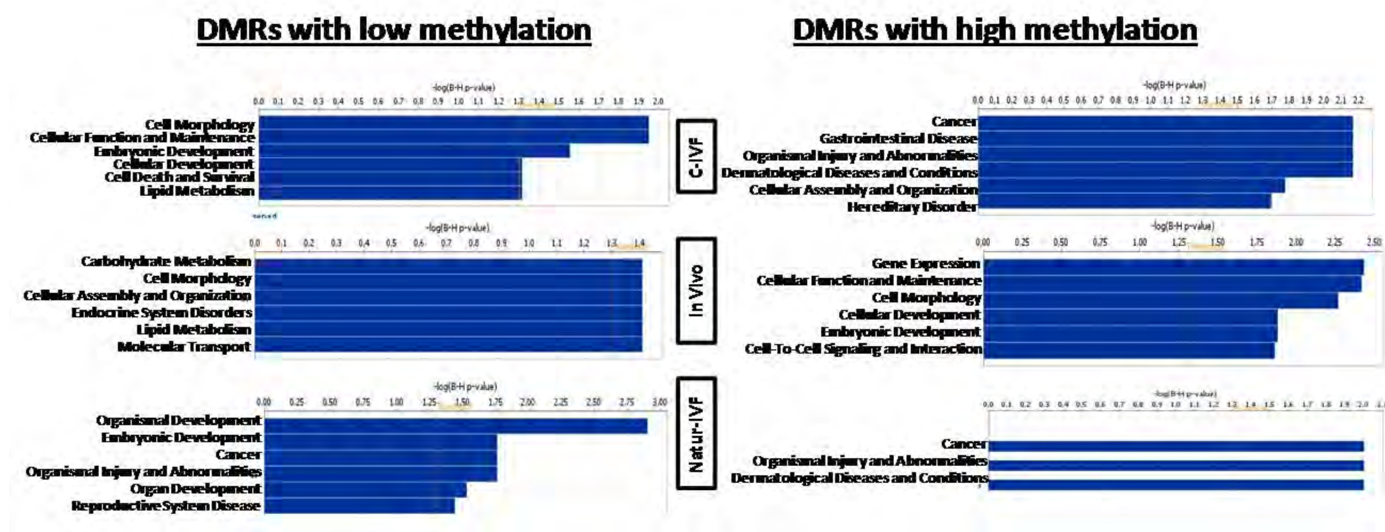

344
345 **Figure 6.** Top Diseases and Bio Functions linked by Ingenuity Pathways Analysis to DMRs
346 exclusive for each group with low or high methylation.
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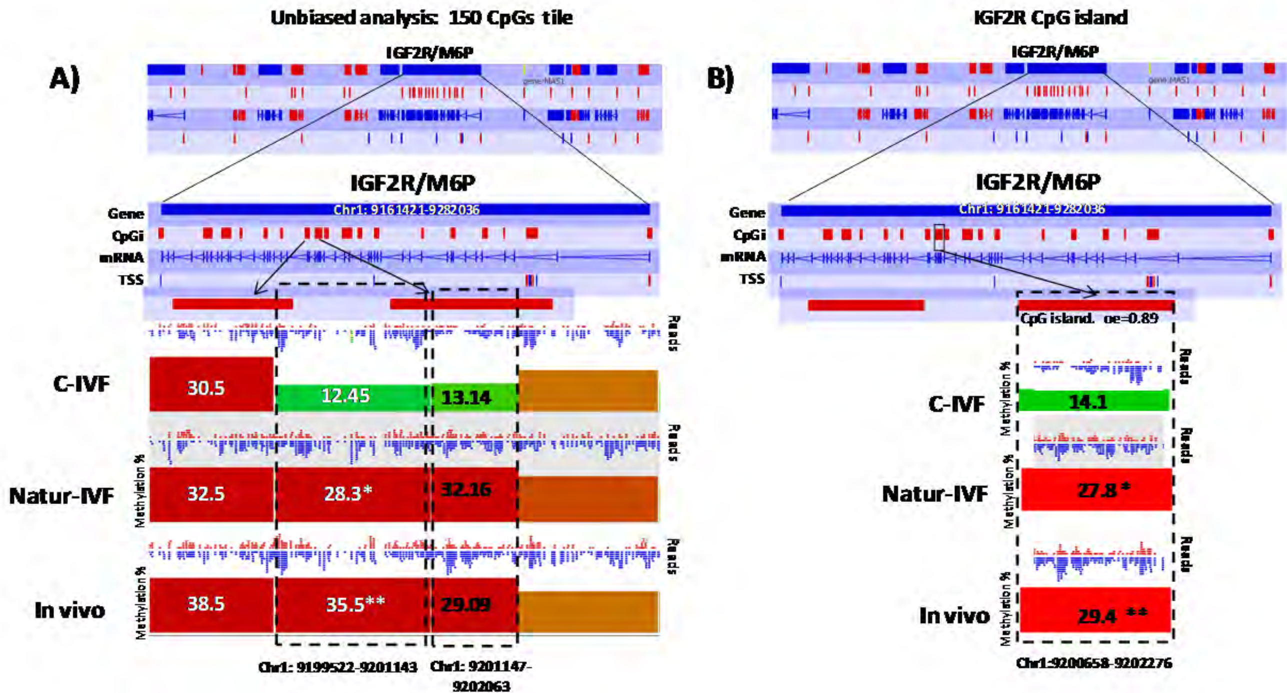
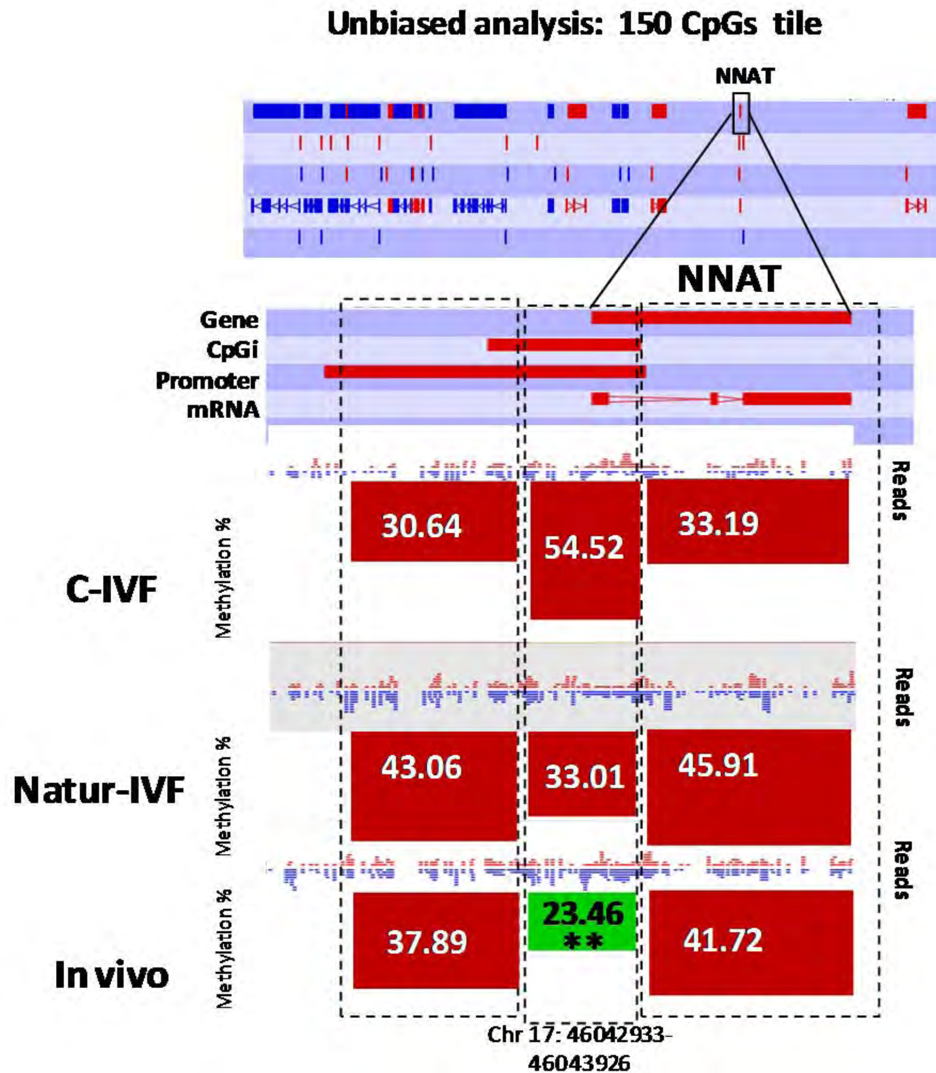


349 **Figure 7. A)** Methylation quantitation at *IGF2R* from the **unbiased analysis** of genome
350 methylation in SeqMonk with a fixed size of 150 CpG windows. Mean percentages of
351 methylation are shown by the bars for each group. Blue (unmethylated) and red (methylated)
352 dots represent methylation reads. Asterisks indicate that methylation at the indicated region
353 showed significantly different values (P<0.05) in Natur-IVF (*) and *In-vivo* (**) *vs*. C-IVF. TSS:
354 transcription starting site. **B)** Detailed view and methylation quantitation of the CpGi at the
355 identified IGF2R DMR. Red rectangles represent, as indicated, CpG islands of the genes. Black
356 boxes indicate the position of the targeted features, whose mean percentages of methylation are
357 shown by the bars for each group. Blue (unmethylated) and red (methylated) dots represent
358 methylation reads.
359

360 *Three imprinted genes were differentially methylated in C-IVF, but not in Natur-IVF blastocysts,*
361 *compared to in vivo blastocysts*

362 Following the finding of a DMR at *IGF2R*, targeted analysis of candidate imprinted genes was
363 done, as the differentially methylated regions of imprinted gene (igDMRs) are expected to
364 maintain constant methylation in preimplantation embryos to ensure faithful imprinted
365 expression of the associated genes throughout development. Therefore they represent sites of
366 methylation in preimplantation of clear biological significance. To identify putative igDMRs in

367 the pig genome, all mouse igDMRs were lifted-over onto the pig genome. Where this was not
368 possible, a gene-by-gene approach was taken to find the best possible fit for a candidate igDMR
369 based on the known organisation of the corresponding mouse imprinted gene. All the genomic
370 regions were then inspected manually to confirm that the correct regions had been found (Table
371 4A). It is not possible to conclude that all regions were actually igDMRs (as this would require
372 methylation information from oocyte and sperm) and, indeed, the methylation values indicated
373 that for some of the genes there was no conserved DMR (i.e., methylation in blastocysts was far
374 below the theoretical 50%) and the associated locus was unlikely to be imprinted. This would
375 seem to be the case, for example, for the genes *IMPACT*, *ZFP787* and *ZFP777*. For some, there
376 was difficulty in finding possible homologous igDMRs, probably because of gaps in the porcine
377 genome assembly (such as *SNRPN*, *KCNQ1* and *GRB10*), and there were a number of others that
378 were excluded because the homologous pig region had no suggestion of a CpG island in the
379 region equivalent to the igDMR in mouse (e.g., *U2AF1-RS1, MCTS2/H13*). Comparison of
380 methylation in the three groups of blastocysts for the resulting 14 candidate igDMRs (with
381 sufficient read coverage) revealed differences for *ZAC1* and *PEG10*, which were more
382 methylated (P<0.05) in the C-IVF than in *In-vivo* group, and *PEG10* and *NNAT*, which were
383 more methylated (P<0.05) in the C-IVF than in Natur-IVF and *In-vivo* groups (Table 4B). No
384 statistical differences were found between Natur-IVF and *In-vivo* groups. Of these three
385 igDMRs, the one at *NNAT* coincides with the promoter CpG island [31] and, in addition, one 150
386 CpG tile overlapping NNAT had methylation higher than 50% in C-IVF in the unbiased analysis
387 (Figure 8).
388



389

390 **Figure 8.** Methylation quantitation at *NNAT* from the **unbiased analysis** of genome methylation
391 in SeqMonk with a fixed size of 150 CpG windows. Black boxes indicate the position of the
392 selected 150 CpG windows, whose mean percentages of methylation are shown by the bars for
393 each group. Blue (unmethylated) and red (methylated) dots represent methylation reads.
394 Asterisks indicate that methylation at the indicated region (black box) showed significantly
395 different values ($P<0.05$) in Natur-IVF (*) and *In-vivo* (**) *vs*. C-IVF.

396

397 **Discussion**

398 The milieu in which fertilization and embryo development takes place is crucial for healthy
399 foetal and offspring growth, as revealed by developmental and epigenetic alterations as a
400 consequence of *in vitro* culture and ARTs [12, 32-34]. However, the progress made by ART
401 during the past two decades make a future without their use inconceivable, thus it is necessary i)
402 to characterize the real epigenetic cost of ART, separated from other factors and ii) to develop
403 new protocols to safeguard against possible negative impacts in offspring. Our study evaluated,
404 by single blastocyst profiling, the genetic and epigenetic impacts of modified protocols to
405 produce embryos *in vitro* that mimic, as far as possible, the physiological conditions of
406 fertilization and early embryo development. This imitation of the natural environment was first
407 approached in both gametes separately: in the male gamete, by using, sperm selection procedures
408 that avoided centrifugations, and sperm washing and processing media containing oviductal fluid
409 from the pre-ovulatory phase of the cycle; and, in the female gamete, by preincubating the
410 oocytes within the precise fluid they encounter when, after ovulation, they are transported
411 through the ampulla of the oviduct to the fertilization site, at the ampullar-isthmic junction [35].
412 Secondly, two experimental groups were established for a comparison with the *in vivo*
413 specimens, where either BSA or reproductive fluids (obtained sequentially at the corresponding
414 phases of the cycle) were added at every step of the IVF and EC procedures.

415 The results showed that reproductive fluids improve the outcome of IVF and the quality of pig
416 blastocysts produced *in vitro*. The approach used, with spermatozoa coming from boars selected
417 by their excellent reproductive performance, avoids the possibility of aberrations due to a
418 paternal factor, which cannot be avoided in the human model, and helps to elucidate the
419 epigenetic cost of ART independently of any paternal pathology. The figure of >40%

420 progression of the cleaved embryos to blastocysts *in vitro* means an improvement over the best
421 previous results [36]. Nonetheless, the most remarkable findings were that Natur-IVF blastocysts
422 attained a more advanced developmental stage and that the mean number of cells per blastocyst
423 was the same as *In-vivo* embryos and 61% higher than C-IVF ones, which it is also above some
424 of the best data previously reported in pigs [36]. These results indicate that the use of
425 reproductive fluids as additives, even at the low dose used in this study (1%) is beneficial for *in*
426 *vitro* development of pig embryos so that it is now possible to obtain similar or even higher
427 yields in the pig (45%) than in the bovine species. Although the possibility of transferring these
428 methods to the human clinic might seem far off, the fact that nowadays other natural fluids such
429 as breast milk for baby feeding or blood serum for transfusions are collected and stored at
430 biobanks, make it possible to predict the future availability of human reproductive fluids
431 obtained from oocyte donors during interventions at human infertility clinics [37]. In fact, the
432 first samples of these fluids are already stored at Biobanc-Mur in Spain (National Register of
433 Biobanks Nº B.0000859).

434 Our study also showed that Natur-ART blastocysts are closer to the gene expression profile of
435 the *In-vivo* blastocysts than C-IVF blastocysts. Amongst the most striking differences found was
436 the expression of genes related to epigenetic reprogramming. It has been shown in mice and
437 human that during the transition from zygote to blastocyst there is a massive loss of DNA
438 methylation, with the exception of imprinted genes and some repetitive elements [29, 38]. In
439 agreement with this observation, the global methylation level in the three groups of pig
440 blastocysts analyzed was below 15%, suggesting that they had largely undergone a
441 reprogramming event. This globally low methylation level compared to somatic cells or gametes,
442 made it difficult to find high quantitative differences between embryos. Despite this, methylation

443 percentage was higher in C-IVF embryos than in the other two groups, in agreement with
444 previous studies indicating that ART-derived blastocysts displayed higher levels of methylation
445 than *in vivo* derived ones [39]. This difference appeared to be global, with all features affected
446 and, no evidence of multiple sub-groups over different genomic regions; therefore, there was no
447 indication of specific regions resisting reprogramming. At the same time, genes for DNMT1 and
448 the binding protein of its crucial cofactor UHRF1, which are considered responsible for
449 maintenance of methylation patterns in replicating DNA and for maintaining imprints during
450 preimplantation embryonic stages, were less expressed in C-IVF blastocysts, as was *DNMT3B*,
451 required for *de novo* remethylation from this stage onwards. Differences in cell numbers, as a
452 result of a probable additional round of cell division in *In-vivo* and Natur-IVF embryos compared
453 to C-IVF, is unlikely to explain a shift from ~11-12% to ~15% global methylation. All together,
454 these data suggest an impaired demethylation in the C-IVF group. Analysis of hemimethylated
455 CpG dyads by deep hairpin bisulfite sequencing, as recently reported in mouse [40], could help
456 to clarify this issue.

457 A second key finding in this study was that the methylation levels in the samples analyzed
458 showed much lower overall methylation levels (mean across all samples was 13.1%) than would
459 be expected from somatic tissues. Furthermore, there were differences in the global mean
460 methylation levels between different samples, ranging from 8.9% to 18.5%. Taken together these
461 observations suggest that the samples were collected during a time of global methylation
462 reprogramming. The variability in global methylation levels would have confounded a direct
463 comparison focussing on locus specific methylation differences, so to account for this a quantile
464 normalisation was required to allow for a direct quantitative comparison of methylation levels.

465 Given that these samples are undergoing active reprogramming it is also not unreasonable to
466 think that some previously reported DMRs may not be established yet, or that the strength of the
467 DMRs would be reduced. Despite this, we were able to find candidate DMRs between the groups
468 with a reasonable statistical significance, although the magnitude of the methylation differences
469 was low. Considering that previous studies have shown extremely close correlations between
470 qPCR and RNA-seq data [41-43] and that validation by qPCR has its own probe-bias based on
471 what region of the cDNA is amplified, we deem, in contrast to microarrays data, that there is not
472 solid evidence that validation of the RNA-Seq and DNA methylation results by qPCR will
473 provide extra significance to our results. For this reason, we did not perform qPCR validation in
474 this study.

475 Another key observation in this study was that the *in vitro* culture affects imprinted gene
476 expression and methylation. Plasticity of the preimplantation embryo could enable a recovery of
477 alterations in methylation and further expression of non-imprinted genes during development,
478 but any erosion of methylation marks at imprinted genes are unlikely to be corrected. In our data,
479 from the 10 candidate imprinted regions retaining more than 30% of methylation in the pig
480 blastocysts, we found three in C-IVF (*ZAC1*, *PEG10* and *NNAT*) with significantly different
481 methylation compared to *In-vivo* blastocysts, and two (*PEG10* and *NNAT*) compared to Natur-
482 IVF. Knockout mice lacking *PEG10* showed early embryonic lethality with placental defects,
483 indicating the importance of this gene in embryonic development [44]. The protein encoded by
484 *NNAT*, on the other hand, may be involved in the regulation of ion channels during brain
485 development and may also play a role in forming and maintaining the structure of the nervous
486 system. Defects in methylation at *ZAC1* and *IGF2R* have been found in patients with the
487 imprinted disorders transient neonatal diabetes mellitus (TNDM) or Silver-Russell syndrome

488 (SRS), respectively, including those born following the use of ART [45]. In addition, genes
489 related to the IGF axis, *IGF2BP2* and *IGF2BP2-IMP2*, were up-regulated in C-IVF, and *IGF2R*
490 in both C-IVF and Natur-IVF embryos. Altered *IGF2BP2* expression in C-IVF is of interest,
491 since reduced abundance of IGF2 has been associated with lower foetal weight after *in vitro*
492 culture [5]. The imprinting status of *IGF2R* in the pig is unclear [46, 47] but, independently of
493 this uncertainty, our data indicated higher expression of this gene in the two *in vitro* groups of
494 blastocysts, which would be in agreement with previous reports in other species and could
495 indicate a possibility of LOS related alterations observed in abnormal *in vitro* and cloned
496 embryos [16]. At the same time, the reduced methylation in *IGF2R* specifically in the C-IVF
497 group could suggest that this group is more likely to be susceptible to sustained deregulation of
498 *IGF2R* expression and a greater probability of LOS-like syndromes.

499 Altered expression in both groups of blastocysts produced under *in vitro* conditions was
500 observed in some genes related to embryonic development, but some aberrations were absent in
501 Natur-IVF embryos. In human blastocysts it has been observed that those with higher
502 implantation rate and higher number of cells per embryo showed up-regulation of *DNMT3A* [48].
503 In our data, the *In-vivo* and Natur-IVF blastocysts showed a higher number of cells than those
504 from the C-IVF group, in which expression of *DNMT3A* was decreased. We also observed higher
505 expression of *CDKN1A* in the two *in vitro* groups, with an intermediate value in Natur-IVF.
506 CDKN1A inhibits embryonic cell proliferation in response to DNA damage and it is considered
507 one of the key genes responsible for the abnormalities in ART embryos since an aberrant
508 increase of CDKN1A expression might be related to the growth-defect phenotype [49].
509 Methylation of the *CDKN1A* gene, however, was similar in all three groups, between 5-7%.
510 Other genes involved in DNA repair and cell cycle regulation were found to be altered, such as

511 *MDM2* (in C-IVF) and *TP53INP* (up-regulated in Natur-IVF and C-IVF) and *HSPA4L,*
512 *HSP40B1, HSPH1, HSP90* (down-regulated only in C-IVF). Altered expression of these genes
513 may limit the ability of the embryo to respond to DNA damage, such that *in vitro* culture may
514 lead to dysregulation of such genes thus affecting long-term embryo viability [50]. The same
515 situation was found for *SLC2A3* (Glut-3) and *SLC2A2*, which have been related to LOS [51] and
516 were highly up-regulated in the two *in vitro* groups. Again, no differences at the methylation
517 level were found for any of these genes. Although DNA methylation at the promoter/gene bodies
518 is directly/indirectly correlated with gene expression, this is not strictly true during the periods of
519 dramatic loss of DNA methylation, as occurs during early embryo development or primordial
520 germ cells (PGC) formation. For example, Gounktela *et al*. [52] showed a general uncoupling
521 between DNA methylation and gene expression during demethylation of PGCs, commenting
522 "Our data reveal a remarkable and pervasive loss of DNA methylation in human PGCs and
523 AGCs during prenatal life that has almost no relationship to changes in gene expression".
524 Comparative analyses between our methylation and gene expression data also showed this lack
525 of correlation. In our opinion, at this stage of development and with this low level of
526 methylation, this was an expected result.

527 Finally, the exclusive alteration in C-IVF of genes such as *KIT*, whose knock-out in mouse
528 results in multiple alterations including embryonic lethality [53], or others genes such as *UBR2,*
529 whose deletion results in female embryonic lethality and growth arrest [54], or *ISOC1*, whose
530 mutation produces phenotypes with body weight loss [55], support the hypothesis that offspring
531 produced with Natur-IVF conditions would be healthier than those produced with C-IVF,
532 although additional studies are necessary to confirm this finding.

533 In conclusion, we report here the first time genome-wide DNA methylation and transcription
534 analysis in single blastocysts (*in vivo* and *in vitro*) of a mammalian species and propose a new
535 strategy for prevention of aberrant epigenetic and gene expression profiles induced by ART. This
536 strategy, based on the addition of reproductive fluids in the culture media used during the ART
537 procedures, can be applied in other animals as well as in humans, after safety concerns of
538 transmission of diseases have been properly addressed. The design of new culture media
539 containing all the proteins that are naturally present in the original biological fluid, represents not
540 only a technical challenge but a biomedical responsibility that must be addressed to prevent
541 future pathologies both in animals and humans. In addition, we offer a new protocol for the *in*
542 *vitro* production of pig embryos with a significant improvement over the previous data
543 published. Our study represents a new form of thinking in the field, far from the chemically
544 defined culture media, and could help to face one of the biggest milestones of the current
545 reproductive medicine: safer ART.
546

547 **Materials and Methods**

548 Culture media

549 Unless otherwise indicated, all chemicals and reagents were purchased from Sigma-Aldrich
550 Quimica S.A. (Madrid, Spain).

551 Collection and processing of follicular, oviductal and uterine fluids

552 Fluids were obtained from animals raised at a commercial farm (CEFU, S.A., Murcia, Spain) and
553 slaughtered in an abattoir belonging to a food industry (El Pozo, S.A) near the University of
554 Murcia. For the collection of follicular fluid, ovaries from 6 months old Large White animals

555 weighing 100-110 kg were transported to the laboratory in saline containing 100 μg/ml
556 kanamycin sulfate, washed once in 0.04% cetrimide solution (alkyltrimethylammoniumbromide)
557 and twice in saline within 30 min of slaughter. The content of follicles between 3-6 mm
558 diameter, from at least 50 ovaries (25 females), was quickly aspirated, centrifuged at 1800 *g* for
559 30 min at 4°C and the supernatant filtered through 0.22 μm diameter filter [56]. One ml follicular
560 fluid (FF) aliquots were stored at -80°C until their use as additives for the IVM medium.

561 For the collection of oviductal (OF) and uterine (UF) fluids, genital tracts from cyclic Large
562 White sows (2-4 years old) were obtained at the abattoir and transported to the laboratory on ice
563 within 30 min of slaughter. The cyclic stage of animals was assessed once in the laboratory, on
564 the basis of ovarian morphology on both ovaries from the same female. Oviducts and uteri were
565 classified as early follicular, late follicular, early luteal or late luteal phase [57]. Both oviducts
566 and uteri coming from the same genital tract were classified as in the same stage of the cycle.
567 Once classified, oviducts and uteri were separated and quickly washed once with 0.4% v/v
568 cetrimide solution and twice in saline. Oviducts and uteri were dissected on Petri dishes or trays,
569 respectively, sitting on ice. Once dissected, OF were collected by aspiration with an automatic
570 pipette by introducing a 200 μl pipette tip into the ampulla and manually making an increasing
571 pressure gradient from the isthmus to the ampulla. The UF was collected by making a manual
572 increasing pressure gradient from the proximal end to the distal end (utero-tubal junction) of the
573 uterine horn and letting the fluid drop into a sterile 50 ml Falcon tube. Once recovered, samples
574 (OF and UF) were centrifuged twice at 7000 *g* for 10 min at 4°C to remove cellular debris. Then
575 the supernatant was immediately stored at -80°C until use. Oviducts from animals at the late
576 follicular phase (POF-LF) and at the early luteal phase (POF-EL) gave a mean volume of around
577 50 μl and 40 μl, respectively per oviduct. At the early luteal phase, approximately 10 ml of UF

578 per uterine horn were collected each time. Aliquots of 50 µl OF and 50 ml UF of pooled samples
579 from at least 20 animals for OF and 5 animals for UF were used. Only samples that passed
580 quality controls (pH 7.0-7.6, osmolality 280-320 mOsm/kg, endotoxin <0.10 EU/mL, a
581 minimum 90% of Metaphase II oocytes after IVM with FF and ZP hardening for oocyte
582 preincubation in POF-LF > 1 hour) were used for experiments.

583

584 Oocyte collection and *in vitro* maturation

585 Ovaries from 6 months old animals weighing 100-110 kg were transported to the laboratory in
586 saline containing 100 µg/ml kanamycin sulfate at 38ºC, washed once in 0.04% cetrimide solution
587 and twice in saline within 30 min of slaughter. Cumulus–oocyte complexes (COCs) were
588 collected from antral follicles (3–6 mm diameter), washed twice with Dulbecco's PBS (DPBS)
589 supplemented with 1 mg/ml polyvinyl alcohol (PVA) and 0.005 mg/ml red phenol, and twice
590 more in maturation medium previously equilibrated for a minimum of 3 h at 38.5ºC under 5%
591 CO_2 in air. Maturation medium was NCSU37 supplemented with 0.57 mM cysteine, 1 mM
592 dibutyryl cAMP, 5 mg/ml insulin, 50 µM β-mercaptoethanol, 10 IU/ml equine chorionic
593 gonadotropin (eCG; Foligon; Intervet International BV, Boxmeer, Holland), 10 IU/ml human
594 chorionic gonadotropin (hCG; Veterin Corion; Divasa Farmavic, Barcelona, Spain), and 10%
595 porcine follicular fluid (v/v). Only COCs with complete and dense *cumulus oophorus* were used
596 for the experiments. Groups of 50 COCs were cultured in 500 µl maturation medium for 22 h at
597 38.5ºC under 5% CO_2 in air. After culture, oocytes were washed twice in fresh maturation
598 medium without dibutyryl cAMP, eCG, and hCG and cultured for an additional period of 20–22
599 h.

600

601 *In vitro* fertilization

602 Before IVF, mature oocytes were preincubated in 100% porcine oviductal fluid (POF) from the
603 late follicular (LF) phase (NaturARTs® POF-LF) for 30 minutes [58] and then washed three
604 times in TALP medium**.** TALP medium consisted of 114.06 mM NaCl, 3.2 mM KCl, 8 mM Ca-
605 lactate.5H_2O, 0.5 mM $MgCl_2$.6H_2O, 0.35 mM NaH_2PO_4, 25.07 mM $NaHCO_3$, 1.85 mM Na-
606 lactate, 0.11 mM Na-pyruvate, 5 mM glucose, 2 mM caffeine, 1 mg/ml PVA and 0.17 mM
607 kanamycin sulfate. Either 3 mg/ml BSA-FAF (A-6003) or 1% of NaturARTs® POF-LF was
608 included as additives in the IVF medium for the first 8 h of coincubation (C-IVF and Natur-IVF
609 groups, respectively). Ejaculated spermatozoa from boars of proven fertility (12-24 months old)
610 were transported to the laboratory and 1 ml of semen was lay below 1ml of NaturARTs® PIG
611 sperm swim up medium (http://embryocloud.com) at the bottom of a conical tube. After 20
612 minutes of incubation at 37ºC (with the tube at a 45º angle), 0.75 ml from the top of the tube
613 were aspirated and used for insemination of the IVF dishes (10^5 cells/mL) with the oocytes. For
614 the density gradient group aliquots of the semen samples (0.5 ml) were centrifuged (700 *g*, 30
615 min) through a discontinuous Percoll® (Pharmacia, Uppsala, Sweden) gradient (45 and 90% v/v)
616 and the resultant sperm pellets were diluted in TALP medium and centrifuged again for 10 min
617 at 100*g*. Finally, the pellet was diluted in TALP and 250 µl of this suspension were added to the
618 wells containing the oocytes, giving a final concentration of 10^5 cells/mL.

619 Spermatozoa and oocytes were incubated at 38.5ºC under 5% CO_2 for 8 hours. Later on, the
620 putative zygotes were transferred to embryo culture medium. At this point, a sample of the
621 putative zygotes from each group was collected, fixed and stained as previously described [58] to
622 assess the fertilization rates (percentage of penetrated oocytes, percentage of monospermy, mean

623 number of spermatozoa penetrating each oocyte and mean number of spermatozoa attached to
624 the zona pellucida). Penetration rate was defined as the proportion of oocytes penetrated by one
625 or more spermatozoa.

626

627 *In vitro* culture of putative zygotes

628 Media for embryo culture were NCSU23 supplemented with sodium lactate (5 mM), pyruvate
629 (0.5 mM) and non-essential amino acids (NCSU23-A, for the first 48 h) or NCSU23
630 supplemented with glucose (5.5 mM) and essential and non-essential amino acids (NCSU23-B,
631 48-180 h). At 8 hour post insemination (hpi), putative zygotes were transferred to culture dishes
632 containing NCSU23-A medium and 0.4% BSA in the C-IVF group or 1% POF from the early
633 luteal (EL) phase of the estrous cycle (NaturARTs® POF-EL) in the Natur-IVF group. At 48 hpi,
634 the cleavage was assessed under the stereomicroscope and the 2-4 cell stage embryos were
635 transferred to NCSU23-B with 0.4% BSA (C-IVF group) or 1% of porcine uterine fluid (PUF)
636 from early luteal phase (NaturARTs® PUF-EL, Natur-IVF group). On day 7.5 (180 hpi),
637 blastocyst stage morphology was assessed under the stereomicroscope and later on a sample was
638 fixed and stained [58] and the remaining blastocyst were washed in PBS and frozen in PCR tubes
639 in the minimum volume of medium. The parameters assessed in the stained blastocysts were
640 development stage (2-4 cells, 8-16 cells, morula or blastocyst), mean number of cells per
641 blastocyst, and ability for hatching (rhythmic movements of expansion and contraction before
642 going out of the zona pellucida). The blastocysts frozen for genetic and epigenetic study were
643 passed through liquid nitrogen vapours for 5 seconds and immediately introduced in the freezer
644 at-80ºC until the day of use for RNA extraction or bisulphite treatment.

645 Statistical analysis of IVF data

646 Data are presented as mean ± SEM and all percentages were modeled according to the binomial
647 model of variables and arcsin transformation to achieve normal distribution. The variables in all
648 the experiments were analyzed by one-way or two-way ANOVA. When ANOVAs revealed a
649 significant effect, values were compared by the Tukey test. A *P* value <0.05 was taken to denote
650 statistical significance.

651 Collection of blastocysts *In-vivo*

652 Ten sows 18 month old were weaned 21 days after second parturition and 5 days later showed
653 signs of standing estrous. Animals were inseminated in the collaborative farm and
654 slaughterhoused 7.5 d after. Genital tracts were collected and transported to the laboratory where
655 uterine horns were briefly dissected and washed with PBS within 2 hours from slaughtering.
656 Blastocysts were identified under the stereomicroscope, collected and immediately frozen as
657 described for the *in vitro* produced embryos. A portion of these blastocysts was fixed in
658 glutaraldehyde and stained with Hoechst for cell counting.

659

660 Experimental groups

661 C-IVF group (C-IVF): 6 blastocysts classified as 7A according to Bo and Mapletoft [25] (#34,
662 35, 36, 93, 94 and 96) were produced *in vitro* with BSA as the only protein source. Sperm were
663 processed by swim up in NaturARTs® sperm medium with BSA (Swim-up-BSA). IVF medium
664 consisted of TALP (0-8 h) and embryo culture medium consisted of NCSU23-A (8-48 h) and
665 NCSU23-B (48-180 h). Natur-IVF group: 6 blastocysts classified as 7A (#55, 85, 86, 27, 54 and
666 60) were produced *in vitro* with NaturARTs® POF and PUF as the protein source. Sperm were
667 processed by swim up in NaturARTs® sperm medium with NaturARTs® POF-LF (Swim-up-

668 Fluid). IVF medium consisted of TALP + 1% NaturARTs® POF-LF (0-8h) and embryo culture
669 medium consisted of NCSU23-A + 1% NaturARTs® POF-EL (8-48h) and NCSU23-B + 1%
670 NaturARTs® PUF-LL (48-180 h). For both groups, before IVF oocytes were pre-incubated for
671 30 min in preovulatory oviductal fluid (NaturARTs® POF-LF). *In-vivo* group: 6 blastocysts
672 classified as 7A (#186, 193, 197, 189, 190 and 191) were collected by flushing the uteri of
673 animals within 2 hour of slaughtering. The animals were under natural heat after weaning and
674 insemination was performed 7 days before slaughtering.

675 RNA isolation and RNA-Seq

676 ARCTURUS® PicoPure® RNA Isolation Kit (KIT0204, Life Technologies) was used to extract
677 the RNA from individual blastocysts. RNA-Seq libraries were generated using Ovation RNA-
678 Seq System V2 (NuGEN, Cat. 7102-08) for low amount of starting material and further
679 amplified with NEB Next DNA Library Prep Master Mix for Illumina (NEB, Cat. E6040S). All
680 steps were performed according to manufacture guidelines. iPCRTag reverse primer with
681 individual index was used to generate three independent biological replicates from each
682 condition. 100 bp single end reads were sequenced on Illumina HiSeq 1000. Sequencing data
683 were processed. For RNA-Seq libraries, raw sequence reads were trimmed using Trim Galore to
684 remove adapter contamination and reads with poor quality defined by low PHRED score.
685 Mapping was performed using Tophat software (http://tophat.cbcb.umd.edu/) and data were
686 visualised with Seqmonk (RRID:SCR_001913,
687 http://www.bioinformatics.babraham.ac.uk/projects/seqmonk/). RNA quality was assayed by
688 Bioanalyzer and even though each sample came from a single blastocyst, RIN score was between
689 6.1-8.2.

690 Analysis of RNA-Seq data

691 Annotated pig mRNA features were quantitated with raw read counts in SeqMonk and these
692 were fed into DESeq2 for differential expression analysis using a P-value cut off of 0.05 and not
693 applying independent filtering. Reads were subsequently re-quantitated as log2RPM (reads per
694 million reads of library) and globally normalised to the 75th percentile of the data. Significant
695 effect sizes were selected using the Seqmonk intensity difference filter where the difference in
696 expression in each gene was compared to the set of differences in the 1% of the data with the
697 most similar average expression level as the gene being tested. Only genes with significantly
698 higher changes ($P<0.05$ after Benjamini and Hochberg correction) were kept.

699 Bisulfite sequencing based on post-bisulfite adapter tagging

700 An adaptation of whole genome bisulfite sequencing that involves post-bisulfite adapter tagging
701 (PBAT) [27] was used to analyse the methylome of individual pig blastocysts at single-base
702 resolution on a genome-wide scale. Further modification of the method described in Smallwood
703 *et al.* [59] was used to generate BS-seq libraries. Briefly, an individual blastocyst was lysed for 1
704 hour in 1% SDS with proteinase K and treated with bisulfite reagent using Imprint DNA
705 modification kit (Sigma, MOD50). DNA was eluted in EB buffer and one round of first strand
706 synthesis was performed using a biotinylated oligo 1 (5-
707 [Btn]CTACACGACGCTCTTCCGATCTNNNNNNNNN-3). Samples were further treated with
708 Exonuclease I, washed and eluted in 10 mM Tris-Cl and incubated with washed M-280
709 Streptavidin Dynabeads (Life Technologies) to pull down the biotinilated fraction of DNA.
710 Second strand synthesis was performed using oligo 2 (5'-
711 TGCTGAACCGCTCTTCCGATCTNNNNNNNNN -3') and samples were amplified for 15
712 PCR cycles using indexed iPCRTag reverse primer [60] with KAPA HiFi HotStart DNA

713 Polymerase (KAPA Biosystems) and purified using 0.8× Agencourt Ampure XP beads
714 (Beckman Coulter). Libraries were assessed for quality and quantity using High-Sensitivity DNA
715 chips on the Agilent Bioanalyser, and the KAPA Library Quantification Kit for Illumina (KAPA
716 Biosystems). Three libraries generated from individual blastocysts for each experimental
717 condition were prepared for 100 bp single-end sequencing on Illumina HiSeq 1000 and
718 sequenced at three samples per lane.

719 Analysis of methylation data

720 For the unbiased analysis, tiles were defined in SeqMonk using the read position tile generator
721 tool and selecting 1 read count per position and 150 valid positions per window, in all the 9
722 individual data sets (286,136 tiles). Then, the bisulphite quantitation pipeline was run over
723 existing tiles, 1 minimum count to include position and 20 minimum observations to include
724 feature. To remove the tiles without data, the filter on values for individual tiles was applied,
725 where values had to be between 0 and 100 for exactly 9 of the 9 selected data stores. Then, tiles
726 with data for all the samples were obtained (N=258,885 tiles). Bisulphite quantitation pipeline
727 was run again over the new tiles and data were normalized by the match distribution quantile
728 normalisation tool. Finally, every pair-wise comparison was filtered to require a consistent 5%
729 change between all replicates of the first and second condition, and then replicate sets stats was
730 applied where every comparison had a significance below 0.05 after Benjamini and Hochberg
731 correction. For the targeted analysis of the candidate imprinted regions a Chi-Square test
732 ($P<0.05$) was applied for every comparison.

733

734 **Ethics**: This study was carried out in strict accordance with the recommendations in the Guiding
735 Principles for the Care and Use of Animals (DHEW Publication, NIH, 80–23). The protocol was

736 approved by the Ethical Committee for Experimentation with Animals of the University of

737 Murcia, Spain (Project Code: 192/2015).

738 **Acknowledgments:** The authors thank CEFU, S.A. and El Pozo, S.A. for providing the
739 biological material; Juan Antonio Carvajal and Soledad Rodriguez for collecting the oviducts
740 and ovaries at the slaughterhouse; Carmen Matás for technical support with IVF and Kristina
741 Tabbada for sequencing RNA-Seq and BS-seq libraries. **Funding:** Work in GK's laboratory was
742 supported by the UK Biotechnology and Biological Sciences Research Council and Medical
743 Research Council. Work in PC's laboratory was supported by grants AGL2012-40180-C03-01
744 and AGL2015-66341-R from the Ministry of Economy and Competitiveness (Spain), and
745 20040/GERM/16 from Fundación Séneca. PC stay at The Babraham Institute was funded by a
746 mobility grant of the Spanish Ministry of Education, Culture and Sports (PRX14/00348).
747

748 **Supplementary information.**

749 **Source data 1**. Differentially Expressed Genes (DEGs) for pair-wise comparisons (C-IVF *vs.*

750 Natur-IVF, *In-vivo vs.* Natur-IVF, C-IVF *vs. In-vivo*) and list of all gene expression values. This

751 data relates to Figure 3C.

752 **Source data 2.** DEGs exclusives for each group: 328 DEG exclusive *In-vivo*, 7 DEGs exclusive

753 Natur-IVF and 13 DEGs exclusive C-IVF. This data relates to Figure 3C.

754 **Source data 3.** All differentially Methylated Regions (DMRs) for each pair-wise comparison (C-

755 IVF *vs.* Natur-IVF, *In-vivo vs.* Natur-IVF, C-IVF *vs. In-vivo*). This data relates to Figure 5B.

756 **Source data 4**. Differentially Methylated Regions (DMRs) exclusive for each group (C-IVF,

757 Natur-IVF, *In-vivo*). This data related to Figure 5B, C, D and E.

758

759 **Supplementary file 1.** Top Canonical Pathways, Physiological Systems and Molecular and

760 Cellular Functions related to DEGs between blastocysts produced *in vitro* under two different

761 systems.

762 **Supplementary file 2.** Functions associated with the down regulated genes in porcine
763 blastocysts produced without reproductive fluids (C-IVF), compared to blastocyts produced
764 using Natur-IVF system or collected *in vivo*.
765 **Supplementary file 3.** Percentages of specific features included in the 150 CpG size DMRs
766 exclusive for each of three groups.
767 **Supplementary file 4.** Top Molecular and Cellular Functions and representative genes related to
768 DMRs with higher or lower methylation in each group (C-IVF, Natur-IVF and *In-vivo*).
769 RNA-Seq and DNA methylation data available from the Dryad Digital Repository:
770 http://dx.doi.org/10.5061/dryad.n77r3
771

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900
901

902 **Table 1**. IVF results after using 3 different sperm processing protocols (Density gradient, Swim-
903 up-BSA and Swim-up-Fluid) as represented in Figure 1. [a, b]: Different letters in the same column
904 indicate values statistically different ($P<0.05$). Penetration: proportion of oocytes penetrated by
905 one or more spermatozoa. Monospermy: Monospermy percentage, calculated from penetrated
906 oocytes, represents the proportion of penetrated oocytes with only one spermatozoon inside the
907 ooplasm. Spermatozoa/Oocyte: Mean number of sperm per penetrated oocyte. Spermatozoa/ZP:
908 Mean number of spermatozoa attached to ZP per oocyte. Yield: Percentage of putative zygotes
909 per oocyte.

910

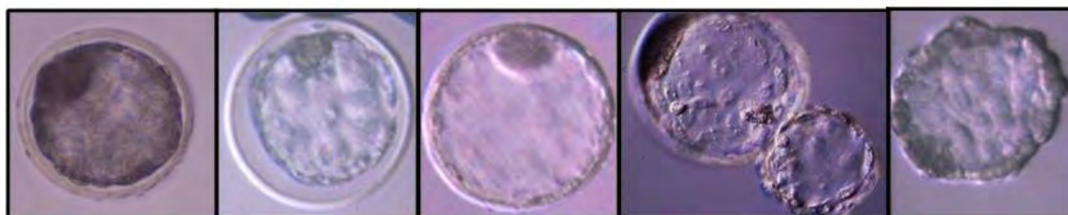
Sperm processing method	N	Penetration (%)	Monospermy (%)	Spermatozoa/ Oocyte	Spermatozoa /ZP	Zygote Yield (%)
Density gradient centrifugation	105	84.3±3.6a	17.4±4.1a	8.4±0.7a	17.3±2.3a	14.6±0.1a
Swim-up-BSA	180	69.6±3.5b	42.7±4.6ab	2.1±0.1b	7.2±0.5b	29.7±0.2b
Swim-up-Fluid	183	71.1±3.4b	49.6±4.5b	2.7±0.1b	8.6±0.5b	35.2±0.2c

911
912

913 **Table 2**. **A)** Comparative results of IVF yield by using BSA (C- IVF) or reproductive fluids (Natur-IVF) as
914 additives in the culture medium for 7.5 days. **B)**. Results of blastocyst development (for each type) using BSA (C-
915 IVF) or reproductive fluids (Natur-IVF) as additives in the culture medium for 7.5 days. Columns from "Early
916 blastocyst" to "Hatched blastocyst" indicate the percentage of each type of blastocyst from Total blastocyst (Table
917 2.A), classified according to Bo and Mapletoft[25]. [a, b]: Different letters in the same column indicate values statistically
918 different (P<0.05). Cleavage: Cleavage percentage from N. Total Blastocysts: Percentage of blastocysts calculated
919 from cleaved embryos. Yield: Percentage of putative blastocysts from N. Cell/blastocyst: mean number of cells per
920 blastocyst.
921
922

A)

Group	N	Penetration (%)	Monospermy (%)	Cleavage (%)	Total Blastocysts (%)	Blastocyst Yield (%)	Cell/ blastocyst
In-vivo	41						87.0±7.2b
C- IVF	903	395 (43.7±0.1a)	656 (72.7±0.1)	429 (47.5±1.6a)	178 (41.4±2.4)	19.6±1.3	49.9±3.7a
Natur-IVF	961	640 (66.6±0.1b)	755 (78.6±0.1)	405 (42.1±1.6b)	180 (44.5±2.5)	18.7±1.2	81.8±7.2b



923

B)

Group	N	Early Blastocyst (%)	Blastocyts (%)	Expanded Blastocyst (%)	Hatching blastocyst (%)	Hatched Blastocyst (%)
C- IVF	178	57 (31.7±6.1)a	50 (28.3±5.9)	71 (40.0±6.4)	0 (0)a	0 (0)a
Natur -IVF	180	23 (12.8±5.4)b	55 (30.8±7.5)	65 (35.9±7.8)	28 (15.4±5.9)b	9 (5.1±3.6)b

924
925
926

927 **Table 3.** Percentages of methylation over genome features in porcine blastocysts produced *in*
928 *vitro* (C-IVF and Natur-IVF) or collected *in vivo* (*In-vivo*).
929

	% Methylation		
	In-vivo	**C-IVF**	**Natur-IVF**
CpG islands	9.69	11.80	10.11
Promoters	9.26	11.61	9.11
TU	12.84	15.47	12.36
Intergenic	11.75	14.48	11.37
LINE1	12.63	15.43	12.02
LTR	12.77	15.53	12.06
SINE	12.45	15.30	11.94
GLOBAL	12.33	15.02	11.09

930
931

932 **Table 4.** Targeted analysis of candidate imprinted genes. **A)** Predicted imprinted regions in the pig genome by lifted-over mouse igDMRs the pig genome and manually inspected. **B)** Pair-wise comparison of methylation by Analysis Chi-Square in the three groups of blastocysts for the resulting 14 candidate igDMRs. ***C-IVF *vs. In-vivo*:** $P<0.05$ with 20 minimum observations and 10 minimum percentage of difference % methylation. **** C-IVF *vs.* Natur-IVF:** Analysis Chi-Square $P<0.05$ with 20 minimum observations and 10 minimum percentage of difference % methylation. **Natur-IVF *vs. In-vivo*:** no statistical differences.
939

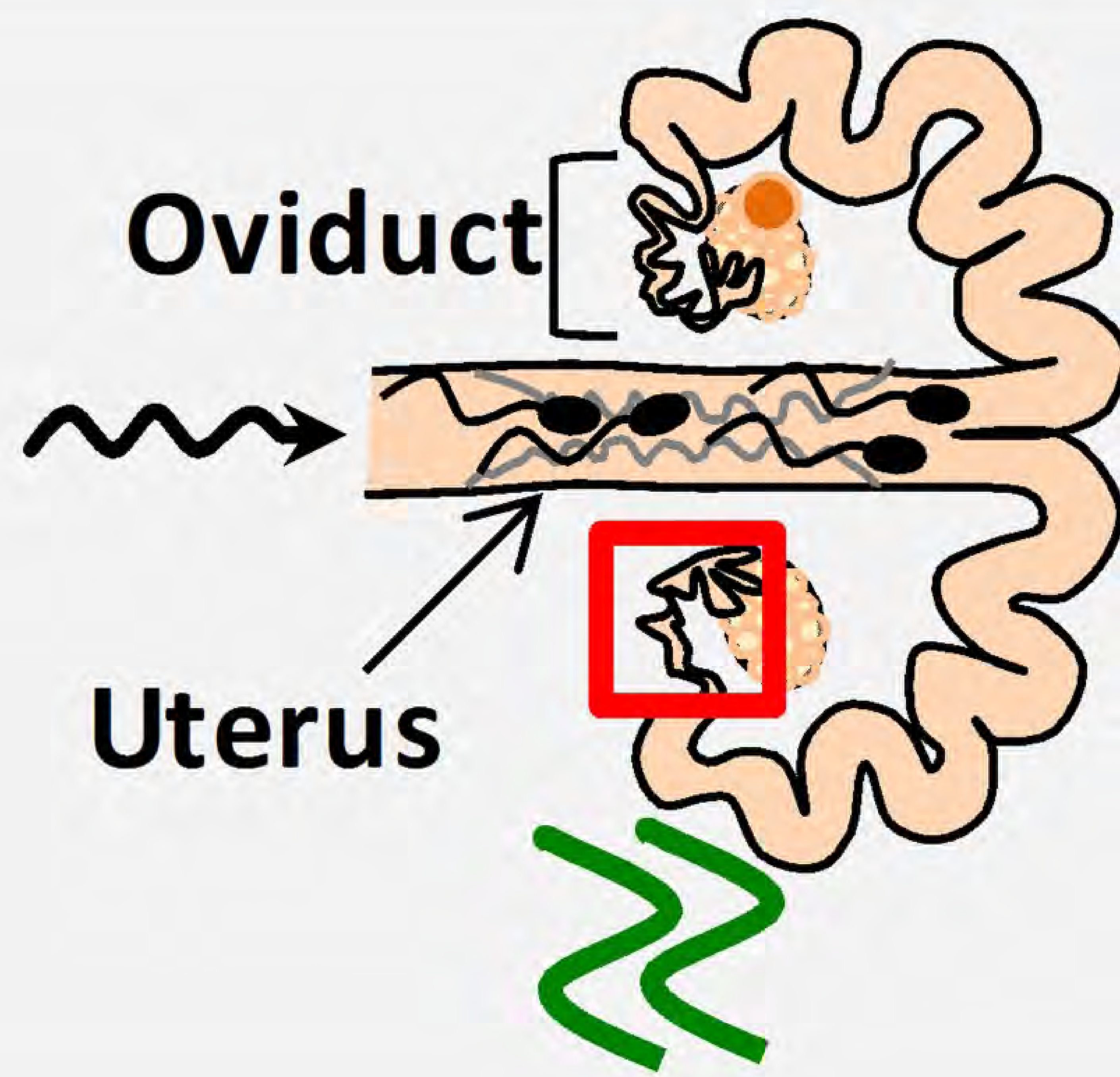
940 **A)**

Tile	Chromosome	Start	End
IGF2R/AIR	1	9,244,239	9,248,054
ZAC1	1	23,638,887	23,643,228
SOCS5	3	99,885,360	99,887,132
ZFP787	6	55,574,080	55,575,926
ZIM2	6	56,641,190	56,644,823
IMPACT	6	102,001,929	102,002,533
NAT1l5	8	139,773,830	139,775,461
PEG10	9	81,642,957	81,644,146
INPP5FV2	14	141,186,219	141,188,231
NNAT	17	46,041,843	46,045,629
NESPAS	17	66,313,673	66,320,932
GNAS-exon1a	17	66,348,009	66,352,062
MEST	18	19,340,335	19,345,549
ZFP777	18	60,941,421	60,943,096

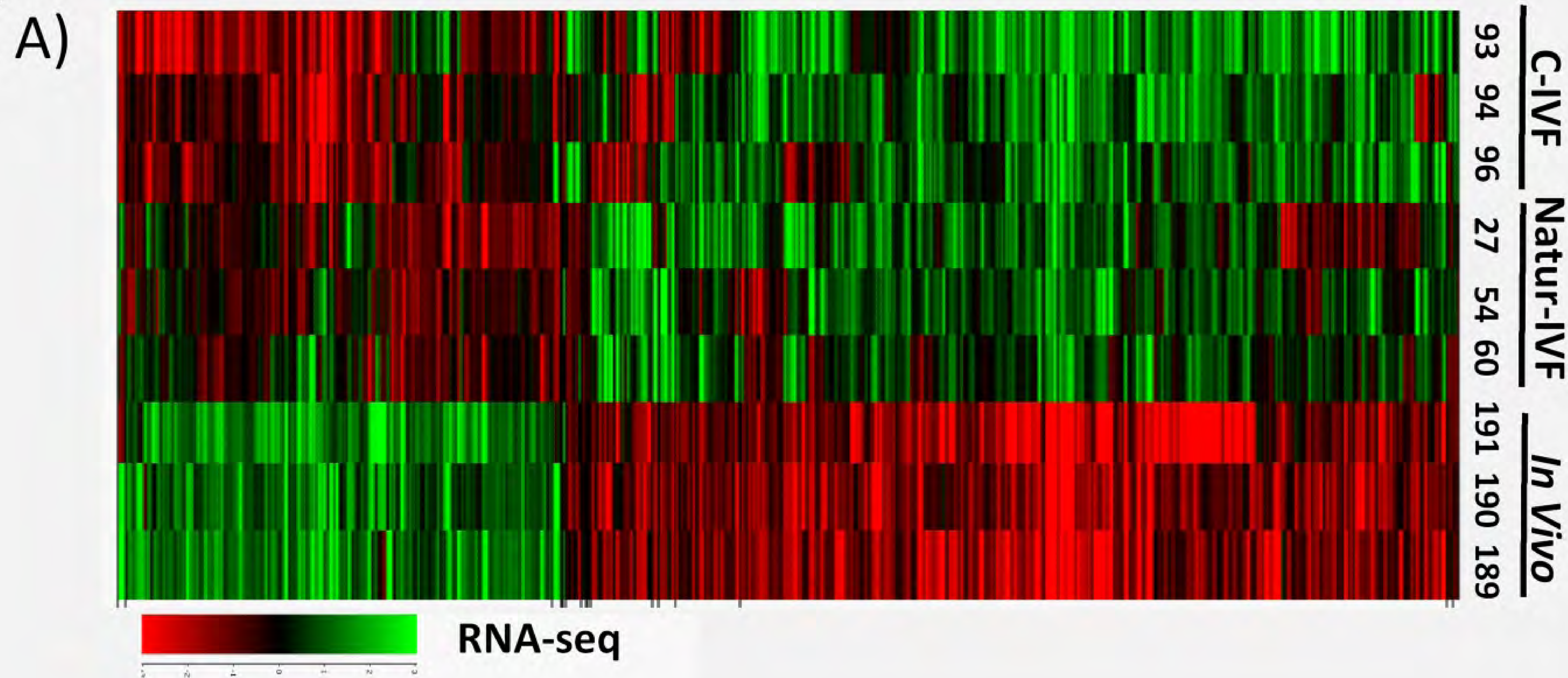
941
942
943 **B)**

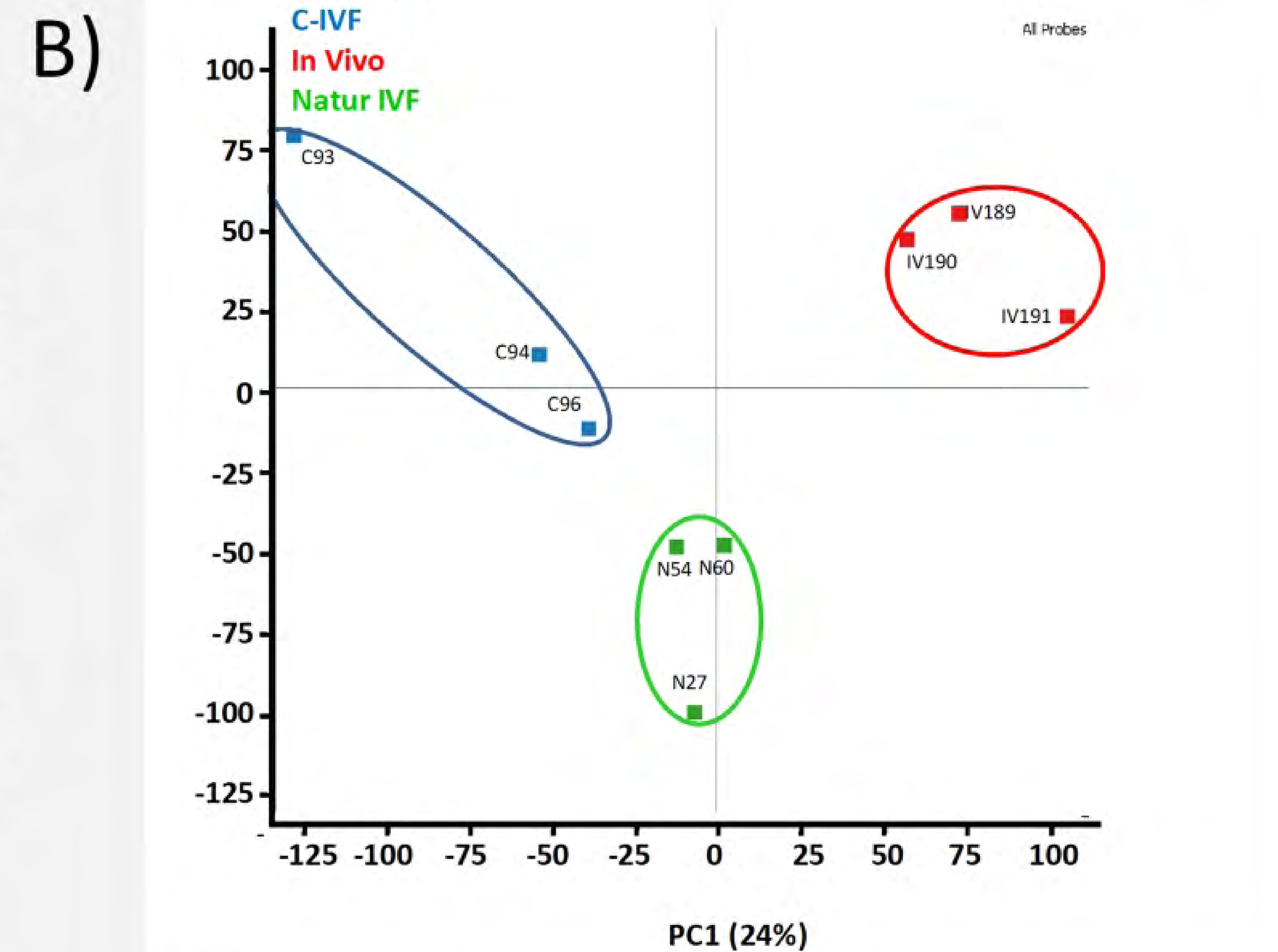
Tile	Chromosome	C-IVF	Natur-IVF	*In-vivo*
ZAC1	**1**	42.41	33.55	23.87*
PEG10	**9**	47.75	36.91**	30.90*
NNAT	**17**	34.63	19.22**	23.28*

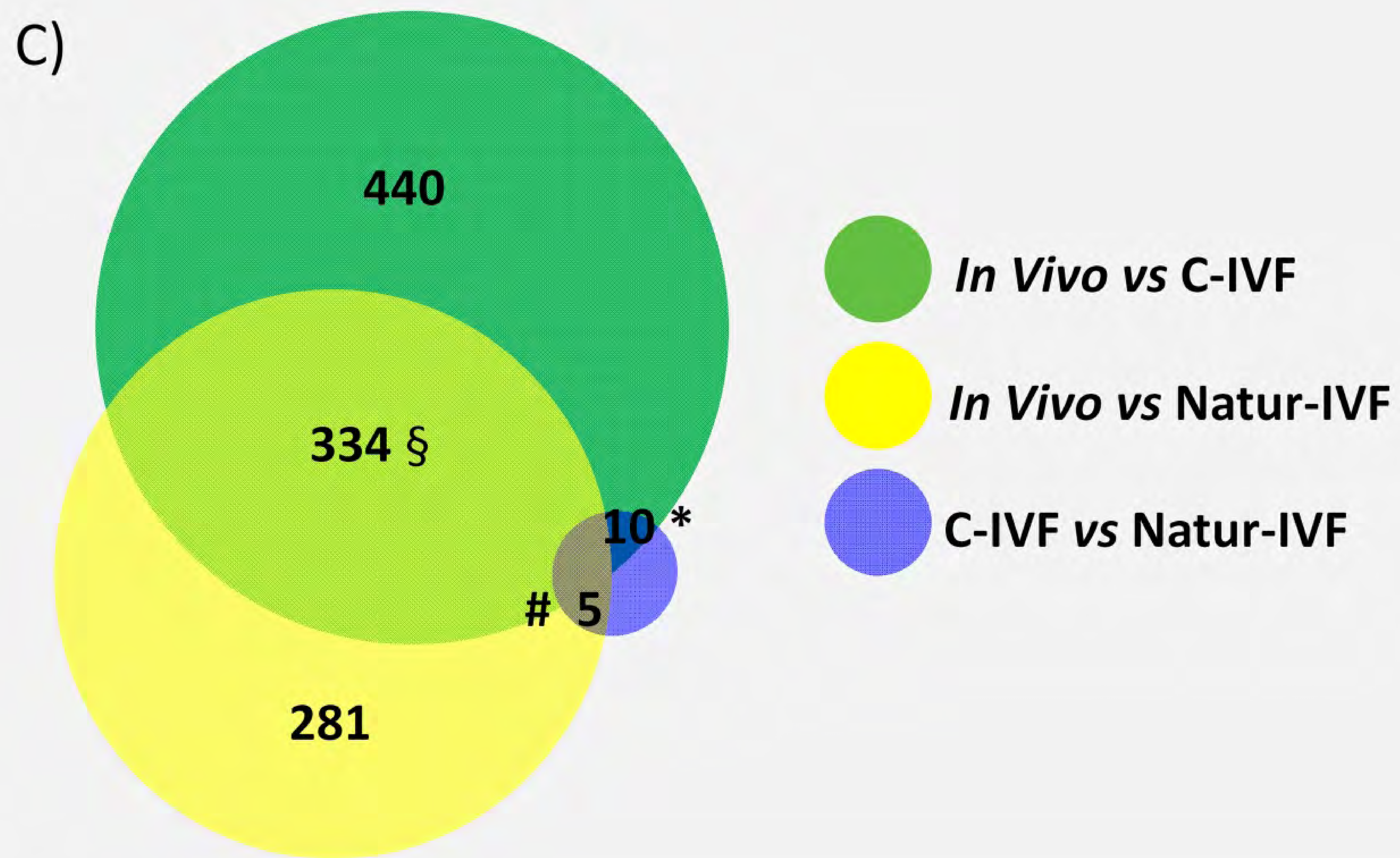
944

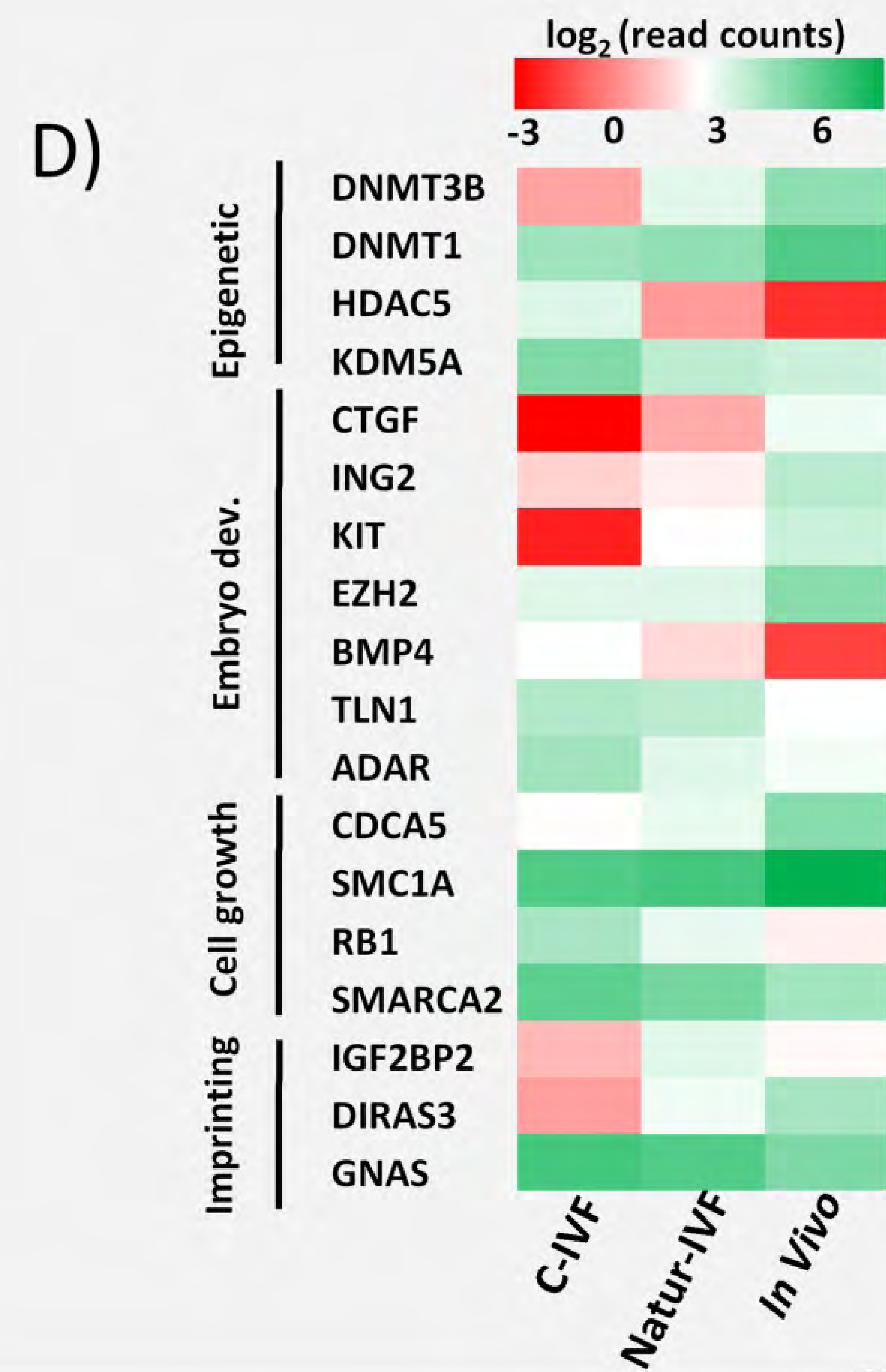

Oviduct
Uterus
Swim-up-Fluid
Swim-up-BSA
Density gradient
centrifugation
Conventional IVF
Table 1

Oviduct
Uterus
O*
O**
IVF
0 h
8 h
48 h
7 d
24 h
Swim-up-BSA
Swim-up-Fluid
Oocytes preincubated in OF-LF for 30 min
C-IVF zygotes
TALP + BSA
NCSU23a + BSA
NCSU23b + BSA
Natur-IVF zygotes
TALP + POF-LF
NCSU23a + 1% OF-EL
NCSU23b + 1% UF-EL
C-IVF Blastocyst
Natur-IVF Blastocyst
IVF control
Table 2

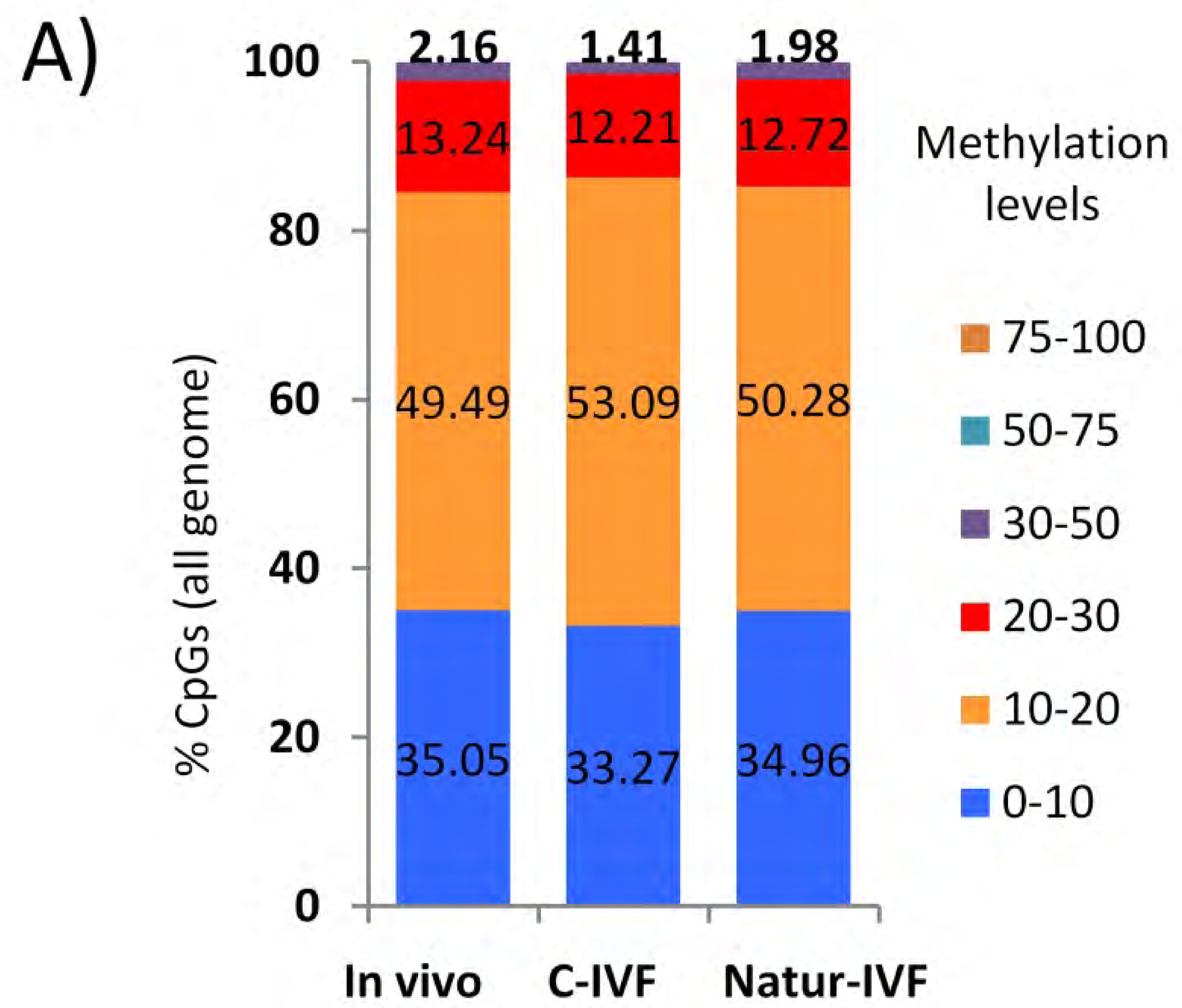

A)
93
94
96
27
54
60
191
190
189
C-IVF
Natur-IVF
In Vivo
RNA-seq


B)
PC2 (16%)
C-IVF
In Vivo
Natur IVF
All Probes
C93
C94
C96
IV189
IV190
IV191
N54
N60
N27
PC1 (24%)


C)
440
334 §
10 *
5
281
In Vivo vs C-IVF
In Vivo vs Natur-IVF
C-IVF vs Natur-IVF


D)
log2 (read counts)
-3
0
3
6
Epigenetic
DNMT3B
DNMT1
HDAC5
KDM5A
Embryo dev.
CTGF
ING2
KIT
EZH2
BMP4
TLN1
ADAR
Cell growth
CDCA5
SMC1A
RB1
SMARCA2
Imprinting
IGF2BP2
DIRAS3
GNAS
C-IVF
Natur-IVF
In Vivo

A)



B)

gene

CpG islands

186 *In-vivo*

193 *In-vivo*

197 *In-vivo*

34 C-IVF

35 C-IVF

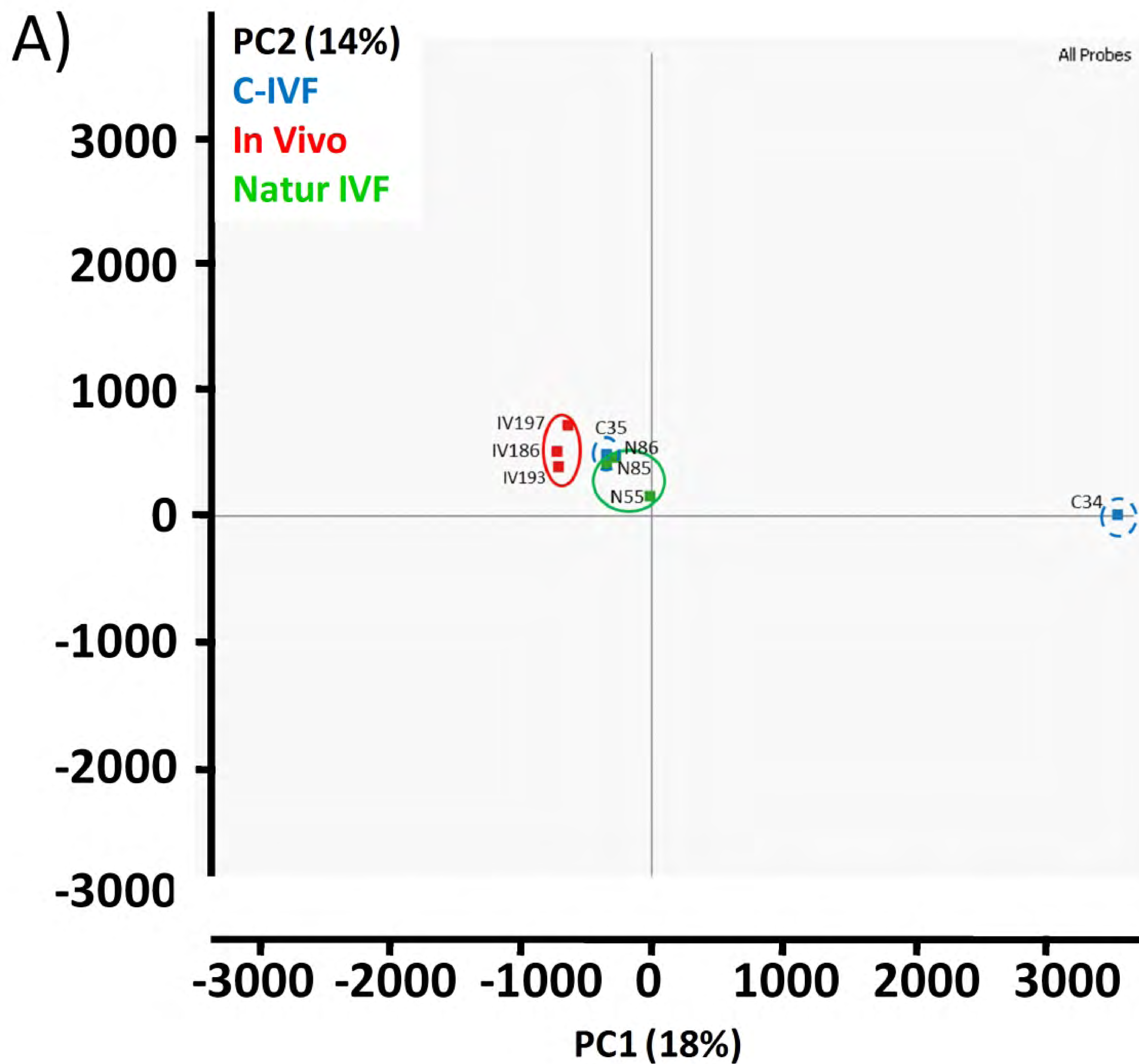
36 C-IVF

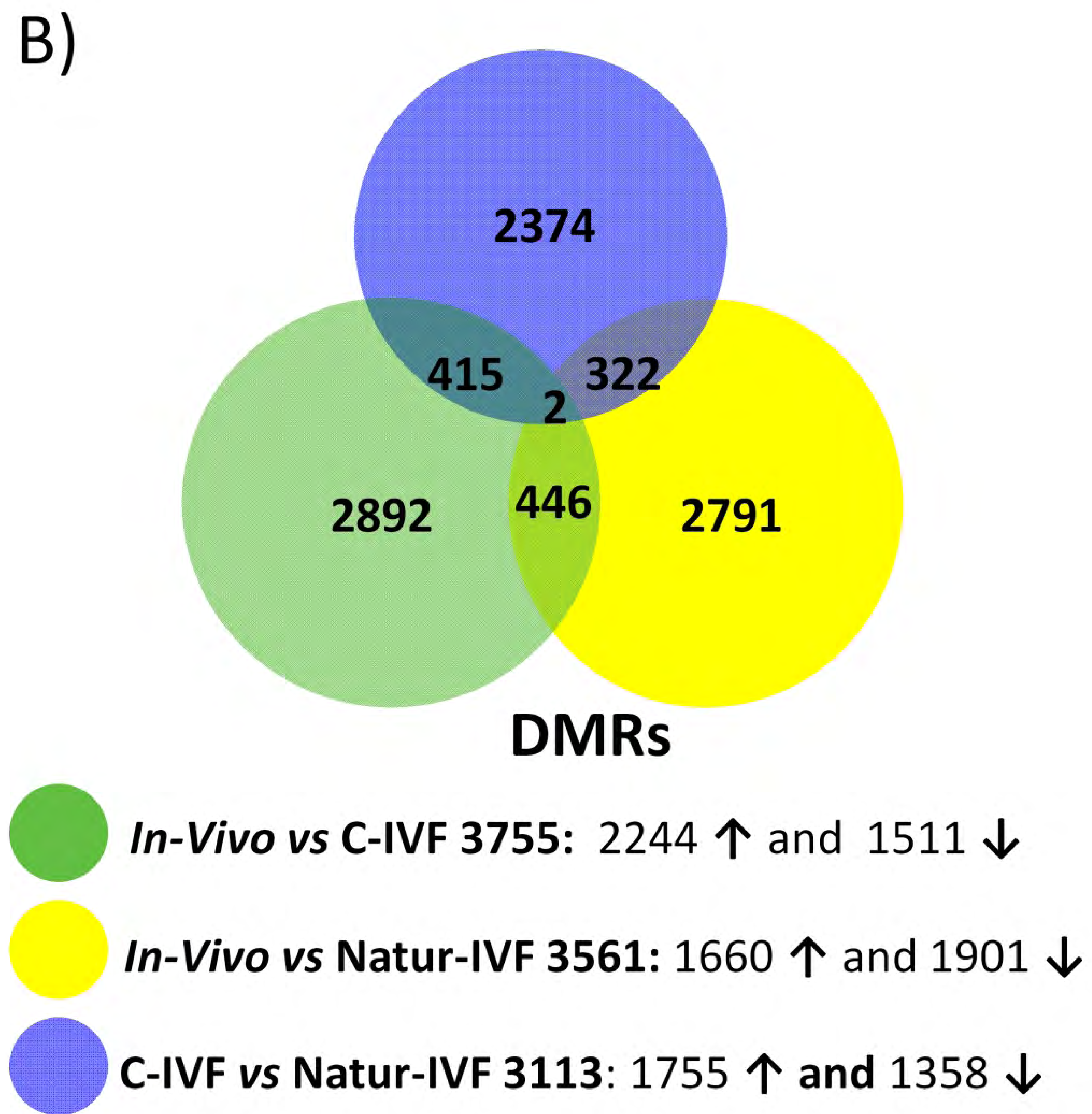
55 Natur-IVF

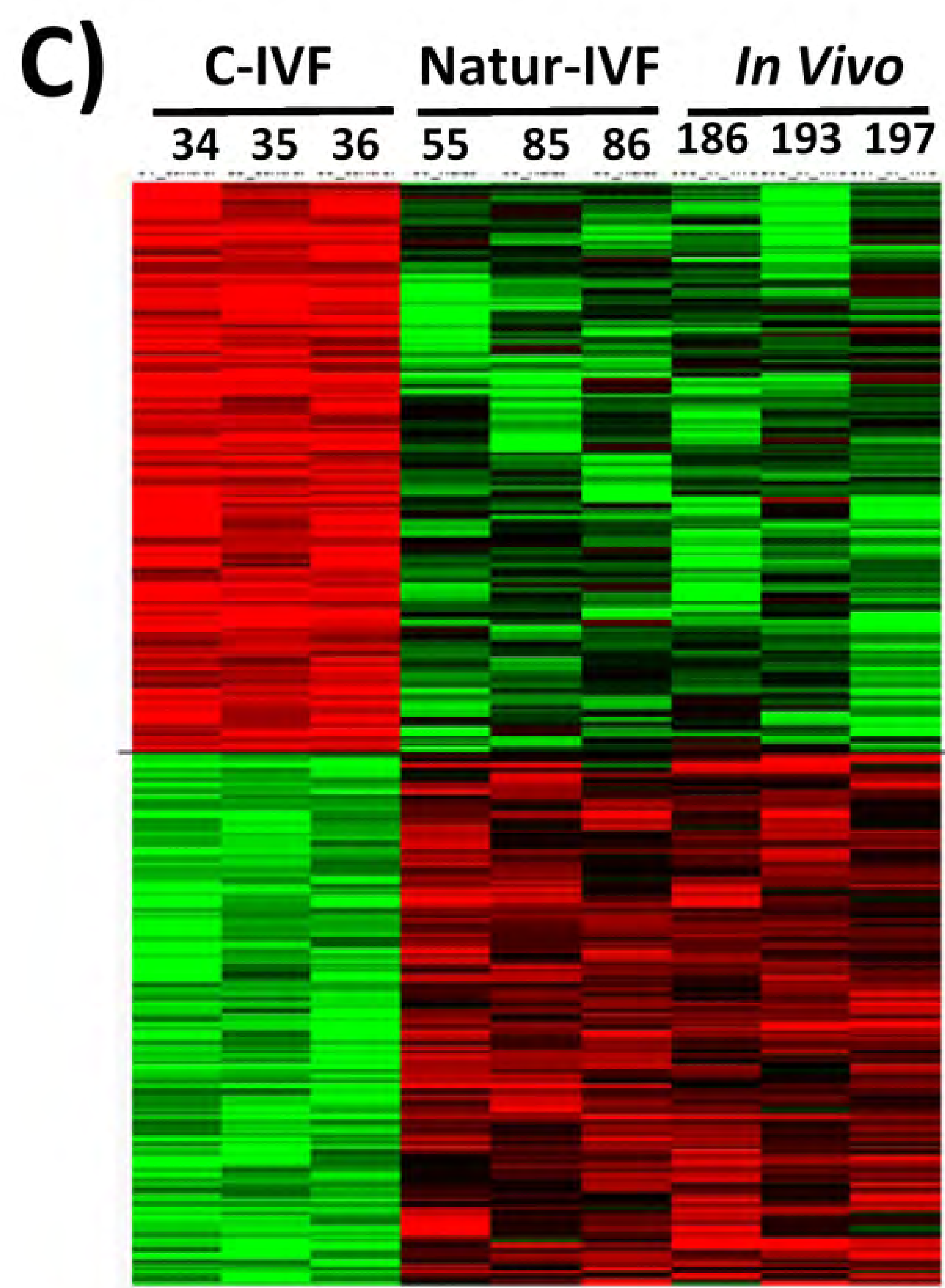
85 Natur-IVF

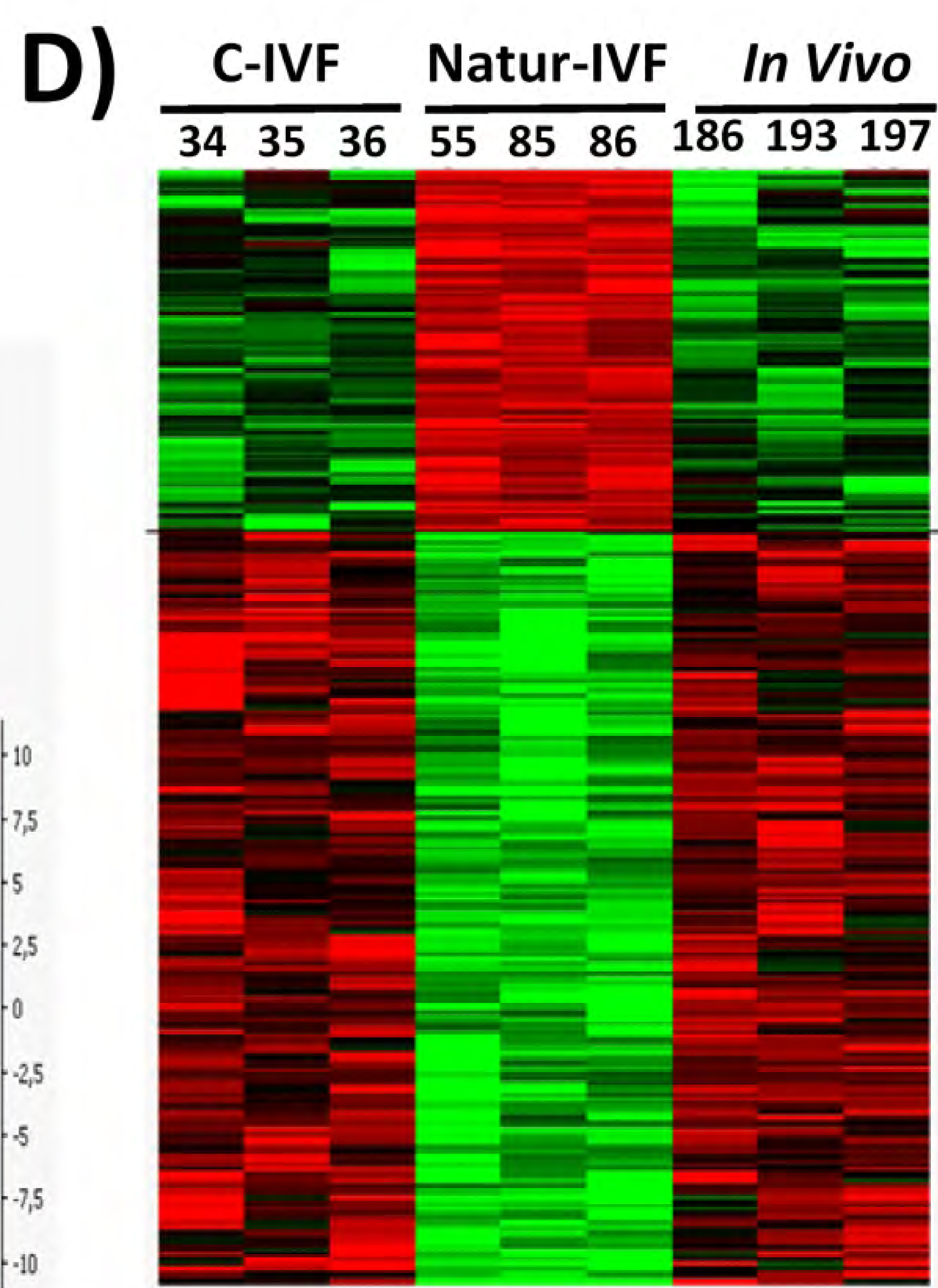
86 Natur-IV

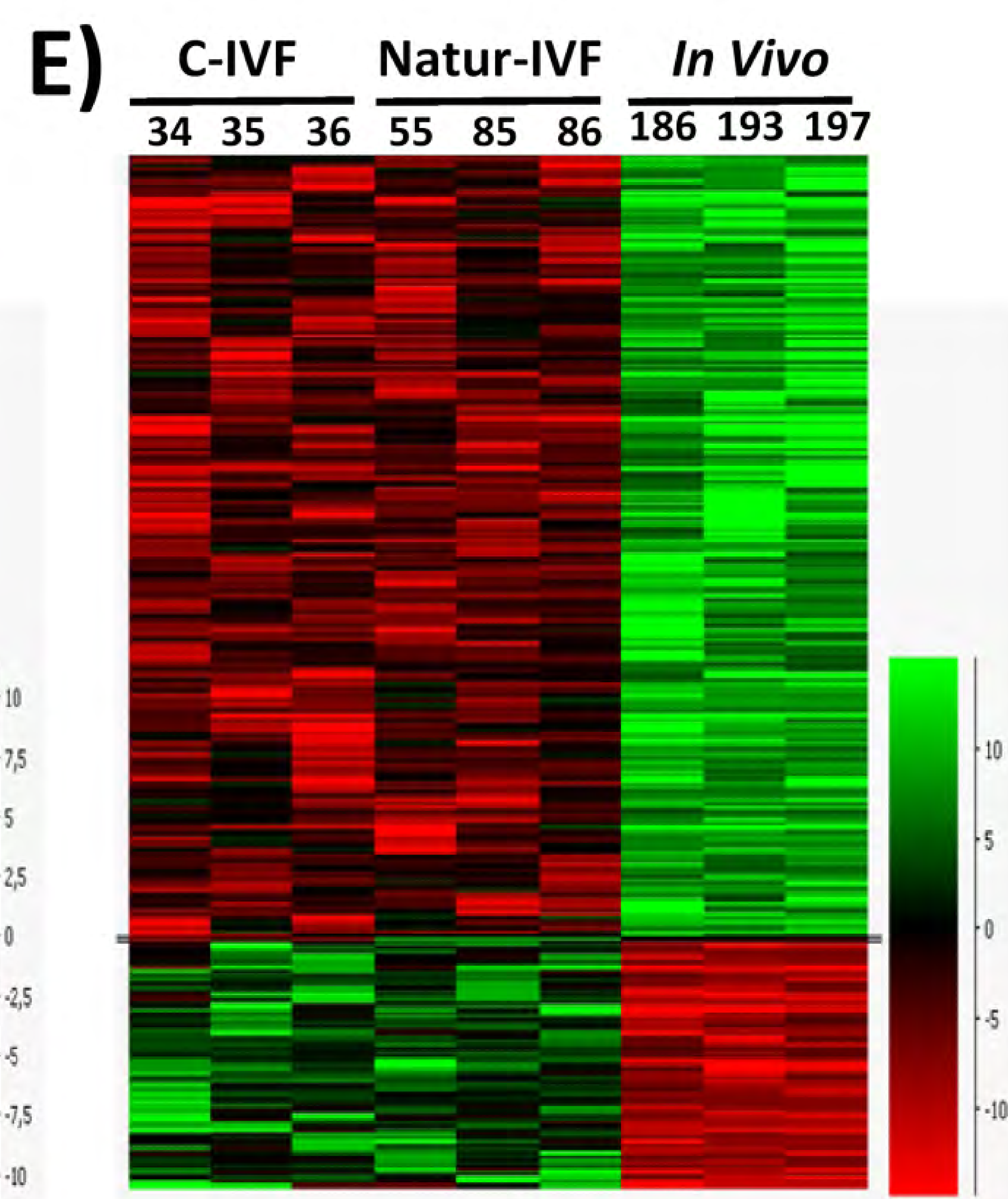
40,000,000 50,000,000 60,000,000 70,000,000 80,000,000 90,000,000 100,000,000 110,000,000


A)
PC2 (14%)
C-IVF
In Vivo
Natur IVF
All Probes
IV197
IV186
IV193
C35
N86
N85
N55
C34
3000
2000
1000
0
-1000
-2000
-3000
-3000 -2000 -1000 0 1000 2000 3000
PC1 (18%)


B)
2374
415
322
2
2892
446
2791
DMRs
In-Vivo vs C-IVF 3755: 2244 ↑ and 1511 ↓
In-Vivo vs Natur-IVF 3561: 1660 ↑ and 1901 ↓
C-IVF vs Natur-IVF 3113: 1755 ↑ and 1358 ↓

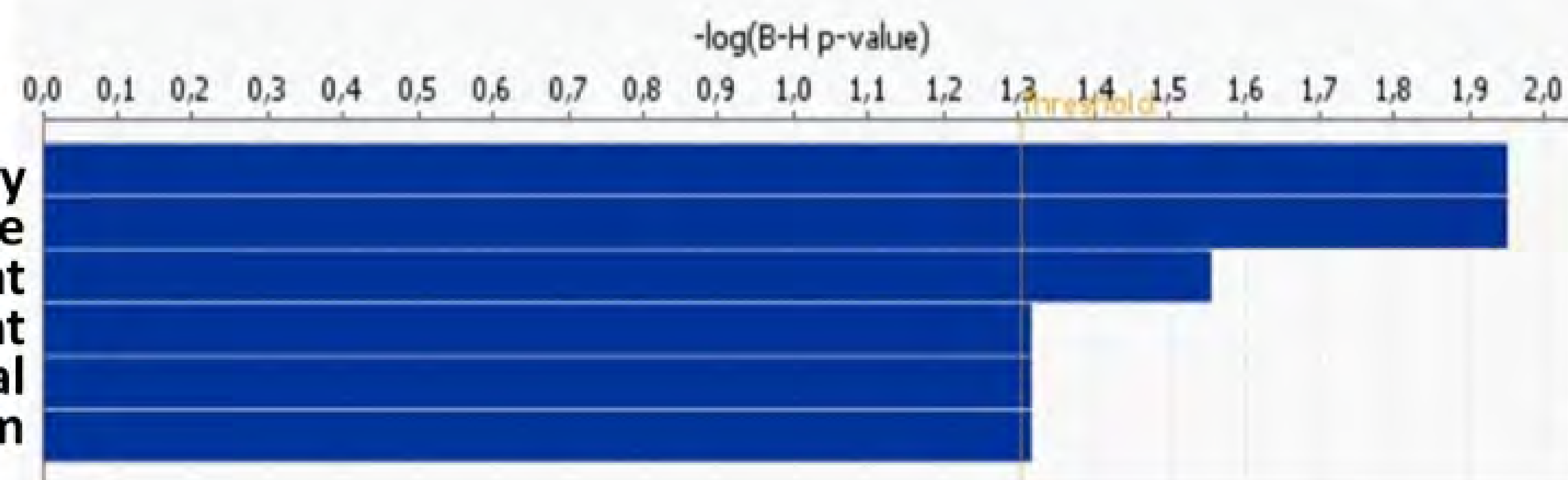

C)
C-IVF
Natur-IVF
In Vivo
34 35 36
55 85 86
186 193 197


D)
C-IVF
Natur-IVF
In Vivo
34 35 36
55 85 86
186 193 197

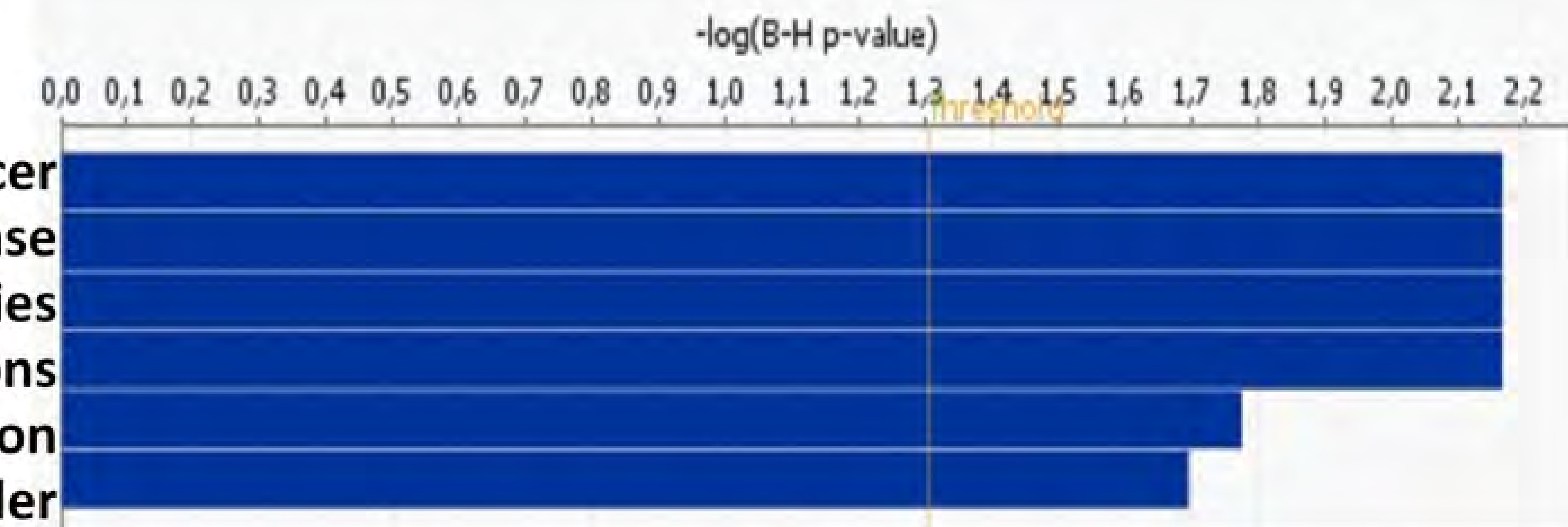

E)
C-IVF
Natur-IVF
In Vivo
34 35 36
55 85 86
186 193 197

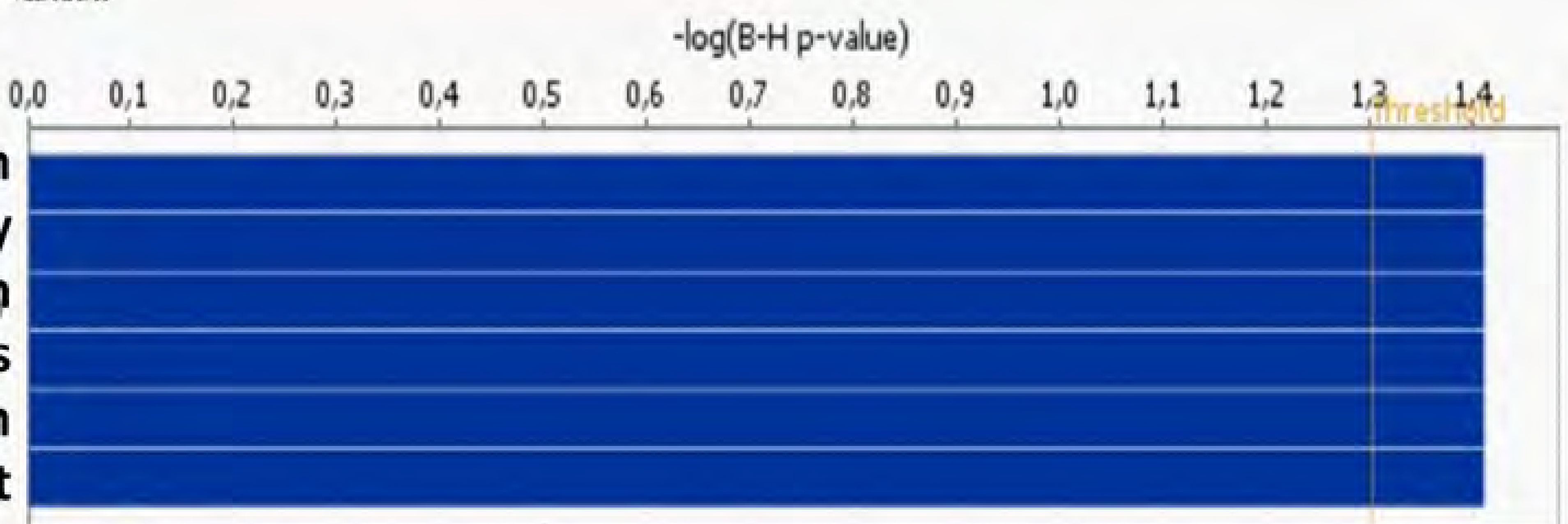
DMRs with low methylation

DMRs with high methylation

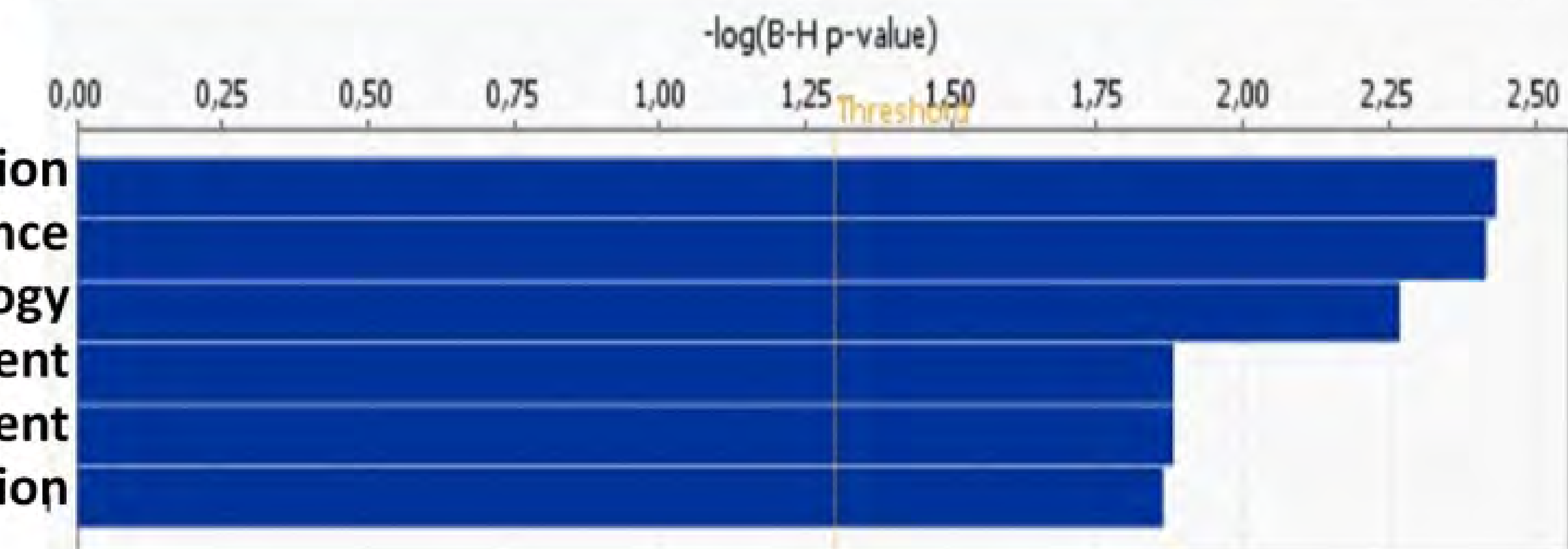


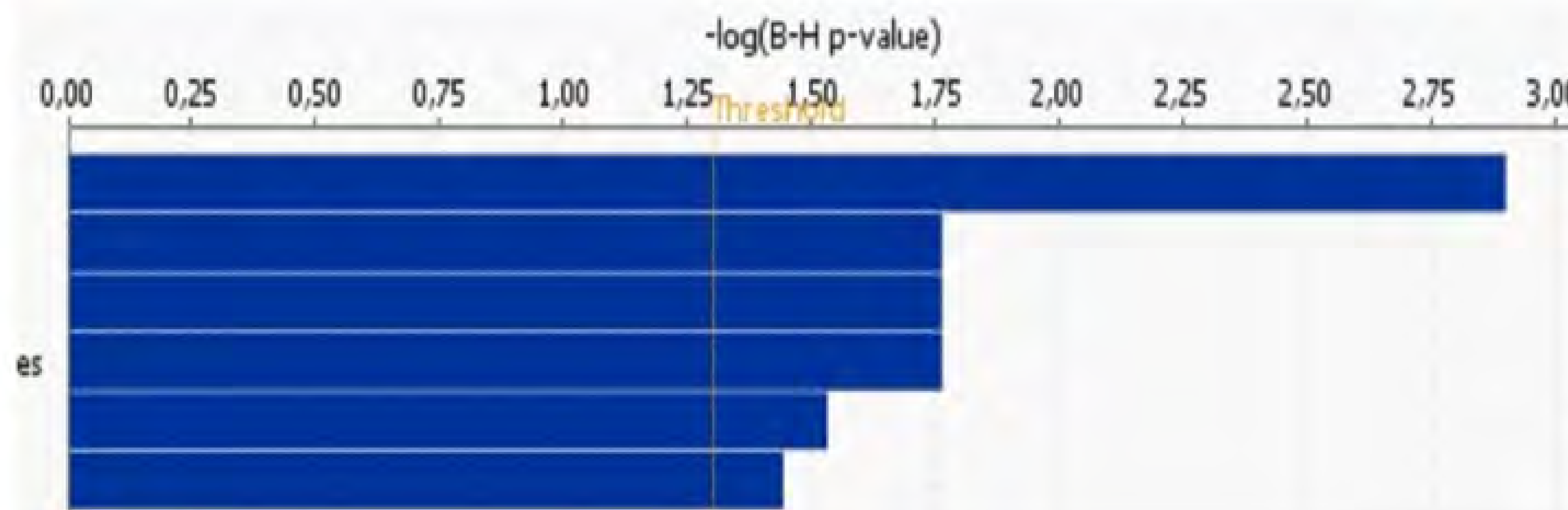
C-IVF



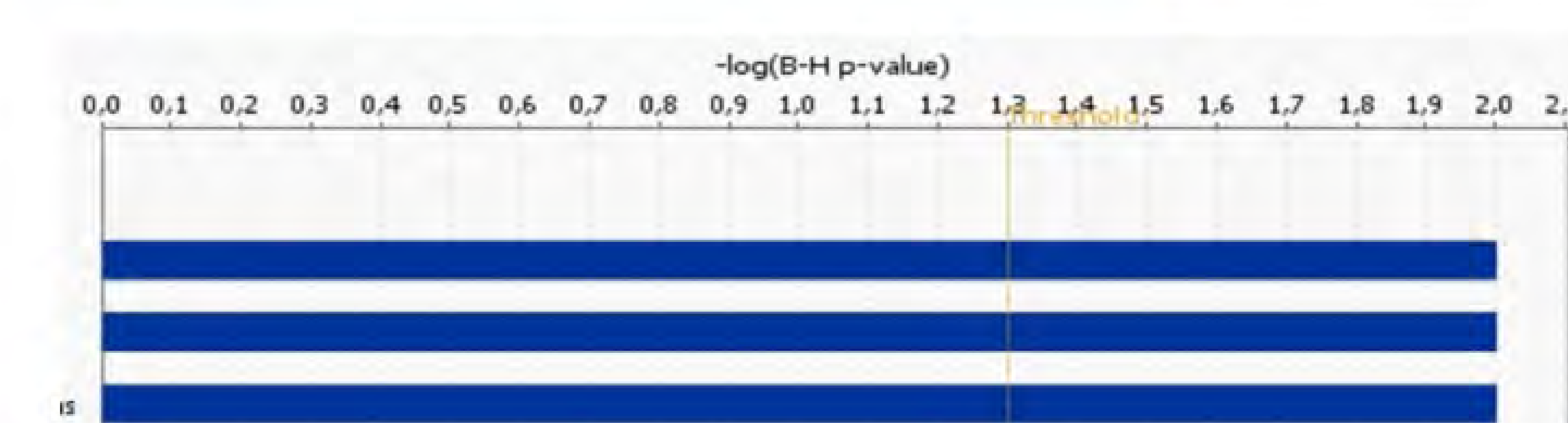


In Vivo





Natur-IVF



A)

IGF2R/M6P

gene:MAS1

IGF2R/M6P

Gene

Chr1: 9161421-9282036

CpGi

mRNA

TSS

Reads

C-IVF

30.5

12.45

13.14

Reads

Natur-IVF

Methylation %

32.5

28.3*

32.16

Reads

In vivo

Methylation %

38.5

35.5**

29.09

Chr1: 9199522-9201143

Chr1: 9201147-9202063

B)

IGF2R/M6P

gene:MAS1

IGF2R/M6P

Gene

Chr1: 9161421-9282036

CpGi

mRNA

TSS

CpG island. oe=0.89

Methylation %

Reads

C-IVF

14.1

Reads

Natur-IVF

Methylation %

27.8 *

Reads

In vivo

Methylation %

29.4 **

Chr1:9200658-9202276

Unbiased analysis: 150 CpGs tile