Video Article

Sperm Collection of Differential Quality Using Density Gradient Centrifugation

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Abstract

In sexual reproduction, a male gamete or sperm cell fuses with a female gamete to bring about fertilization. However, a large number of sperm cells with fertilizing ability are required to interact with a female gamete to ensure fertilization. As such, the fertilizing ability of individual sperm cells is critical for successful reproduction. Density gradient centrifugation has been utilized for several decades as a reproducible, fast, efficient, effective and extremely adaptable method to collect only high-quality sperm to be used in assisted reproductive technology. The protocols we described herein focus on the utilization of the discontinuous Percoll density gradient centrifugation (PDGC) technique to isolate three distinct populations of rooster sperm by their quality. We were able to collect low-, medium- and high-quality sperm. We also describe reproducible protocols that entail determining fertility potential of sperm by assessing their viability, mobility and penetrability. Collection of sperm by their quality using PDGC technique would be useful to accurately and thoroughly characterize sperm with differential fertility potential.

Video Link

The video component of this article can be found at https://www.jove.com/video/58833/

Introduction

In vertebrates, male gametes undergo intense selective pressure; therefore reproductive fitness of a male is pivotal for achieving successful fertilization. Males of any given vertebrate species must be able to produce sperm cells in large quantities and of sufficient quality in order to meet the needs of fertilization. Sperm cells, having both a sperm head and a flagellum, are the most polarized cells in the body. They are also very heterogeneous in quality of sperm (live and dead, morphologically normal and abnormal, and immobile, low mobile and high mobile), which is revealed through the wide variation in reproductive efficiency of the males. The larger the proportion of high-quality sperm, the fewer the number of matings required to successfully fertilize the ovum. However, to achieve fertility, morphologically normal sperm cells rely on propulsive forces generated by their flagella to reach the site of fertilization as well as to penetrate the zona pellucida[1] (ZP; in the case of mammals) or inner perivitelline layer[2] (IPVL; in the case of birds and reptiles) of the ovum following natural mating or artificial insemination (AI). Determining sperm quality is necessary for use in assisted reproductive technologies[3] (ART) and selection of breeding males to be used in AI programs[4]. On the other hand, the success of ART solely relies upon the accurate evaluation of sperm quality. A number of laboratory tests have been developed to determine the functional characteristics of sperm. The most important parameters are sperm morphology, viability, mobility, capacitation (avian sperm do not require capacitation[5]), acrosome reaction (AR; exocytosis and release of a proteolytic enzyme from the acrosome of the sperm head), sperm penetration of ZP or IPVL, and fertilization[6,7,8,9,10,11]. Measures of fertility alone do not provide an accurate evaluation of the fertilizing ability of a sperm population[11]. Measures of the several events leading up to fertilization of an egg allow for an appropriate representation of the performance of individual spermatocytes[7].

The methodology developed for measuring sperm function is primarily species-specific. For example, in avian sperm, viability, mobility and penetration of IPVL are the most common parameters used to assess sperm quality[8,11,12]. The number of live sperm in the ejaculate plays a crucial role for the survival of sperm because the presence of a large number of dead sperm in the semen affects the quality of sperm. This enhances the production of reactive oxygen species in the semen and causes oxidative damage to the live sperm[13]. Sperm mobility, the capacity for flagellar movement of avian sperm against resistance at body temperature, is known to play a direct role in bringing about fertilization[8]. It is well established that mobility is positively correlated with fertility and is, therefore, a primary determinant of fertility[8]. However, a mobile sperm must also have the ability to undergo an AR and to penetrate the IPVL[11]. IPVL penetration assays take account for every sperm that participates in the process of fertilization[11].

In the application of ART, ejaculate is usually processed in order to maximize the concentration of high-quality sperm and minimize concentration of low-quality sperm. After collection of semen, the proportion of high-quality sperm can be enriched through sperm separation procedures commonly used in both industry and research practices. Many of these procedures have been developed, all with respective benefits and limitations, but all utilize the heterogeneous nature of sperm to collect only the sperm with high fertilizing ability. These procedures include sperm migration methods, adherence column filtration and density gradient centrifugation (DGC)[14,15,16,17,18,19,20]. Among the available techniques, DGC has been found to be very simple, repeatable, cost-effective and efficient in isolating the maximum amount of high-quality sperm for use

in ART with the goal of maximizing chance of fertilization[14,15]. In addition, DGC is not injurious to the sperm cell membrane. In contrast, sperm migration methods collect only progressively mobile sperm[18,19], but the quantity of sperm collected is very low, making it inefficient in collecting large volumes of sperm[18,20]. Adherence column filtration is very efficient in filtering highly mobile sperm from semen[17]; however, it tends to be injurious to sperm membranes[20,21].

In the DGC technique, the most commonly used substrate for generating the density gradient is Percoll, which consists of colloidal silica particles coated in polyvinylpryrolidone. Percoll density gradient centrifugation (PDGC) can either be continuous or discontinuous but a discontinuous gradient is most commonly used for high yield isolation of highly mobile sperm[13,16,20]. In a discontinuous gradient, lower density media floats above higher density media, creating a gradient that increases in density from the top to the bottom of a conical tube. This creates boundaries at the interface between the two media of differing density. The efficiency of PDGC is derived from two factors: 1) the propulsive ability of individual sperm cells and 2) the tendency of sperm cells with high structural integrity to have an increased density. Sperm with higher mobility are better able to cross from lower density media and penetrate into a higher density media. Lower mobility sperm are more likely to become trapped at the boundary created by the interface between media of differing density. Sperm cells with high structural integrity and mobility tend to have a higher density than dead, abnormal or low mobile sperm cells. When centrifugal force is applied in PDGC, this facilitates movement of sperm with different densities to their respective place in the gradient.

In general practice, after PDGC is performed, the soft pellet of sperm with high fertility potential at the bottom of the conical tube is collected, and the remainder is discarded. However, an underutilized advantage of this technique is its ability to separate sperm cells into several groups based on the quality differences. For research purposes, separation of sperm by degree of quality utilizing the PDGC technique allows for study of sperm quality as it pertains to physiologic, metabolomic and proteomic differences. Here, we aim to detail how this technique may be used to separate sperm by quality, as well as demonstrate these differences in quality, using the previously established eosin-nigrosin vital staining for viability, Accudenz assay for mobility, and sperm-IPVL interaction assay for penetrability.

Protocol

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Georgia.

1. Washing using Traditional Centrifugation

1. Prepare phosphate buffer solution (PBS). Add 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 to 800 mL of distilled water (dH_2O). Adjust the pH to 7.4 using 0.1 N HCl and bring the solution to 1 L using dH_2O.
2. Prepare motility buffer. Add 6.5 g of NaCl, 4.5 g of glucose, 0.444 g of $CaCl_2$ and 11.5 g of N-tris-[hydroxymethyl] methyl-2-amino-ethanesulfonic acid (TES) to 800 mL of dH_2O. Adjust the pH to 7.4 using 1 M NaOH and bring the solution to 1 L using dH_2O.
3. Pipette 0.5 mL of semen into a polypropylene microcentrifuge tube. Add 1.0 mL of PBS and mix gently.
4. Centrifuge at 1,500 x g for 10 min at room temperature (RT) and discard the supernatant. Resuspend the sperm pellet with PBS up to 1.5 mL.
5. Centrifuge at 1,500 x g for 10 min at RT. Resuspend the sperm pellet with motility buffer up to 0.5 mL.

2. Performing the PDGC technique

NOTE: Perform the entire process of PDGC at room temperature.

1. Make 3.0 mL of 1.08 g/mL and 1.07 g/mL Percoll solutions in two separate tubes.
    1. In a clean test tube, add 1.712 mL of the 1.13 g/mL original Percoll to 0.3 mL of 1.5 M NaCl solution. Add 0.988 mL of dH_2O and mix by gentle inversion to make 3.0 mL of a 1.08 g/mL density solution.
    2. In a clean test tube, add 1.482 mL of the 1.13 g/mL original Percoll to 0.3 mL of 1.5 M NaCl solution. Add 1.218 mL of dH_2O and mix by gentle inversion to make 3.0 mL of a 1.07 g/mL density solution.
2. In a clean test tube, dilute 1.0 mL of semen sample 1:2 with 2.0 mL of PBS. Mix gently by pipetting.
3. Pipet 3.0 mL of the 1.07 g/mL density solution into a sterile 15 mL conical tube. Carefully pipet 3.0 mL of the 1.08 g/mL density solution beneath the 1.07 g/mL density solution. Ensure that the two layers do not mix. A long-form (9 in) Pasteur pipette can make this step easier.
4. Pipet 3.0 mL of diluted semen sample overtop the PDG. To ensure that the semen sample does not mix with the PDG, gently tilt the conical tube containing the PDG at a 45° angle. Pipet the sample along the wall of the tube and allow it to flow down the tube and over the PDG.
5. Prepare a blank tube to match the mass of the PDG with overlaid sample. Centrifuge both tubes at 1500 x g for 20 min. Be careful to maintain the discontinuous gradient while transferring the tubes from the bench to the balance and then to the centrifuge.
   NOTE: Do not use the brake at the end of centrifugation.
6. Observe the results. Ensure that three distinct semen layers have formed in the tube, as seen in **Figure 1**.
7. Aspirate isolated semen layers with a pipette. Collect the top layer of semen first, the middle layer second, and last the hard pellet at the bottom of the tube. Transfer each to a clean and sterile polypropylene*microcentrifuge tube*.
8. Dilute each sample to 1.5 mL with PBS. Centrifuge at 1500 x g for 10 min.
9. Pour off the supernatant. Reconstitute sperm pellet with motility buffer by gentle pipetting.
   NOTE: Alternative densities may be used to suit investigator needs. Determine amounts of ingredients used with the following equation, where v_0 is volume of stock density solution used, v is final volume of solution desired, p is density of final density solution desired and p_0 is the density of the stock density solution:
   $$v_0 = v * \frac{p - 1.0058}{p_0 - 1}$$
   Always use 0.3 mL of 1.5 M NaCl in preparation of density solutions to match the NaCl concentration of physiological saline.

3. Determining Sperm Quality

1. Calculate the sperm concentration as previously described[22].
2. Perform eosin-nigrosin vital staining as previously described[12] with the following modifications:
    1. Prepare 100 µL of sperm solution at a concentration of 1 x 10^8 cells/mL.
    2. Pipet 50 µL of sperm solution in a polypropylene microcentrifuge tube containing an equal volume of eosin-nigrosin stain. Incubate the mixture for 5 min at room temperature.
    3. Place a 20 µL drop of stained sperm sample at one end of a glass slide and smear uniformly in a manner similar to that used for blood smears. Air-dry the smeared slides at room temperature for 3-5 min.
    4. Observe the smear under microscope. Count the number of live sperm (no stain) and dead sperm (stained pink) and calculate the percentage of live sperm.
3. Perform the Accudenz assay, as previously validated for the chicken sperm, to objectively assess the sperm mobility[8] with the following modifications:
    1. Pipet 1.0 mL of 6% assay solution into polystyrene cuvettes, as illustrated in **Figure 2**. Incubate to 41 °C.
    NOTE: 41 °C is used to match the internal temperature of a hen. The incubation temperature should match that of the female reproductive tract of the species being investigated.
    2. Overlay the preheated assay solution with 100 µL of semen sample at a concentration of 5 x 10^8 cells/mL.
    3. Place the cuvette containing overlaid sperm sample in the spectrophotometer. Record the absorbance value at 550 nm.
4. Perform IPVL-penetration assay as previously described[11] with the following modifications:
    1. Cut a piece (0.5 cm x 0.5 cm) of non-germinal disc region of intact IPVL.
    2. Adjust sperm concentration to 4 x 10^6 cells/mL
    3. Incubate sperm in motility buffer with IPVL in a small glass vial for 15 min at 37 °C, as illustrated in **Figure 3**.
    4. Immerse the IPVL piece in 3% NaCl to stop the interaction between the IPVL and sperm.
    5. Mount the IPVL piece on a microscope slide and stain with Schiff's reagent for 10 min following fixation with 10% formalin for 20 s.
    6. Observe the IPVL under a microscope for successful sperm penetration holes and count the number of all visible holes per 0.25 mm^2 at 40X magnification.

Representative Results

The PDGC technique resulted in distinct separation of three layers of sperm by degree of quality across all parameters. Sperm separates into a high-quality layer below the higher density solution, a medium-quality layer between the higher and lower density solution and a low-quality layer above the lower density solution. These differences in quality are evidenced by clear differences in viability (**Figure 4**), mobility (**Figure 5**) and penetrability (**Figure 6**). Sperm isolated from the high-quality layer of the PDG exhibited increases in all three parameters relative to those of the traditionally washed sample. Those layers noted as medium- and low-quality upon separation by PDGC display moderate and dramatic, respectively, decreases across all test parameters.

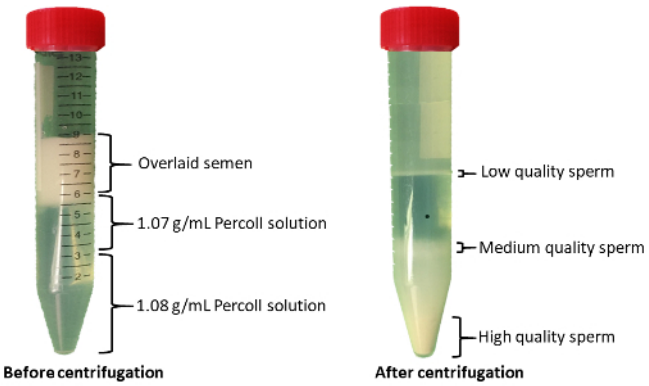

Figure 1: Isolation of sperm with differential quality using PDGC. Overlay 3 mL of sperm solution on prepared PDG solution. Centrifuge at 1500 x g for 20 min. Collect three distinct groups of sperm with low-, medium- and high-quality. Please click here to view a larger version of this figure.

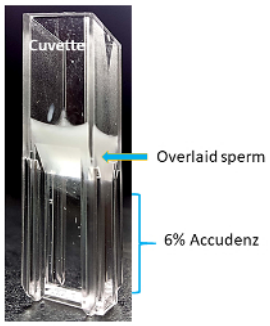

Figure 2: Determination of sperm mobility using Accudenz assay. Overlay 100 µL of sperm sample on a 6% assay solution in a cuvette. Incubate at 41 °C for 5 min. Record the mobility of sperm as a function of absorbance at 550 nm. Please click here to view a larger version of this figure.

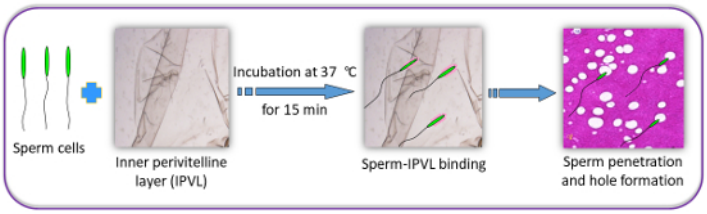

Figure 3: A model of formation of sperm penetration holes in IPVL. Incubate sperm with a small section of IPVL at 37 °C for 15 min. Upon contact with IPVL, sperm cells bind with the IPVL, undergo an acrosome reaction and penetrate the IPVL, creating penetration holes. Count the number of penetration holes and measure the penetrability of sperm. Please click here to view a larger version of this figure.

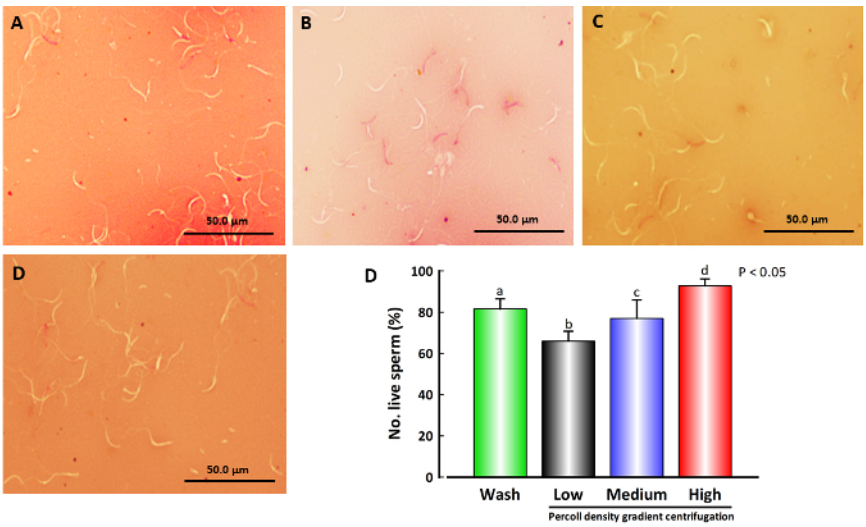

Figure 4: Viability of sperm as assessed by eosin-nigrosin vital staining. Digital micrographs showing eosin-nigrosin vital staining of sperm obtained through a process of traditional wash (A) and PDGC (B, low motile sperm; C, medium motile sperm; D, high motile sperm). Scale bar = 50 µm at 40 x magnification. Pink staining indicates dead sperm which have taken up eosin stain. Data were subjected to one-way ANOVA, followed by the Tukey-Kramer test. Error bars indicate the standard error of the mean (SEM). Values are presented as the mean ± SEM (n = 5). $^{a-d}$ Values without a common superscript differed (P < 0.05). Sperm isolated by PDGC into low-, medium- and high-quality groups (E) reveal significant differences in percentage viability, 66.0 ± 4.7, 77.0 ± 8.9 and 92.8 ± 3.4, respectively. Sperm washed by traditional methods displayed an 81.6 ± 4.9% viability, lower than the high-quality group but higher than the medium- and low-quality groups. Please click here to view a larger version of this figure.

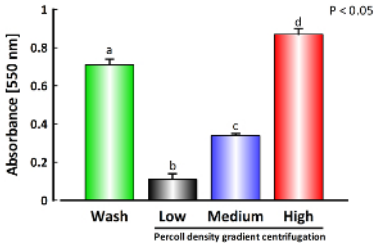

Figure 5: Mobility of sperm as assessed by Accudenz assay. Absorbance values act as a proxy measure for sperm mobility in assessment by the Accudenz assay. Data were subjected to one-way ANOVA, followed by the Tukey-Kramer test. Error bars indicate the standard error of the mean (SEM). Values are presented as the mean ± SEM (n = 5). [a-d] Values without a common superscript differed (P < 0.05). Sperm isolated into low-, medium- and high-quality groups by the PDGC technique exhibited markedly different low (0.11 ± 0.03), medium (0.34 ± 0.01) and high (0.87 ± 0.03) absorbance values, respectively. Sperm washed by traditional methods exhibited an absorbance of 0.71 ± 0.03, an absorbance lower than that of the high-quality group, but higher than that of the medium- and low-quality groups. Please click here to view a larger version of this figure.

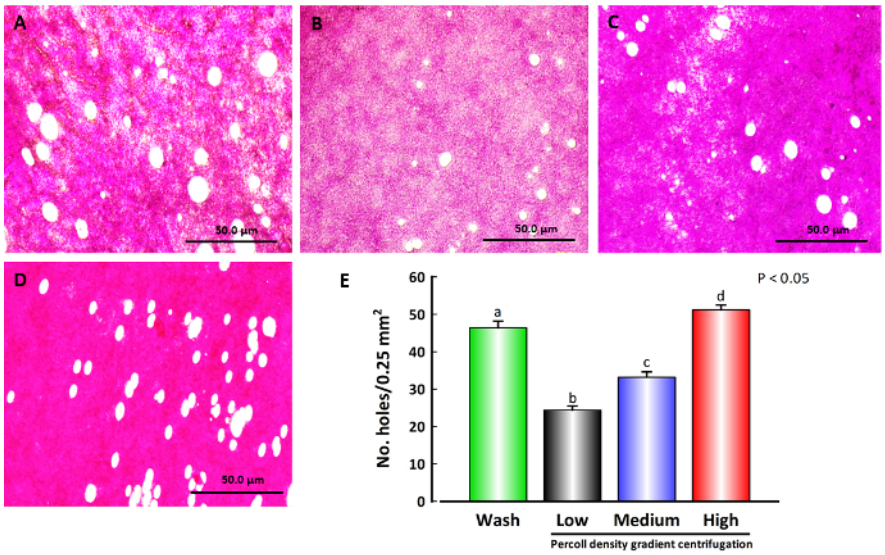

Figure 6: Penetrability of sperm as assessed by sperm-inner perivitelline layer (IPVL) interaction assay. Digital micrographs showing *in vitro* formation of holes on the surface of the IPVL by chicken sperm (4 x 10^6 cells/mL) during incubation in motility buffer at 37 °C for 15 min. Micrographs represent IPVL penetrated by sperm having undergone traditional wash (A) and sperm from low- (B), medium- (C) and high- (D) quality groups from PDGC. Scale bar = 50 µm at 40X magnification. Data were subjected to one-way ANOVA, followed by the Tukey-Kramer test. Error bars indicate the standard error of the mean (SEM). Values are presented as the mean ± SEM (n = 5). [a-d] Values without a common superscript differed (P < 0.05). The number of penetration holes per 0.25 mm^2 (E) differed between the sperm isolated from the different gradients of the density media. Sperm isolated into low-, medium- and high-quality groups by PDGC produced a low (24.40 ± 1.1), medium (33.20 ± 1.4) and high (51.20 ± 1.3) number of penetration holes, respectively. The sperm washed using the traditional method produced 46.40 ± 1.8 penetration holes, a value lower than the high-quality group but higher than the medium- and low-quality groups. Please click here to view a larger version of this figure.

Discussion

Fertility not only determines the profitability of animal production but also acts as a means of natural selection of species for existence. The ultimate function of a sperm cell is to fertilize an ovum. The oviduct of a female selects only those fittest sperm in order to ensure fertilization of the ovum[23,24]. *In vitro* studies have also revealed a close correlation between qualitative sperm traits and fertilization success[4,11,23,24]. Reduced fertility is associated with poor quality of sperm[4,11,25]. As such, it necessitates processing semen for improvement of sperm quality. In ART, PDGC has been applied to collect only the high-quality sperm to supplement fertility of humans and agriculturally-important animals[13,16,20,26]. Using this technique, we, however, demonstrated a gentle, non-invasive technique of separating low-, medium- and high-quality sperm in the domestic chicken model.

The presented protocol of PDGC, when performed correctly, allows separation of not only high-quality sperm, but also effective separation of low- and medium-quality sperm. The degree of success of PDGC is reliant on careful preparation of the discontinuous gradient as well as equally careful collection of isolated layers. Diameter of the conical tube used also influences success of PDGC. We have observed that a larger tube diameter results in decreased efficiency of the technique. It is important that PDGC is performed at room temperature in order to maintain integrity of the PDG. Any previously refrigerated samples should be adjusted to room temperature before being placed on the PDG. The most common issue that could be observed after performing PDGC is inefficient separation; the isolated layers will not be distinct from one another.

If this issue occurs, in addition to addressing the above critical steps, it may be corrected by reducing sample dilution, increasing the volumes of density solutions used and ensuring the volumes of solutions used are equal.

PDGC is very adaptable. Any range of densities between 1.00 and 1.13 g/mL may be used for preparation of the gradient. We have found that 1.07 and 1.08 g/mL density solutions work well for separation of chicken sperm by quality, but these densities may need adjustment for other sample types or experimental purposes. The number of density solutions used in the gradient may also be adjusted to suit the needs of the experimenter. The number of quality groups isolated will always be one more than the number of density solutions used.

PDGC is much more effective than alternative methods of sample separation in situations where large quantities of sample are needed[13,16,20,26]. Migration assays effectively separate the highest quality sperm from a sample[18,19], but they cannot be used to resolve the sample any further. Migration assays are also incapable of preparing large volumes of sample[18,20]. Adherence column assays effectively separate large volumes of sample[17]; however, the process is deleterious to the quality of the sperm collected, making confirmation of the quality groups after separation difficult[20,21]. PDGC, however, does have its own limitations. In the case of mammalian sperm, it should not be used for preparation of samples for *in vitro* fertilization, due to the possible contamination with endotoxins[27]. The effect of Percoll contamination with endotoxins has not been investigated in other animal classes. Sperm performance is not affected by endotoxins, meaning the PDGC is still appropriate for research purposes.

Collection of sperm by their quality using the PDGC technique will be instrumental for a wide range of applications including studies involving sperm physiologic, metabolomic, transcriptomic and proteomic research. Application of this technique will contribute to the discovery of biomarkers of sperm quality for usage in marker-assisted fertility improvement programs.

Disclosures

The authors have nothing to disclose.

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