Archives of Razi Institute Journal
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Research Paper

Diagnostic Performance of RT-PCR for Fetal Sex Determination Using Cell-free Fetal DNA in Maternal Plasma During the Last Third of the First Trimester in Mares

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How to cite this article Tabatabaee S, Ahmadi-hamedani M, Narenji Sani R, Staji H. Diagnostic Performance of RT-PCR for Fetal Sex Determination Using Cell-free Fetal DNA in Maternal Plasma During the Last Third of the First Trimester in Mares. Archives of Razi Institute Journal. 2026; 81(4):909-916. https://doi.org/10.32598/ARI.81.4.3734

https://doi.org/10.32598/ARI.81.4.3734

Article info:
Received: 11 Jan 2026
Accepted: 15 Mar 2026
Published: 01 Jul 2026

Keywords:
cffDNA, Fetal sex determination, Mare, RT-PCR, *SRY* gene

ABSTRACT

Introduction: Accurately determining fetal sex early in mares is essential for horse breeding. In this study, we investigated the detection of cell-free fetal DNA (cffDNA) in horse plasma using real-time polymerase chain reaction (RT-PCR) during pregnancy.

Materials & Methods: Blood samples were obtained from 30 pregnant mares in the last third of the first trimester of gestation at stables located in the provinces of Semnan, Golestan, and Tehran. In addition, blood samples were taken from one male foal and one female foal with no history of mating to serve as positive and negative controls, respectively. The phenotypic sex of the foals was recorded after parturition and compared with RT-PCR results performed on maternal plasma to evaluate the accuracy of fetal sex determination using the sex-determining region Y (SRY), equine Y-linked microsatellite marker YH12 (Eca.YH12), and X-linked microsatellite locus TKY270 markers.

Results: A total of 30 pregnant mares were sampled, with 27 carrying to term and three aborting. Fetal sex was confirmed postnatally (11 males, 16 females). The SRY marker had a sensitivity of 72.7%, specificity of 100%, and accuracy of 88.9%. The Eca.YH12 marker yielded the same results as *SRY* and did not enhance sensitivity when used in combination. The X-linked marker TKY270 was detected in all samples, indicating that the samples were of high quality and could serve as an internal control. These markers supported our results, strengthening the diagnostic process.

Conclusion: RT-PCR for the *SRY* gene detected cffDNA in the last third of the first trimester and worked well, especially for identifying female fetuses. These findings were further supported by the results obtained from the Eca.YH12 and TKY270 genetic markers.

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1. Introduction

Sex determination is a fundamental biological process in all sexually reproducing eukaryotes [1]. In many species, sex is genetically established through the expression of one or more genes located on the sex chromosomes. Among vertebrates, the most common systems are male heterogamety (XX females and XY males) and female heterogamety (ZW females and ZZ males). Sex chromosomes have evolved independently multiple times, typically emerging when a member of an autosomal pair acquires a gene or allele responsible for sex determination. The frequency of new sex chromosome emergence varies greatly across species. Notably, frequent turnovers, defined as changes in the location or identity of sex-determining genes, have been observed in several taxa, including many species of fish and amphibians [2, 3]. Over the years, scientists have developed several methods to determine genetic sex, including karyotyping, testing for H-Y antigens, and analyzing enzymes associated with the X chromosome [4]. In addition to molecular methods, some studies have examined hormonal indicators such as maternal testosterone concentration to predict the sex of the fetus in horses [5]. However, polymerase chain reaction (PCR)-based methods have taken the lead, since they are faster, more accurate, and more reliable than the older techniques. Additionally, PCR is relatively easy to use and budget-friendly, making it a popular choice—especially in commercial settings [4]. Fetal DNA fragments, originating from the trophoblastic cells covering the chorionic villi, are released into the maternal circulation following trophoblast apoptosis. Additionally, mRNA and DNA fragments derived from placental cell apoptosis contribute to a minor portion of the fetal genetic material detectable in maternal blood [6]. It has been well established that circulating cell-free fetal DNA (cffDNA) can be detected in maternal blood throughout all stages of pregnancy [7]. cffDNA becomes detectable as early as the fifth week of gestation and remains present until parturition [8]. Due to its short half-life (approximately 16.3 minutes), cffDNA is rapidly cleared from the maternal bloodstream within a few hours postpartum, minimizing interference from previous pregnancies. This transient nature makes cffDNA an ideal target for non-invasive prenatal genotyping studies [9]. De Leon et al. (2012) [10] provided the first evidence of circulating cffDNA in the plasma of pregnant mares, opening a new avenue for non-invasive prenatal diagnostics, including fetal sex determination. Molecular techniques based on cffDNA in maternal blood present a highly accurate and non-invasive alternative to traditional invasive procedures. Beyond sex determination, these methods have the potential to facilitate early diagnosis of genetic disorders and pregnancy-related complications [11].

Early, non-invasive determination of fetal sex in horses has the potential to become a valuable tool in equine breeding and genetic diagnostics. By analyzing cffDNA present in maternal plasma, fetal sex can be identified during the first trimester of gestation. Determining a foal's sex early is economically crucial in the horse world, as it significantly impacts a pregnant mare's value. This study investigates the diagnostic performance of real time-PCR (RT-PCR) to detect cffDNA in the maternal plasma of pregnant mares during the last third of the first trimester of gestation.

2. Material and Methods

2.1. Study population

The study population consisted of horse breeding farms located in the provinces of Semnan, Golestan, and Tehran. Sampling was conducted over a 16-month period (two phases), from December 2019 to March 2021. A total of 30 pregnant mares were examined to determine fetal sex during the last third of the first trimester of pregnancy. The phenotypic sex of the fetus (male or female) was recorded after parturition and used to evaluate the accuracy of the test.

2.2. Sampling

Blood samples were collected from 30 pregnant mares during the last third of the first trimester of gestation to determine fetal sex. Additionally, blood was drawn from one male and one female foal with no prior breeding history to serve as positive and negative control samples, respectively. The actual fetal sex (male or female) was recorded after parturition to assess the accuracy of the test. Blood was collected from the jugular vein and transferred into tubes containing ethylenediaminetetraacetic acid (EDTA) anticoagulant. The samples were quickly transported to the laboratory on ice. To isolate the plasma, the EDTA-treated blood samples were centrifuged at 4,000 rpm for 10 minutes.

2.3. Polymerase chain reaction analysis

The separated plasma was stored at -80 ˚C until DNA extraction. DNA was extracted from the plasma samples, as well as from the genomic DNA of the control male and female blood samples, using a commercial DNA ex-

traction kit (GeNet Bio, Korea). The amount and purity of the extracted DNA were assessed using a NanoDrop spectrophotometer. To identify the *SRY* gene marker, PCR was carried out on DNA obtained from the plasma of pregnant mares. The primers used for amplification of the *SRY* gene were forward primer 5′-CGCCAGCATAGATCACAGAA-3′ and reverse primer 5′-CGCAAGGTAGCTGAAAGACC-3′, as previously described by De Leon et al. (2012) [10].

To verify the reliability of the *SRY* gene detection, additional genetic markers were included in the analysis: ECAY (a Y-chromosome-specific marker), ECAX (an X-chromosome marker), and an autosomal marker serving as a positive control for PCR. Specifically, the Eca.YH12 marker (291 bp), a Y-linked sequence specific to horses, was used as a secondary indicator of male DNA; TKY270 (274 bp) was used to detect X-chromosomal sequences; and the *GAPDH* gene (150 bp), a commonly used housekeeping gene, served as an autosomal internal control.

The primer sequences for these markers were as follows:

The primers used for TKY270 were 5′-CTGCTTTAGAGAAACAAACT-3′ (forward) and 5′-CCATGGTGAGAAAAATGAGA-3′ (reverse), as described by Wallner et al. [12]. For *GAPDH*, the forward primer was 5′-GCCGTAACTTCTGTGCTGTG-3′, and the reverse primer was 5′-AATGAAGGGGTCATTGATGG-3′, as reported by Kakoi et al. [13].

For *GAPDH*, the primers used were 5′-GCCGTAACTTCTGTGCTGTG-3′ (forward) and 5′-AATGAAGGGGTCATTGATGG-3′ (reverse), as described by De Leon, Campos [10].

2.4. Statistical analysis

The outcomes of RT-PCR fetal sex determination were systematically compared with the phenotypic sex identified at birth to evaluate test performance. The results were subsequently presented as percentages to facilitate clear interpretation. To evaluate the performance of the *SRY* gene detection method in the last third of the first trimester of gestation, the following metrics were calculated using SAS software (version 9.1, SAS Institute, 1997): sensitivity, specificity, positive predictive value, negative predictive value, overall accuracy, and method efficiency.

3. Results

A total of 30 blood samples were collected from mares during the latter portion of the first trimester of gestation, with pregnancy confirmation achieved through ultrasonography. Among these mares, 27 successfully delivered foals, while three unfortunately experienced abortions. The findings related to mare parturition, along with the outcomes of gene amplification for *SRY*, TKY270, Eca.YH12, and *GAPDH*, derived from the analyzed blood samples, are detailed in Table 1.

An analysis of fetal sex following parturition indicated the presence of 11 male foals and 16 female foals. A comparison between the postnatal sex and the gene amplification results for *SRY* and TKY270 was not conducted for the three mares that aborted. In male foals (n=11), SRY, a Y-chromosome marker, was amplified in 8 of 11 cases (72.7%), with no amplification in the remaining three. Eca.YH12, another Y-linked marker, showed identical results. The X-linked marker TKY270 and the autosomal control *GAPDH* were amplified in all male samples. In female foals (n=16), neither SRY nor Eca.YH12 was amplified, but both TKY270 and GAPDH were present in all samples. These findings confirm that TKY270 and *GAPDH* are reliable internal controls for DNA quality and amplification (Table 1). Diagnostic performance was then calculated. Using *SRY* alone, sensitivity for detecting male fetuses was 72.7%, specificity was 100%, positive predictive value (PPV) was 100%, negative predictive value (NPV) was 84.2%, and overall accuracy was 88.9% (Table 2).

When combining *SRY* and Eca.YH12 in an "either/or" approach (male if any Y marker was positive), the results were identical in this dataset, as Eca.YH12 did not rescue the three *SRY*-negative male cases (Table 3).

Figures 1 and 2 illustrate the amplification curves for samples containing the *SRY* and TKY270s genes in male fetuses. In contrast, Figure 3 presents the amplification curve for samples with the Eca.YH12 gene in female fetuses.

4. Discussion

The presence of cell-free nucleic acids in blood was first reported by Mandel and Metais in 1948. Although initially regarded as a significant scientific discovery, it received limited attention until 1994, when the detection of mutated *RAS* gene fragments in the plasma of cancer patients brought renewed interest to the field [14]. Lo et al. [9] successfully isolated fetal-derived

Table 1. Results of mare parturition and the outcomes of gene amplification for *SRY*, TKY270, Eca.YH12, and *GAPDH* from blood samples collected from pregnant mares

Female					Male				
No	***SRY***	**TKY270**	**Eca.YH12**	***GAPDH***	**No**	***SRY***	**TKY270**	**Eca.YH12**	***GAPDH***
1	-	+	-	+	1	-	+	-	+
2	-	+	-	+	2	+	+	+	+
3	-	+	-	+	3	+	+	+	+
4	-	+	-	+	4	+	+	+	+
5	-	+	-	+	5	-	+	-	+
6	-	+	-	+	6	+	+	+	+
7	-	+	-	+	7	+	+	+	+
8	-	+	-	+	8	-	+	-	+
9	-	+	-	+	9	+	+	+	+
10	-	+	-	+	10	+	+	+	+
11	-	+	-	+	11	+	+	+	+
12	-	+	-	+					
13	-	+	-	+					
14	-	+	-	+					
15	-	+	-	+					
16	-	+	-	+					

Table 2. Fetal sex determination in 27 pregnant mares using RT-PCR targeting the *SRY* and Eca.YH12 genes from cffDNA in maternal plasma during the last third of the first trimester of gestation and confirmed after parturition

Marker	True Positive (Male Correctly Identified)	False Negative (Male Misclassified)	True Negative (Female Correctly Identified)	False Positive (Female Misclassified)	Total
SRY	8	3	16	0	27
Eca.YH12	8	3	16	0	27

Table 3. Sensitivity, specificity, PPV, NPV, and accuracy of fetal sex determination based on the detection of cffDNA by RT-PCR during the last third of the first trimester of gestation in pregnant mares

Marker	%				
	Accuracy	**Sensitivity**	**Specificity**	**PPV**	**NPV**
SRY	88.9	72.72	100	100	84.21
Eca.YH12	88.9	72.72	100	100	84.21

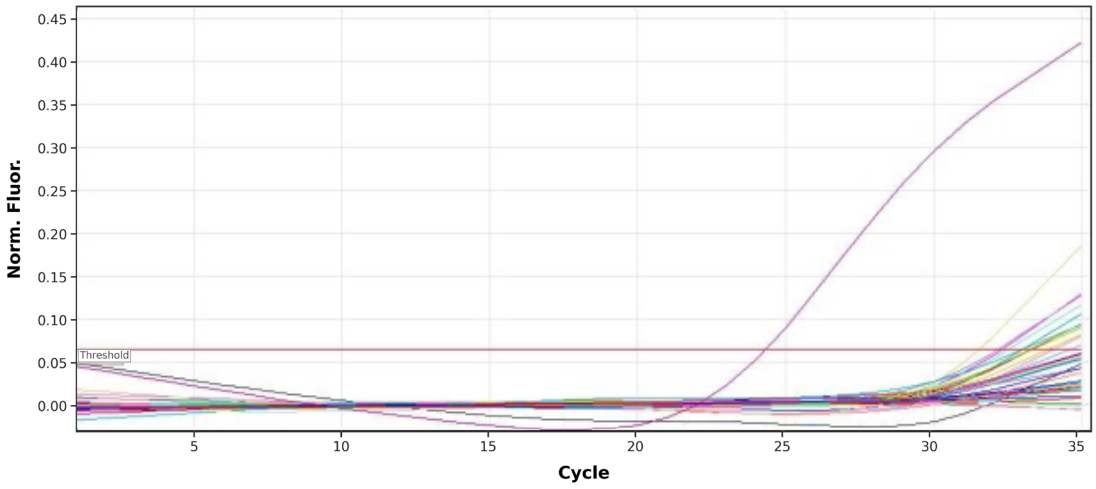

Figure 1. RT-PCR analysis of some fetal DNA samples extracted from maternal plasma: Amplification plot of *SRY*

nucleic acids from maternal plasma using Y-chromosome-specific sequences. They demonstrated that these nucleic acids could be detected in maternal blood as early as the initial weeks of gestation and were rapidly cleared postpartum, minimizing interference from previous pregnancies. This fetal DNA enters maternal circulation primarily through apoptosis and immunologic or physical degradation of placental and fetal cells.

Molecular approaches utilizing fetal DNA in maternal plasma provide a non-invasive and reliable alternative to traditional invasive procedures. Such techniques not only enable prenatal sex determination but also have broader applications in detecting genetic abnormalities and pregnancy-related complications [11]. In equine reproduction, non-invasive fetal sexing based on the analysis of cffDNA extracted from maternal plasma is a promising tool that can contribute to early genetic diagnosis. De Leon et al. [10] introduced a notable approach for fetal sexing in horses using cffDNA. In their study, *SRY* gene amplification via PCR in 20 pregnant mares during the third trimester yielded 72.7% sensitivity and 85% overall accuracy. When second-round PCR and quantitative PCR (qPCR) were employed, these values improved to 90.9% sensitivity and 95% accuracy, respectively.

Kadivar et al. [15] further expanded this research by employing RT-PCR to detect SRY in 28 pregnant Arabian mares between 8 and 20 weeks of gestation. The test achieved an overall accuracy of 88%, with 85.7% sensitivity and 90.9% specificity. The PPV and NPV were reported at 92.3% and 83.3%, respectively.

Similar advancements have been reported in bovine fetal sex prediction using maternal plasma DNA. JF [16] successfully identified fetal sex by amplifying the *SRY* gene in pregnant cows between days 30 and 59 of gesta-

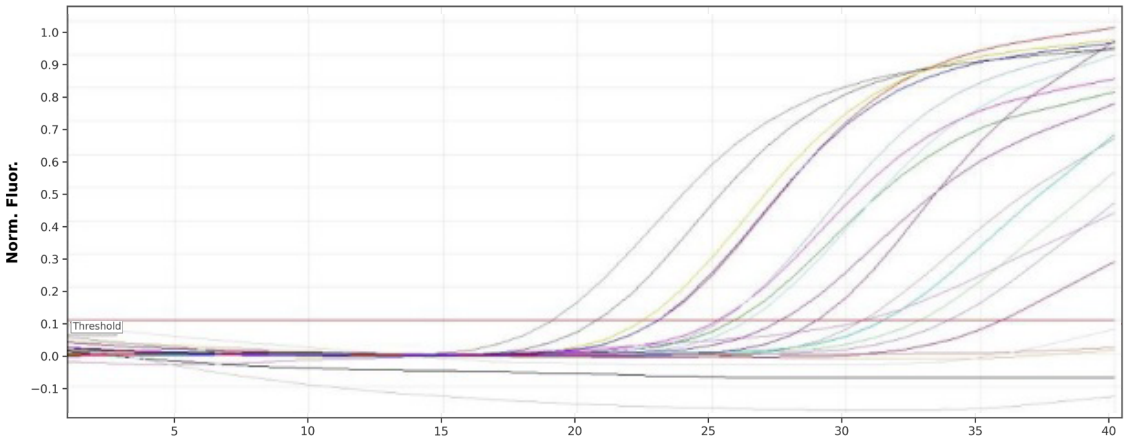

Figure 2. RT-PCR analysis of some fetal DNA samples extracted from maternal plasma: Amplification plot of TKY270

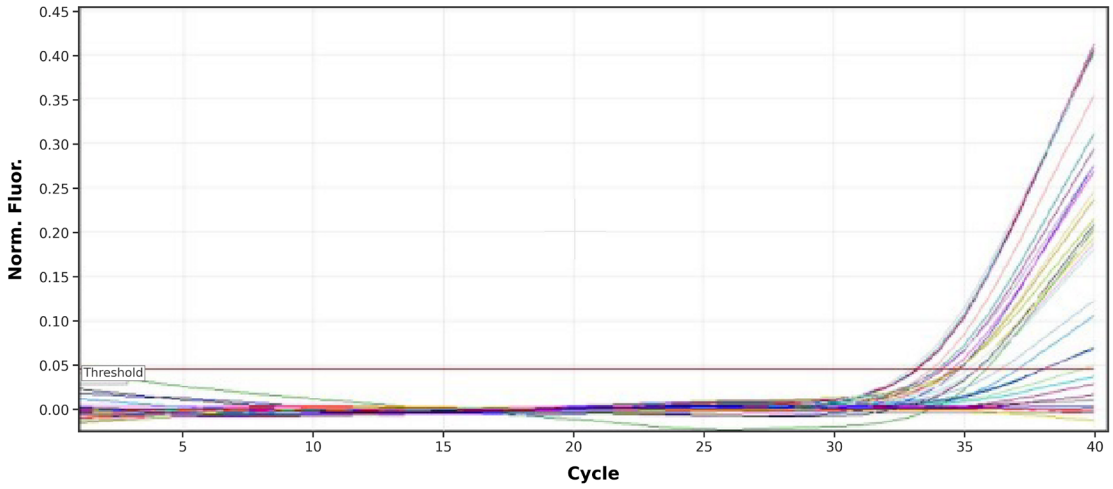

Figure 3. RT-PCR analysis of some fetal DNA samples extracted from maternal plasma: Amplification plot of Eca.YH12

tion, with an overall accuracy of 60%. Later, Wang et al. [17] employed nested PCR on 110 plasma samples from pregnant cows and reported fetal sexing accuracies of 100% for male fetuses (43/43) and 91% for female fetuses (61/67). Likewise, Da Cruz et al. [18] achieved 88.6% accuracy in sex prediction by amplifying Y-specific sequences in plasma from 35 pregnant cows. The estimated accuracy rose to 99.9% when testing was conducted at or after day 55 post-fertilization.

Further validation was provided by Lemos et al. [19], who used *TSPY* gene primers to accurately match PCR results with phenotypic sex in all 47 male and 37 female bovine fetuses studied.

In sheep, Kadivar et al. [20] reported the successful application of RT-PCR targeting the *SRY* gene for sex determination in 46 pregnant ewes between the second and fifth months of gestation. Their approach showed 100% sensitivity and specificity, with no false positives or negatives. Quantitative RT-PCR further demonstrated a significant increase in fetal DNA levels as pregnancy progressed. Similarly, biometric variations in the genitalia of other livestock species, such as the Arbia goat, have been examined to enhance understanding and diagnosis of reproductive status and pregnancy [21]. Kadivar et al. [22] introduced a novel approach using the amelogenin gene for fetal sexing in sheep. In a group of 45 pregnant ewes (8–18 weeks of gestation), the test demonstrated 96.5% sensitivity, 87.5% specificity, and 93.3% overall accuracy. The relative quantification showed a significantly higher level of cffDNA in ewes beyond 12 weeks of gestation compared to earlier stages.

Our findings revealed that no false-positive results occurred in fetal sex determination using cffDNA during the last third of the first trimesters, aligning with previous research in mares [23, 4] and ewes [20, 22], which also utilized the detection of *TKY270* and *Eca.YH12* gene fragments alongside the *SRY* marker. However, some other studies have reported false-positive results [10, 13, 15, 16]. On the other hand, the false-negative results we found were similar to those reported in previous studies [10, 13, 15, 23]. Interestingly, these false negatives became less common as pregnancy progressed, which helped improve the accuracy of the test. This trend was also supported by our observation that the *Eca.YH12* gene fragment was absent in every sample that gave a false-negative result.

Detection of cffDNA using an RT-PCR assay targeting the *SRY* gene during the last third of the first trimester of pregnancy in the studied mares demonstrated acceptable diagnostic performance (including accuracy, specificity, and positive predictive value), especially for the detection of female fetuses. Additionally, the identification of the Eca.YH12 and TKY270 gene markers has further confirmed these findings.

The main limitation of our study was the occurrence of these false negatives in male fetuses. The most likely explanations include a low concentration of cffDNA during the last third of the first trimester of gestation, as well as the single-copy nature of *SRY* and Eca.YH12, and potential technical constraints in DNA extraction or PCR efficiency. Previous reports in horses have emphasized similar challenges: for example, De Leon et al. [10] reported a sensitivity of 72.7% using *SRY* PCR and an increased sensitivity of up to 90.9% using nested-PCR or

qPCR approaches. Kadivar et al. [15] employed nested RT-PCR for mares between 8 and 20 weeks of gestation, achieving a sensitivity of 85.7% and an accuracy of 88%. Increasing gestational age, adopting quantitative or nested PCR, or utilizing multi-copy Y markers, such as TSPY, may enhance sensitivity in future studies.

The auxiliary markers had distinct roles. Eca.YH12 mirrored the performance of SRY in this dataset and, therefore, did not enhance sensitivity when combined; however, it provided methodological robustness as an independent Y target. TKY270, being X-linked, was consistently amplified in all samples and functioned as a reliable internal control, ensuring that negative Y results reflected the actual absence of the Y-specific signal rather than amplification failure.

5. Conclusion

Taken together, our results support the feasibility of early fetal sex determination in horses using non-invasive cffDNA testing during the last third of the first trimester. However, the occurrence of false-negative results highlight the importance of gestational age and technical optimization. A practical diagnostic system would be as follows: if either marker (*SRY* or Eca.YH12) is detected, the fetus should be classified as male. Conversely, if neither Y marker is detected, the fetus should be classified as female, provided that the internal controls (TKY270 and *GAPDH*) are successfully amplified. Samples with ambiguous results should be retested at a later stage of gestation.

Acknowledgements

The authors thank the Deanship of Scientific Research, Semnan University, Semnan, Iran.

Compliance with ethical guidelines

This study fully complied with ethical standards.

Funding

This research did not receive any grant from funding agencies in the public, commercial, or non-profit sectors.

Authors' contributions

Conceptualization, data curation, formal analysis, and validation: Mahmood Ahmadi-Hamedani, Reza Narenji Sani, and Hamid Staji; Methodology, investigation, resources and project administration: Mahmood Ahmadi-Hamedani, Reza Narenji Sani, and Sina Tabatabaee; Writing the original draft: Mahmood Ahmadi-Hamedani; Review and editing: Mahmood Ahmadi-Hamedani and Hamid Staji.

Conflict of interest

The authors declared no conflict of interest.

Data availability

The data that support the findings of this study are available upon request from the corresponding author.

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