1   **An efficient protocol for chromosome isolation from sterlet (*A. ruthenus*) embryos and larvae**

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24   **Keywords**: Cytogenetics, chromosome preparation, hypotonization, cell fixation, sturgeon.

25

Abstract

26 In the present study, the development of an efficient and feasible protocol for chromosome preparation

28 from sterlet (*A. ruthenus*) embryos and larvae was carried out. In the established protocol, the mean

29 efficiency of chromosome extraction ranged from 70 to 100%. The average number of recorded metaphases

30 per slide was between 9 to 15. In general, the most satisfactory results were obtained for embryos at 6 dpf

31 and larvae at the age of up to 7 dph. In the 24 dpf group, chromosome isolation was possible without

32 immersion in spindle poison, however; in successive developmental stages, the minimal immersion time

33 exceeded 1.5 hours, regardless of chorionation. Immersion for 14 hours did not compromise the efficacy of

34 chromosome isolation. In the current study, successful chromosome isolation was determined mainly by

35 hypotonization conditions. Younger developmental stages generally require the shortest hypotonization

36 times, whereas older larvae require longer hypotonization times. The optimal hypotonization period is 5-

37 15 minutes for embryos at 24 dpf, 40 minutes for embryos at 4dpf, and 50-60 minutes for fish at 6 dpf-7

38 dph. The only exception was the 24 hpf group where only blastula cells were used. An additional overnight

39 fixation step significantly enhanced chromosome quality and supported chromosome counting especially

40 in the 24 dpf group. The quality and quantity of chromosome slides were also significantly determined by

41 tissue type, and the slides prepared from heads (gill cells) produced the best results.

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Introduction

44 Sturgeons are one of the most valuable fish groups in the global aquaculture, and they are cultured

45 for caviar, tasty meat and isinglass. Sturgeons are also one of the most endangered fish species in the world

46 (FishBase 2021). The development of efficient genome engineering techniques for aquaculture and

47 conservation of sturgeons is a highly promising tool in modern fisheries that continues to attract significant

48 interest (Gui and Zhu 2012; Crego-Prieto et al. 2013; Thresher et al. 2014; Chandra and Fopp-Bayat 2020).

49 Biotechnology tools such as nuclear and mitochondrial DNA markers, gene expression, flow cytometry,

50 genome transplantation and chromosome techniques play a fundamental role in taxonomy, inter-specific

51 hybridization and improvement of commercial fish stocks (Chandra and Fopp-Bayat 2020).

52   Genome engineering by means of karyotype manipulation encompasses gynogenesis, androgenesis

53   and polyploidization (especially triploidization and tetraploidization). The induction of development in

54   embryos containing uniparental genetic material is known as gynogenesis (maternal material) and

55   androgenesis (paternal material). In aquaculture, both techniques are highly useful for the production of

56   female or male mono-stocks (Chandra and Fopp-Bayat 2020). In sturgeons, gynogenesis plays a particularly

57   important role in caviar production because it reduces the costs associated with the maintenance of

58   spawning stock before it reaches late sexual maturity. Androgenesis is a very useful tool in the restitution

59   and conservation of endangered species in their natural habitat (Corley-Smith and Brandhorst 1999). The

60   triploidization technique plays the key role in polyploidy induction because some triploid fish (especially

61   females) grow at a faster rate and have better tasting meat due to their sterility (Lakra and Das 1998).

62   Chromosome engineering is also important in developmental biology, reproduction and genetics

63   (Piferrer at al., 2009; Chandra and Fopp-Bayat 2020). It is widely used in research on sturgeons'

64   evolutionary history, including the determination of differences and abnormalities in ploidy level and the

65   mechanisms responsible for karyotype rearrangement. At present, karyotyping is the only direct method for

66   ploidy determination in sturgeons (Fontana et al. 2003; Fontana et al. 2008; Fopp-Bayat et al., 2017, 2018).

67   Chromosome analysis techniques are also widely applied to study the sex determination system and to

68   identify new sex-related chromosomes in sturgeons (Vasil'eva et al. 2009; Chandra and Fopp-Bayat 2020).

69   There is considerable evidence to suggest that chromosomes can be effectively isolated from

70   embryos, larvae and adult fish of various species. The isolation of chromosomes from embryos and hatched

71   larvae poses the greatest challenge and requires sensitive procedures (Shao et al., 2010; Ozouf-Costaz et

72   al., 2015; Katami et al., 2015). Despite the availability of various karyotyping techniques such as tissue

73   cultures (Lomax et al., 2000), squashing techniques (Armstrong and Jones 2003) and cell suspensions of

74   mitotic tissues (Henegariu et al., 2001), only one protocol for isolating chromosomes from sturgeons has

75   been developed to date by Fopp-Bayat and Woźnicki (2006), and it relies mainly on the larval stage.

76   Moreover, the extend of optimal chromosome isolation parameters and the impact of fish age on the efficacy

77   of the isolation procedure have never been comprehensively studied. Therefore, the aim of the present study

78 was to develop a feasible, simple and efficient chromosome preparation protocol for sterlet embryos and

79 larvae.

80

81 **Results**

82 The developed chromosome preparation protocols enabled the isolation of chromosomes of

83 satisfactory quality, but the results differed across groups. The quality and quantity of chromosome slides

84 were determined by tissue type, and the slides prepared from heads (gill cells) produced the most

85 satisfactory results. The only exception was the 24 hpf group where only blastula cells were used. In the

86 present study, the mean efficiency of chromosome extraction ranged from 70 to 100%. The average number

87 of recorded metaphases per slide was between 9 to 15. The smallest number of chromosomes of the lowest

88 quality were isolated for embryos at the age of 24 hpf, whereas the chromosomes obtained from embryos

89 at 6 dpf and larvae up to 7 dph were of the highest quality (Table 1, Fig. 1). In the current study, the optimal

90 developmental stage for the chromosome preparations was before 7 dph (data not shown).

91

92 Table 1. The summary of tested colchicine, hypotonic, fixative treatments, tissue type application and age

93 of fish material utilized together with obtained results. NT: not tested, N/A: not applicable, hpf: hours post

94 fertilization, dpf: days post fertilization, dph: days post hatch. The positive or negative effect of tested

95 variant was marked by plus and minus label, respectively.

Experimental design		Age			
		Blastula stage of developmpent	Post blastula stage of development		Larvae
Chromosome isolation step	Experimantal variant	24hpf	4dpf	6dpf	Up to 7dph

Immersion in 0,025% colchicine	Without immersion	+	-	-	-
	1,5h	+	NT	-	-
	3,0h	+	+	NT	NT
	4,0h	+	+	+	+
	5,0h	+	+	+	+
	14,0h	NT	+	+	+
Hypotonization in 0,075M KCl	5min	+	-	-	-
	15min	+	-	-	-
	30min	-	NT	-	-
	40min	-	+	-	-
	50min	-	+	+	+
	60min	-	NT	+	+
Immersion and hypotonization in solution of 0,1% colchicine and 0,075 KCl	30min	-	-	-	-
	40min	-	-	-	-
	60min	-	-	-	-
Cell fixation	Overnight extension	+	+	+	+
Tissue type	Entire embryo/larvae	+	-	-	-
	Head	N/A	+	+	+
Summary	Efficiency of chromosome extraction (%)	70%	80%	100%	100%

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	Average number of metaphases per slide	9	10	15	13
	Chromosome quaility	++	++	+++	+++

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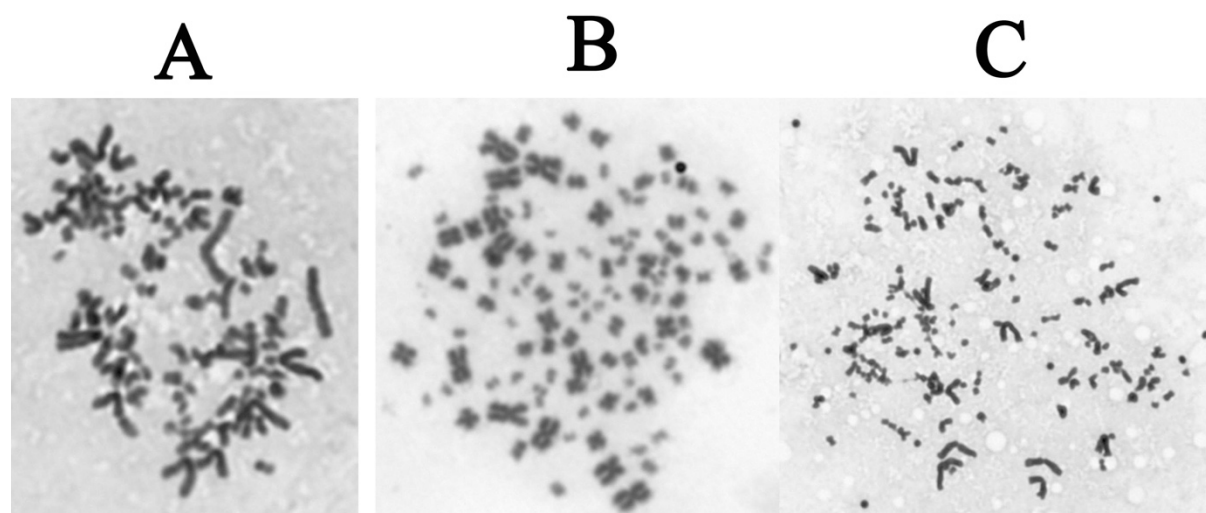

A **B** **C**

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98 Figure 1. The results of chromosome isolation. The medium and the quality of the isolated chromosomes

99 are marked as A and B, respectively. The result of chromosome isolation without colchicine

100 treatment is marked as C.

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102 In the present study, the incubation time of spindle poison had no significant influence on the

103 effectiveness of chromosome isolation from chorionated or chorion-less embryos. In general, the highest

104 plasticity was observed in embryos at 24 dpf, where positive effects of immersion were noted for every

105 incubation and were associated with the highest rate of mitotic division in this group. In the 24 dpf group,

106 chromosome isolation was possible without immersion in spindle poison that characterized by chromosome

107 slides of relatively high quality. In successive developmental stages, the minimal immersion time exceeded

108   1.5 hours, regardless of chorionation. Immersion for 14 hours did not compromise the efficacy of

109   chromosome isolation (Table 1).

110   The optimal hypotonization period was 5-15 minutes for embryos at 24 dpf, 40 minutes for embryos

111   at 4dpf, and 50-60 minutes for fish at 6 dpf-7 dph. The alternative step combining immersion and

112   hypotonization failed to generate positive results (Table 1).

113   The applied 12-14h additional fixation step significantly improved the quality of the isolated

114   chromosomes. The most pronounced improvement was observed in the 24 hpf group, which significantly

115   enhanced chromosome quality and supported chromosome counting. In turns, the least noticeable

116   improvement was recorded in 6 dpf-7 dph groups.

117

118   **Discussion**

119   The majority of chromosomal isolation protocols have been designed for specific species, and the

120   effects of each step in the procedure of isolating chromosomes from adult fish or larvae fish, in particular

121   the omission of embryonic developmental stages, have been examined by different authors. In the present

122   study, an optimal protocol was developed for isolating chromosomes from the embryos and larval

123   developmental stages of *A. ruthenus*. The results supported the identification of three main chromosome

124   preparation approaches that differ in the parameters of each step for fish at: (1) 24 hpf, (2) 4 dpf and (3) 6

125   dpf-7 dph (Table 1; Fig. 1 and 3).
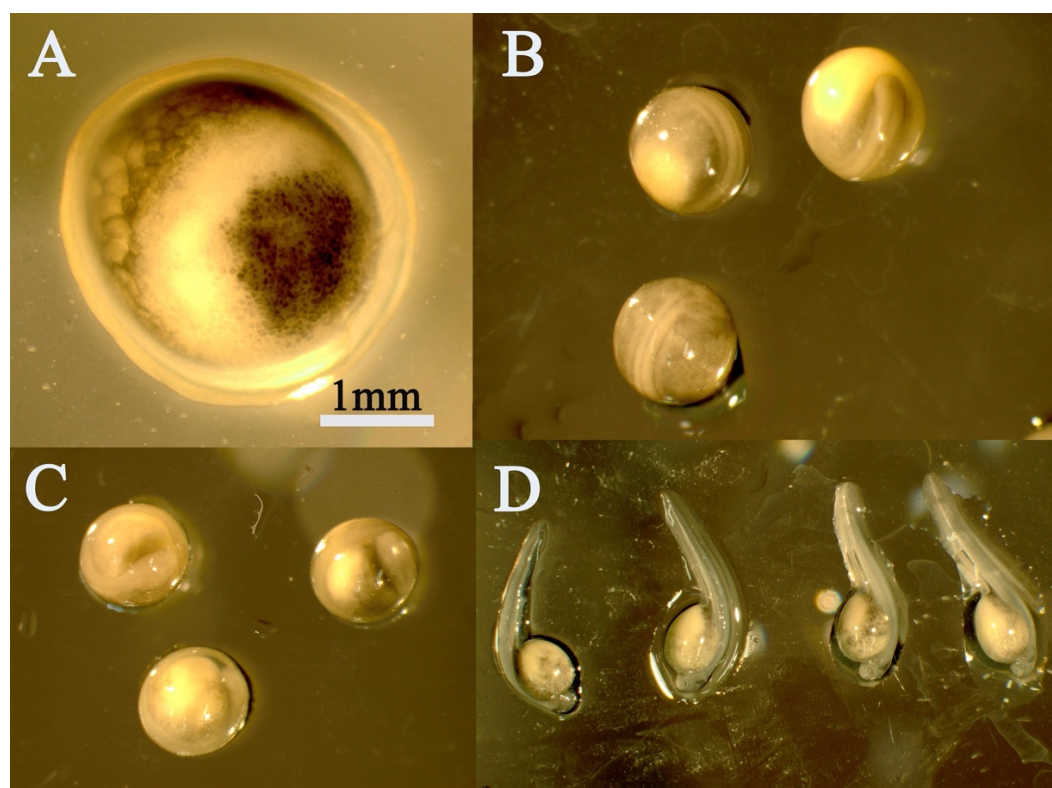
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Figure 2. Fish material used in the study. A: embryos at 24 hours post-fertilization (hpf) (blastula stage), B: 4 days post-fertilization (dpf), C: 6 dpf (before hatching), and D: larvae with the yolk sac up to 7 days post-hatching (dph).

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In most studies, the achievement of clear and identifiable metaphase chromosome spreads requires sufficient concentrations and incubation times of tissues in the spindle poison (Rieder and Palazzo 1992). In general, insufficient concentration and/or duration of exposure to the spindle poison prevents the interception of metaphase cells, whereas very high concentrations and/or prolonged exposure may lead to chromosomal condensation (Wood et al., 2001). The most common microtubular poison for cell interception is colchicine which inhibits spindle microtubules before envelope breakdown (NEB) in metaphase cells (Karami et al., 2015). In other studies, fish age was also correlated with minimal immersion time, which implies that the procedure should be optimized for individual species and age groups (Caperta et al., 2006; Karami et al., 2015). However, according to some authors, killing the larvae and separating the yolk before incubation in colchicine is a more efficient procedure than incubating live larvae (Hussain and

142   McAndrew 1994; Pradeep et al., 2011; Karami et al., 2015). These authors also hypothesized that yolk size

143   and absorption rate are the key facts that differentiate chromosome preparation protocols.

144   After mitotic spindle inhibition, cells and tissues must be incubated in a hypotonic solution to swell

145   the nuclei and spread the chromosomes on the slides (Moore and Best 2001). The hypotonic solution and

146   incubation time have to be optimized to prevent chromosome knotting, overlapping or its partial loss (Baksi

147   and Means 1988). According to some authors, the yolk should be separated from hypostasized tissues

148   because its high lipophilicity can prevent the hypotonic solution from penetrating the cells (Baksi and

149   Means 1988). Potassium chloride (KCl 0.075 M) is one of the most widely used hypotonic solutions. In the

150   current study, successful chromosome isolation was determined mainly by hypotonization conditions.

151   Younger developmental stages generally require the shortest hypotonization times, whereas older larvae

152   require longer hypotonization times (Table 1). Other authors demonstrated that distilled water can produce

153   superior results, which suggests that the type of hypotonic solution should be carefully selected for the

154   analyzed species and age group (Karami et al., 2015).

155   In the next obligatorily step of the chromosome preparation protocol, samples are fixed in Carnoy's

156   fixative solution and a cell suspension is prepared. In this step, the evaporation of the fixative solution and

157   slide preheating can affect the quality and quantity of the isolated chromosomes (Moore and Best 2001). In

158   general, this step is relatively similar in all chromosome isolation protocols for fish. Due to the high

159   effectiveness of this procedure, additional modifications were not required, and the only variation was a

160   12-14 fixation step which significantly improved the quality of the isolated chromosomes. However, the

161   observed improvement varied across groups and was most pronounced in the 24 hpf group and least

162   noticeable in 6 dpf-7 dph groups. In the 24 dpf group, an additional overnight fixation step significantly

163   enhanced chromosome quality and supported chromosome counting.

164   Fish transport and other handling operations exerted a negative effect on chromosome preparation

165   efficacy. Embryos and larvae should not be acclimatized after transport or other significant manipulations

166   because the number of the isolated chromosomes declines steadily over time. The above can be attributed

167   to stress that inhibits cell proliferation (Mohanty et al., 2018). Therefore, chromosome preparations should

168   be made immediately after fish transport and handling. It should also be noted that larval age is yet another

169   critical determinant of chromosome isolation efficacy. The effectiveness of chromosome isolation generally

170   decreases on successive days after hatching because the mitotic rate is affected by species, age and

171   environmental conditions (Wakahara 1972; Shelton et al., 1997; Shao et al., 2010). In African sharptooth

172   catfish (*Clarias gariepinus*) and zebra fish (*Danio rerio*), the samples isolated at 4 dph failed to produce

173   countable mitotic chromosome spreads (Karami et al., 2015). However, this threshold time is species-

174   specific and needs to be experimentally determined. In the current study, the optimal developmental stage

175   for the chromosome preparations was before 7 dph (data not shown).

176   In summary, this study demonstrated that chromosomes can be isolated in every developmental

177   stage of *A. ruthenus*, but the overall quality and quantity of the extracted chromosomes can vary. Under the

178   current study three main chromosome isolation procedures were determined (Fig. 3). In general, the most

179   satisfactory results were obtained for embryos at 6 dpf and larvae at the age of up to 7 dph. In both groups,

180   the quality of the isolated chromosomes supported counting and karyotyping. The chromosomes isolated

181   from 24 hpf and 4 dpf groups were characterized by the lowest quantity and quality. These isolates could

182   be used for chromosome counting only, and high-quality metaphases for karyotyping were rarely obtained.
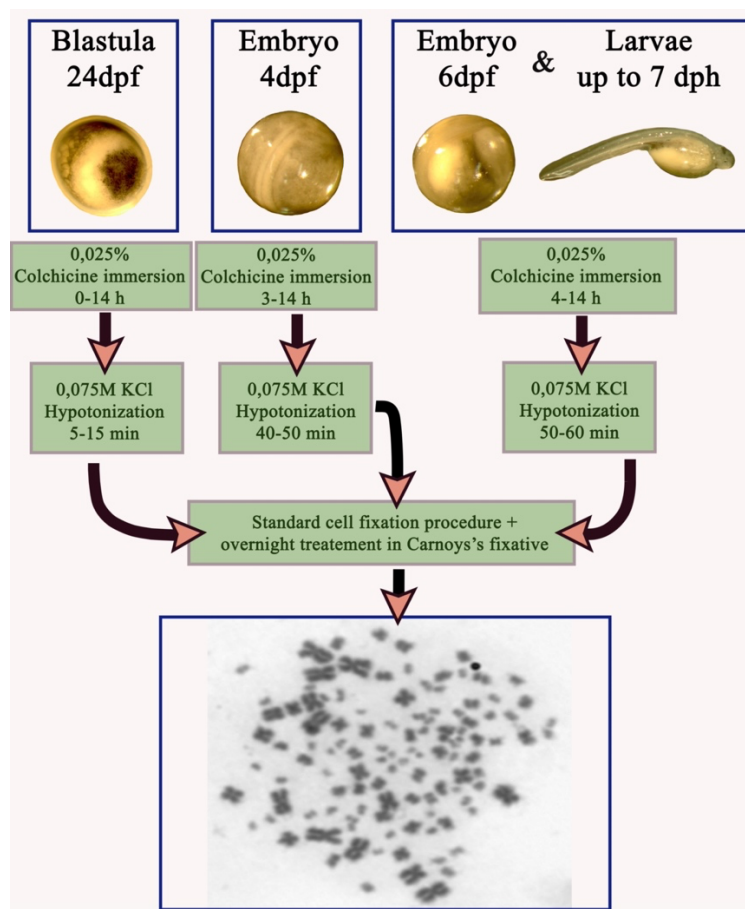
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Figure 3. The graphical summary of determined three main chromosome isolation protocols for sterlet embryos and larvae.

Materials and Methods

A series of experiments based on the chromosome preparation procedure described by Fopp-Bayat and Woźnicki (2006), which is a modified version of the spindle poison technique developed by Kligerman and Bloom (1977), were conceived and conducted to establish the chromosome isolation protocol. To this end, ten embryos and larvae of *A. ruthenus* were used in each experimental variant. The fish material differed in age to determine the influence of developmental stage on the effectiveness of chromosome isolation. Four age variants were used: (1) embryos 24 hours post-fertilization (hpf) (blastula stage), (2) 4 days post-fertilization (dpf), (3) 6 dpf (before hatching), and (4) larvae with a yolk sac up to 7 days post-hatching (dph) (Table 1 and Fig. 1). Fish material was obtained from a fish farm in Wąsosze (southern

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197 Poland), and the study was performed in the Center of Aquaculture and Environmental Engineering in

198 Olsztyn (north-east Poland). The experiments were performed immediately after the delivery of the fish

199 material.

200   The experimental chromosome preparation procedure comprised five main steps, where all

201 variables were optimized in a stepwise manner (Table 1). The following variants were tested:

202   1. Immersion of live embryos (chorionated or chorion-less) and larvae in 0.025% colchicine at

203   16°C for 1.5, 3.0, 4.0, 5.0 and 14.0 hours (h). The variant was also tested without immersion,

204   2. Hypotonization of embryos and larvae in 0.075 M solution of KCl for 5, 15, 30, 40, 50 and 60

205   minutes (min),

206   3. Immersion and hypotonization in a solution of 0.1% colchicine and 0.075 KCl for 30; 40 and

207   60 minutes was tested simultaneously as an alternative to the first and second step,

208   4. Tissue fixation in cooled Carnoy's fixative solution (methanol and acetic acid, 3:1). A fresh

209   solution was prepared each time, and it was triple fixed at -20°C in every experimental variant.

210   Each fixation stage lasted 30, 15 and 15 minutes. A fourth fixation step of 12-16 hours

211   (overnight) was additionally applied to check the effect of extended fixation on the quality of

212   the isolated chromosomes,

213   5. Homogenization of different types of tissues. Blastula cells extracted from the chorion and

214   separated from the yolk sac were used in the homogenization of fish material at 24 hpf. Tissues

215   from the entire embryo/larva and head tissues were used in the remaining age groups (Table

216   1). Tissues were homogenized with a needle in Eppendorf tubes containing a small volume

217   (around 0.1-0.2 ml) of fresh fixative solution.

218   The tissues intended for chromosome preparations were carefully separated from the yolk sac after

219 colchicine immersion. Chromosome slides were prepared in accordance with the standard splash procedure,

220 where several droplets (1-3) of the homogenized cell suspension are dropped onto microscope glass slides

221 from a height of around 30 cm. Before chromosome preparation, the glass slides were washed in solution

222 of 96% ethanol containing several drops of condensed hydrochloric acid (HCl) (Fopp-Bayat and Woźnicki

223 2006). One or two slides were prepared from each embryo or larva.

224   The quality of the obtained chromosome slides was checked under the Zeiss Axio Imager.A1

225 microscope equipped with a digital camera. Chromosome slides were examined under bright light, and the

226 quality of the extracted chromosome was classified into three main categories: (1) high quality results

227 characterized by a large number of slides with successfully isolated chromosomes (above 80%) that are

228 eligible for counting and karyotyping (+++), (2) medium quality results characterized by an average number

229 of slides with successfully isolated chromosomes (30-80%) that can be used only for counting, and (3) no

230 results or low quality results characterized by a small number of slides containing chromosomes (below

231 30%) that cannot be used for cytogenetic analysis. Clear chromosome spreads were counted on each slide

232 to assess the quantity of the chromosomes isolated in each experimental variant.

233

234   **Competing Interests**

235   Authors do not declare any competing interests that may be perceived as contributing to potential

236 bias.

237

238   **Acknowledgements**

247   Initiative of Excellence" for the years 2019-2022, Project No. 010/RID/2018/19, amount of funding

248   12.000.000 PLN". There is no conflict of interest declared in this article.

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