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Sequencing human rhinoviruses: Direct sequencing versus plasmid cloning

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ABSTRACT

Human rhinoviruses (RV) are associated with the majority of viral respiratory illnesses in infants, children and adults. Over the last several years, researchers have begun to sequence the many different species and strains of RV in order to determine if certain species were associated with increased disease severity. There are a variety of techniques employed to prepare samples for sequencing. One method utilizes plasmid-cloning, which is expensive and takes several hours to complete. Recently, some investigators have instead used direct sequencing to sequence RV strains, allowing for omission of the time- and labor-intensive cloning step. This study formally compares and contrasts the sequencing results obtained from plasmid-cloning and direct Sanger sequencing of a 500 base pair PCR product covering the VP4/VP2 region of RV. A slightly longer sequence (by 65 base pairs on average) was obtained when specimens were plasmid-cloned, and the sequences were 86% similar. After trimming the extra base pairs from the cloned sequences, the sequences were 99.7% identical. Overall success of directly sequencing samples was similar to that of cloning, 5% on average failed for each technique. Therefore, in many instances, directly sequencing samples may be considered in lieu of the more expensive and time-consuming plasmid-cloning technique.

1. Introduction

Human rhinoviruses (RV) are medically important pathogens that are associated with upper and lower respiratory infections (Jartti et al., 2004; Lemanske et al., 2005; Miller et al., 2009; Bizzintino et al., 2011; Cox et al., 2013; Linder et al., 2013). Advancement in molecular sequencing methods over the last several years has allowed researchers to genotype hundreds of strains of RV. This led to the discovery of a new species of RV in 2006, now known as RV-C (Lamson et al., 2006; Lau et al., 2007; Lee et al., 2007a; McErlean et al., 2007). Certain species of RV may be associated with varying disease severity. For example, RV-C is associated frequently with lower respiratory infections and asthma exacerbations (Jartti et al., 2004; Lemanske et al., 2005; Miller et al., 2009; Bizzintino et al., 2011; Cox et al., 2013; Linder et al., 2013). In addition, seasonal variation of the RV species differs (Annamalay et al., 2012; Lee et al., 2012; Linder et al., 2013; Pierangeli et al., 2013). Therefore, it is important for researchers to have the ability to identify the strains and species of RV. Currently there are no vaccines available for the >150 types of RV. However, if a treatment or vaccine was available for the more pathogenic strains, rapid identification of the strain of RV infecting a patient could be necessary.

To successfully sequence RV, the genomic RNA (or cDNA) must be amplified to produce an adequate copy number. Initially, researchers cloned RV gene fragments amplified by reverse transcriptase (RT)-PCR into plasmid vectors in order to amplify the sequence and allow for successful cloning (Lee et al., 2007b; Pierangeli et al., 2007; Huang et al., 2009; Olenec et al., 2010; Xiang et al., 2010; Miller et al., 2011; Linder et al., 2013). Plasmid-cloning was desirable because it allowed for amplification of very small quantities of cDNA from samples. This technique was also useful in amplifying large portions of unknown strains of RV genome. However, recently researchers shifted to directly sequencing the cDNA product after purification. Most articles published within the last

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year that sequenced RV utilized this method (Daleno et al., 2013; Garcia et al., 2013; Miyaji et al., 2013; Pilorge et al., 2013). Others utilized direct sequencing in earlier studies as well (Blomqvist et al., 2009; Kneider et al., 2009; Wisdom et al., 2009; Mizuta et al., 2010; Watanabe et al., 2010). Plasmid-cloning typically takes three days to complete due to incubation times; however, direct sequencing is conducted immediately after RT-PCR amplification and purification. Due to labor costs alone, researchers may have preferred direct sequencing to save both time and money. However, direct sequencing of RT-PCR product has not been rigorously compared to plasmid-cloning to the authors knowledge. It is not known if the quality of the resulting sequences is similar in terms of which nucleotides are observed, or if the two techniques have equivalent success rates. In this study, RV fragments that were amplified and plasmid-cloned were compared to RV fragments that were amplified and directly sequenced from the same sample. This allowed for comparison of cost, technique, and quality of results between the two methods to determine the most efficient way to sequence many RV samples.

2. Methods

2.1. Sample collection and processing

Samples utilized for testing were nasal aspirates collected from a cohort of very low birth weight (<1500 g) premature infants (<32 weeks gestational age) in Buenos Aires, Argentina, which had been approved by the Internal Review boards at Vanderbilt University, USA, Pediatric Hospital de J.P. Garrahan, and the Hospital Materno Infantil Ramón Sarda. Parental consent was obtained for enrollment and sampling, and families could refuse sampling or withdraw from the study at any time. Patients were enrolled from June 2011 to October 2012 during the infants' first year of life. Samples were collected using sterile saline with no additives and were immediately frozen until shipment on dry ice to the United States. Total nucleic acids were extracted using the Roche Magna Pure system (kit # 3038505001). Conventional reverse transcriptase (RT)-PCR was then conducted two separate times (once for the plasmid-cloning and once for the direct sequencing) using Qiagen One step RT kit (#210212), including positive and negative controls with each run. Primers (Savolainen et al., 2002) encompassed the VP4/VP2 region and were as follows: RV-Forward GGGACCRACTACTTTGGGTGTC-CGTGT; RV-Reverse GCATCWGGYARYTTCCACCACCADCC, where there were four wobble primers W (A+T) Y (C+T), R (A+G), and D (A+G+T). After processing, samples were sent to the Vanderbilt sequencing core and Sanger sequencing was conducted. Samples were sequenced in one direction. For cloned samples, a T7 primer was used. For directly sequenced samples, the RV-forward or RV-reverse primer was provided with the samples for sequencing.

2.2. Plasmid-cloning

After RT-PCR was completed, 10 μl of each sample was run on a 1% agarose gel. Positive bands (approximately 525 base pairs long) were excised with a clean scalpel and DNA was extracted using the Qiagen Qiaquick gel extraction kit (#28706). Supplemental Fig. 1 illustrates an example of a RT-PCR gel. Running the gel confirmed that there was one band of the correct size. By excising the band, no sample was discarded and only the target nucleotides were processed. Samples were then ligated into the pGEM-T Easy Vector (Promega #A1360), and transformed into DH5 alpha (ampicillin resistant) competent *E. coli* cells. Cells were shaken at 37 °C for 90 min and then plated onto ampicillin-infused agar plates coated with 100 mM IPTG (isopropyl-beta-D-thiogalactopyranoside) and 50 mg/ml of Xgal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside). On day two, colonies that were successfully ligated and transformed (white colonies) were picked with a sterile toothpick and placed into 2 ml of broth containing 4 ul of 100 mg/ml ampicillin. Cultures were shaken at 37 °C overnight. On day three, the plasmids were extracted from the bacteria using the Qiagen Miniprep kit (#74106). Five microliters of each sample was then digested using the EcoR1 restriction digest enzyme and run on a 1% agarose gel to confirm that the correct size of DNA fragment was incorporated into in the plasmid (approximately 525 base pairs). If the RV sequence was not observed in the plasmid after restriction digest, another colony was picked and reprocessed. Supplemental Text 1 details the cloning protocol used in this laboratory. The whole procedure requires approximately 10 h of active labor to process 16 samples, and three days of processing due to incubation times. In this laboratory the material cost (including reagents) to prepare one sample for sequencing was approximately 14 US dollars (USD), not including the cost of the initial RT-PCR, the sequencing costs at the VANTAGE Vanderbilt Sequencing Core, or labor. If labor costs were included, (at a cost of 20 USD an hour for labor), the total was 424 USD to process 16 samples.

Table 1
Comparison of time and material costs between plasmid-cloned and directly sequenced samples. USD = United States Dollars.

	Plasmid-cloning	Direct sequencing
Number of steps	9 Run gel Excise and extract bands Ligate Transform Grow on agar plates Amplify colony Mini prep Digest Run gel	3 Run gel Nanodrop ExoSap-IT[a]
Active time	10 h	2 h
Total time	3 days	3 h
Cost (materials)	14 USD/sample	2 USD/sample
Cost (labor $20/h)	200 USD	40 USD
Total cost (16 samples)	424 USD	72 USD

[a] VANTAGE Vanderbilt Sequencing Core purified samples with ExoSap-IT at a cost of $0.70 per sample.

2.3. Direct sequencing

After RT-PCR was conducted, 10 μl of each sample was run on a 1% agarose gel to confirm a positive band. Positive samples were then quantified using a Nanodrop (Thermo Scientific) and diluted with sterile water to reach a final concentration of approximately 50 ng/μl. Samples were sent to the VANTAGE Vanderbilt Sequencing Core to be treated with ExoSap-IT (Affymetrix #78200) for removal of PCR contaminants, and then sequenced. The cost of ExoSap-IT at the VANTAGE Vanderbilt Sequencing Core was $0.70 per sample. Thus, the material cost (including reagents) of preparing one sample for sequencing was approximately 2 USD, again not taking into account the cost of RT-PCR, the sequencing costs at the VANTAGE Vanderbilt Sequencing Core, or labor. The time required to set up the experiment, run the gel, and prepare the samples for sequencing was approximately 2 h, with labor costs included (20 USD/h) the total cost was 72 USD to process 16 samples. Table 1 shows a comparison of the time and material costs between the two sequencing methods. VP4/VP2 sequences were submitted to GenBank (accession numbers KJ620336–KJ620367).

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2.4. Analysis and statistics

All alignments were conducted using MacVector (version 12.6). Neighbor Joining phylogenetic trees were built in Mega 5.2 (Tamura et al., 2011). *T*-tests were used to compare similarity of directly sequenced and plasmid-cloned samples when examining diversity within RV species. The National Center for Biotechnology Information (NCBI) website 'BLASTN' was used to determine similarity of study sequence nucleotides to known RV sequences in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3. Results

Of the 20 samples tested, two (10%) were unable to be successfully sequenced either by plasmid-cloning or direct sequencing. One RV-B sample (5%) failed by direct sequencing but was successfully plasmid-cloned and sequenced, and one RV-A sample (5%) failed by plasmid-cloning but was successful by direct sequencing, even though the RV segment was confirmed by restriction digest to be in the plasmid. Of the 16 remaining samples, 6 were RV-A, 4 were RV-B, and 6 were RV-C.

The primary difference detected between plasmid-cloned sequences and direct sequences was that the plasmid-cloned sequences were on average 64.7 [standard error (SE) ± 5.71] base pairs longer than the direct sequences (Fig. 1). When the entire sequences were aligned and compared for similarity, for any given sample, average similarity was 86% (SE ± 1.14; Table 2). However, when the 65 base pair 'tail' was removed, and the sequences were again aligned and compared, the similarity between sequencing methods was 99.7% (SE ± 0.08). On average in the directly sequenced samples, the first 64.4 (SE ± 5.10) base pairs were under the quality threshold and trimmed off. On average the plasmid-cloned sequences were 518 base pairs long, and the directly sequenced samples were 453 base pairs long. This demonstrated an approximate 12% loss in the sequence length among directly sequenced samples. Both the direct and cloned sequences were compared to NCBI's database using 'BLASTN'. In 15 of the 16 samples, the cloned and direct sequences matched with the greatest similarity to the same accession number in the NCBI database (Table 2). The cloned version of study sample 14 best matched with a 95% identity to accession number FJ615713.1, where the directly sequenced version matched with a 98% identity to accession number AB904651.1, and 95% identity to FJ615713.1. Both were RV-C strains. Supplementary text 2 provides the VP4/VP2 regions of the study samples and the sequences with which they matched most closely from NCBI.

Next, the responses of both techniques to alignments and phylogenetic analyses were examined. The similarity of samples within RV species was compared to determine if the sequencing method affected the similarity of the results. First, a similarity analysis was run on the plasmid-cloned sequences, and then separately on the directly sequenced samples. These two values were then compared. When analyzing the entire plasmid-cloned sequence, there was greater similarity within the RV-A and RV-C samples than when compared to the directly sequenced samples (Fig. 2, black and gray bars; RV-A, $P < 0.001$ and RV-C, $P = 0.003$). However when the additional base pairs were trimmed off of the plasmid-cloned sequences to make them the same size as the directly sequenced samples, there was no longer a significant difference in similarity between RV species (Fig. 2, black and white bars). Phylogenetic analysis illustrated the diversity of the samples, and that both methods of sequencing yield the same phylogenic tree (Fig. 3).

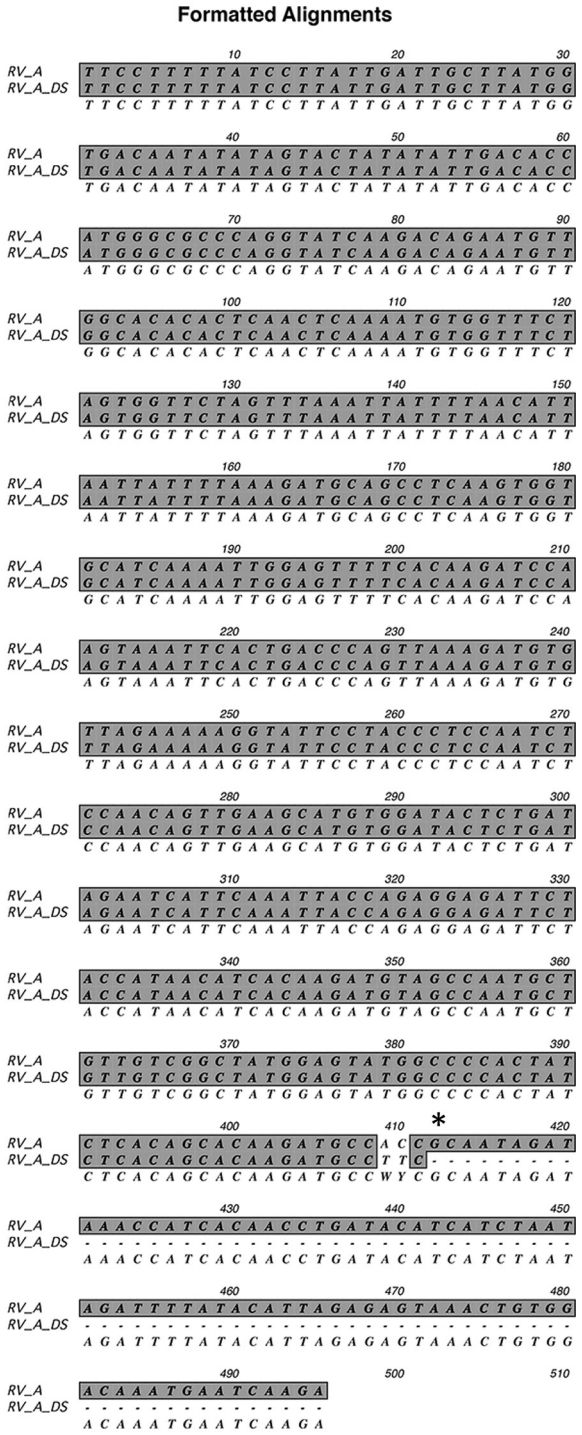

Fig. 1. Sequences of plasmid-cloned and directly sequenced samples were similar except cloned sequences were longer. Top line 'RV-A' is the plasmid-cloned sequence; middle line 'RV-A DS' is the directly sequenced sample, lower line is the consensus sequence between the two samples. *Indicates where direct sequence stopped, and where plasmid-cloned sequences extended.

4. Discussion

Finding the most efficient and cost effective way to sequence many RV specimens without loss of quality is important for current research and medicine. It has been suggested that certain species of RV are associated with more severe disease (Jartti et al., 2004; Lemanske et al., 2005; Louie et al., 2009; Miller et al., 2009; Bizzintino et al., 2011; Cox et al., 2013; Linder et al., 2013) and

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Table 2
Q4 Percent similarity between plasmid-cloned and directly sequenced samples.

Species	ID number	% Similarity[a]	Number of extra base pairs	% Similarity trimmed[b]	Matching accession #
A	1	82.6	85	99.5	JX129444.1
A	2	85.3	73	99	JX129407.1
A	9	82.5	88	100	JN815252.1
A	13	91	45	99.8	FJ615680.1
A	15	94.1	28	99.6	JF781503.1
A	16	87.9	61	100	JX129431.1
B	5	83.6	80	99.5	JN990706.1
B	7	83.7	80	100	JN798588.1
B	10	83.5	81	99.5	KC306789.1
B	11	83.1	81	99.3	FJ950778.1
C	3	86.8	65	99.8	JX129449.1
C	4	94	28	100	HQ444899.1
C	6	93.3	32	99.6	JX876790.1
C	8	83.2	84	100	HQ444899.1
C	12	92.6	37	100	EU840952.2
C	14	82.3	87	100	FJ615713.1 (C) AB904651.1 (DS)
Average[c]		**86.8** (±1.2)	**64.7** (±5.7)	**99.7** (±0.1)	

[a] Compares the entire sequence for a given sample.
[b] The extra base pairs obtained by plasmid-cloning have been removed in this comparison.
[c] Arithmetic mean of 16 samples with standard error (±). Matching accession number refers to the accession number in the NCBI database that matched most closely with the study sample, '(C)' refers to 'cloned sequence', '(DS)' refers to 'direct sequence'.

are detected at different times of the year (Annamalay et al., 2012; Lee et al., 2012; Linder et al., 2013; Pierangeli et al., 2013). The ability to identify species of RV easily could facilitate rapid specimen processing, and if a strain-specific treatment or vaccine were available, could allow for the rapid identification of the infecting strain and alter clinical management. This study found that direct sequencing was associated with an approximate 12% loss of sequence length (Fig. 1; Table 2). This loss of sequence length was likely due to the beginning of the sequence not meeting the quality threshold, which is typical of Sanger sequencing due to primer attachment. The quality threshold was used to distinguish the background base pair reading from base pairs in the target sample. The threshold at the VANTAGE Vanderbilt Sequencing Core used in this study was a quality value of <21. In the plasmid-cloning preparation, the primers attached to the plasmid itself, which was located earlier in the sequence than the target RV portion. This explained why longer RV sequences were obtained with plasmid-cloning. The portion that was under the quality threshold overlapped with the plasmid, not the target RV sequence (as in direct sequencing).

When study samples were compared to the NCBI database it was found that both the direct and cloned versions of the sequences matched to the same accession numbers, except in one sample. In this particular case, though both matched to RV-C, the cloned version had 95% identity to the best match in the NCBI database, FJ615713.1; where the directly sequenced version had 98% identity to accession number AB904651.1 and 95% identity to the FJ615713.1, the most similar to the cloned version. When the authors were contacted, they confirmed that AB904651.1 was also directly sequenced (personal communication), potentially explaining why the directly sequenced version of study sample 14 matched more closely to the shorter AB904651.1 than FJ615713.1. Comparing the study sequences to known sequences in NCBI does have limitations. It is possible that the strain and type information in the database is incorrect, or perhaps in some cases a perfect match may not be found. However, with the current information available,

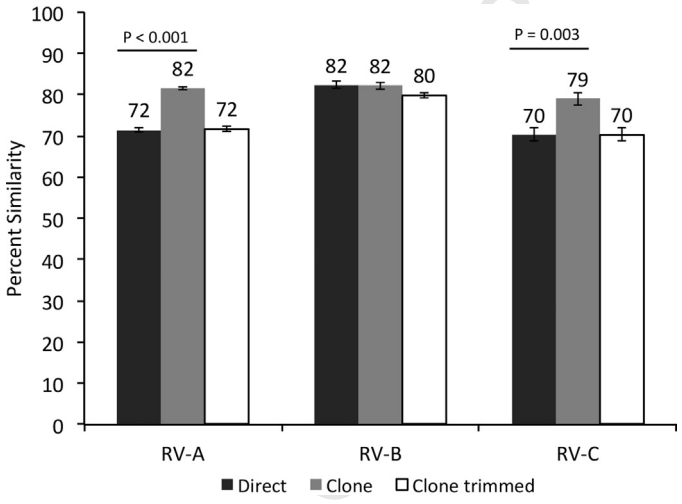

Fig. 2. RV-A and RV-C plasmid-cloned (gray bar) samples are more similar to each other than directly sequenced (black bar) samples. White bars show percent similarity after extra base pairs were trimmed off of the cloned sequences. No significant difference was detected between trimmed cloned sequences and direct sequences.

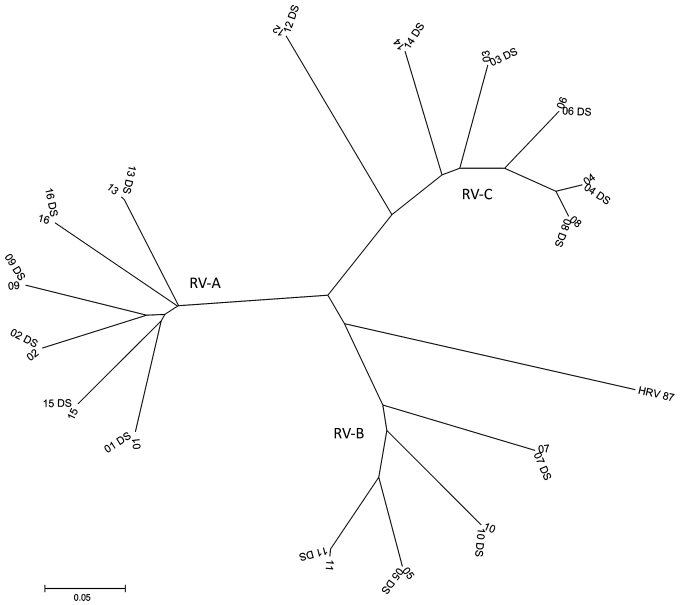

Fig. 3. Neighbor Joining Tree of samples that have been plasmid-cloned and directly sequenced. Samples cluster into three distinct species, RV-A, RV-B, and RV-C. Samples are labeled 1–16, 'DS' indicates the directly sequenced samples.

the authors are fairly confident the study samples match closely to other published sequences.

Failure rates were observed at the same proportion in both methods, and were typical for this laboratory (Linder et al., 2013). This suggests that there was not a discordance in the number of samples that were sequenced successfully by either method. Cloning failure may be due to failure of any step in the procedure, where direct sequencing usually failed if the DNA concentration in the sample was too high or too low. The restriction digest step at the end of the cloning technique is important to confirm that the RV sequence has inserted. However, even when the sequence is observed, sometimes the sample still fails to sequence properly. Failure to sequence in this case may have been related to primer attachment in the sequencing process or purity of the sample; however it is unknown why the samples failed to sequence. One limitation of this study was that only 16 strains were examined. Though more precise percentages of failure rates could have been obtained with larger sample sizes, it is unlikely that sequencing more samples would alter conclusions of this study because sample sequences were very similar between the two methods.

Finally, the cost in time and materials was much greater for plasmid-cloning of samples. It took approximately 10 active work hours spread across three days to plasmid-clone 16 samples, as opposed to 2 h for direct sequencing. Furthermore, plasmid-cloning utilized more labor and material resources, approximately 224 USD in materials for 16 samples and 200 USD in labor, totally 424 USD. Preparing samples for direct sequencing utilized only 40 USD in labor, and cost approximately 32 USD for 16 samples, totaling 72 USD. This led to an overall savings of 352 USD for 16 samples. Though time and money requirements were reduced with direct sequencing, certain situations still favor for plasmid cloning. For example, for a novel strain of RV, not previously sequenced, obtaining a longer sequence would be informative. Also, if the sample was infected with several strains of RV, multiple transformed colonies could be picked and processed using the cloning method, where direct sequencing only sequences the most prominent type. If primers spanning longer segments of the genome were required, the sequence quality may be better if plasmid-cloning were used. However, in this laboratory and for large epidemiological studies that require rapid screening of many RV samples, direct sequencing saves time and money with little difference in quality of results.

5. Conclusion

In conclusion, though plasmid-cloning yielded slightly longer sequences, the similarity of results between the two techniques was quite high. Considering the costs of materials and labor, the preferred use of direct sequencing of RV-positive samples seems prudent. This method saves researchers both time and money, and allows for rapid processing of many RV samples, which furthers studies on respiratory viruses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2014.09.020.

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