

Development of a Parvovirus Assay Using rNS-1 His-tagged Antigen

By ELENA M. SELETSKAIA, J. PAUL COWLEY, MICHELLE L. WUNDERLICH, STEVEN M. JENNINGS, KENNETH S. HENDERSON, WILLIAM R. SHEK, and RAJEEV K. DHAWAN

Parvoviruses are one of the most prevalent infectious agents in the laboratory rodent. Their effect on research can range from immune dysfunction that may mislead researchers when interpreting results to lethal effects on animals. Until recently parvovirus infection in mice was thought to be caused by minute mouse virus (MMV) and in rats by rat viral agents in the KRV or H-1 serogroups. Relatively newly discovered viruses in these groups are mouse (MPV) and rat parvoviruses (RPV-1 and 2).

Parvoviruses are 15–20 nm in diameter and are single-stranded DNA viruses of about 5,000 nucleotides, which replicate through a double-stranded DNA intermediate. The protein composition consists of three structural or capsid proteins providing the viral coat (VP-1, VP-2, and VP-3) and two non-structural proteins involved in viral replication (NS-1 and NS-2). Among the capsid proteins, VP-2 is the major protein. The non-structural proteins NS-1 and NS-2 are well conserved, with greater than 90

percent similarity in their amino acid sequence for all serogroups. At the present time, not enough information is available about the seroconversion to structural and non-structural proteins during infection. In some cases, sera which are positive for capsid-specific assays have low titers or are negative in NS-1 ELISA (enzyme-linked immunosorbent assay). Therefore, it is necessary to screen the sera using MPV and MVM structural antigens (AG) as well as NS-1.

The hemagglutination inhibition assay (HAI) is the traditional serologic method of detecting parvovirus. In addition, ELISAs or immunofluorescence assays (IFA) can be used for detection of antibodies to structural or non-structural proteins. However, conventional AGs are difficult to purify and the partially purified products may have low sensitivity and specificity. Recombinant antigens (rAG) are easier to grow and can be purified when the recombinant protein is linked to a tag. In addition, rAGs are non-infectious by nature. The NS-1 protein was selected as the best candidate for making a rAG for parvovirus detection because the sequence is highly conserved and therefore the protein structure and antigenic activity are assumed to be highly conserved. Parvovirus ELISA using whole virus particles can not detect the presence of NS-1 antibodies. Recombinant NS-1 AG ELISA can screen for these NS-1 antibodies and their presence can be confirmed by using IFA.

Recombinant proteins can be

expressed in a wide variety of systems. However, despite that flexibility, purification can still be difficult. Recombinant techniques permit the construction of fusion proteins in which specific affinity tags are added to the protein of interest. Nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography matrices (Qiagen, Valencia, CA) have selective affinity for biomolecules (i.e., proteins) that have been N- or C-terminally tagged with six consecutive histidine residues (His) and can be selectively purified by virtue of this property. For this and the reasons mentioned above, CRL Diagnostic Reagents R&D department constructed a 6-His-tag NS-1 AG-expressing recombinant baculovirus for a parvovirus screening ELISA.

Materials and Methods

Plasmid and Clone Selection

A plasmid, designated as pV-1, containing a genomic clone of MPV was obtained from Lisa Ball-Goodrich (Yale University, New Haven, CT). PCR primers were designed for the amplification of the MPV NS-1 gene from pV-1 (Fig. 1, see website).

PCR was performed and the resulting PCR product (~2 kb) was separated on agarose gel and isolated. This PCR product was cloned into pNoTA (5'→3') using the Prime PCR cloner cloning kit (Eppendorf – 5 Prime, Boulder, CO). Following *E. coli* transformation, positive clones were selected and plasmid DNA was isolated by mini-

Elena M. Seletskaiia, M.S. is a senior research associate; J. Paul Cowley, M.S. is assistant manager of diagnostic reagents; Michelle L. Wunderlich, M.S. is senior supervisor of diagnostic reagents; Steven M. Jennings is manager of diagnostic reagents; Kenneth S. Henderson, Ph.D. is director of molecular diagnostics; William R. Shek, Ph.D. is senior director of diagnostic services; and Rajeev K. Dhawan, Ph.D. (rdhawan@criver.com) is associate director of diagnostic reagents, diagnostic reagent department, R&D, Charles River Laboratories, Wilmington, MA.

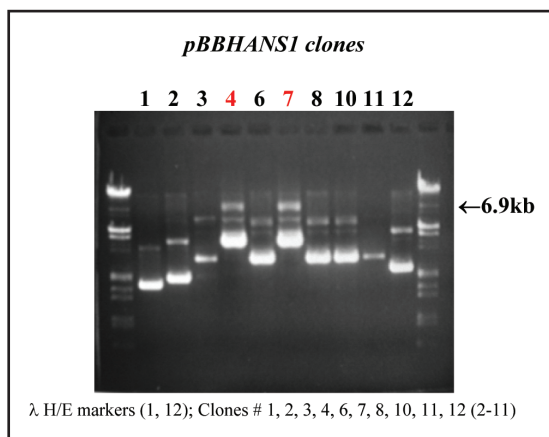


Figure 3. AGE of recombinant clones

prep preparation. Plasmid DNA for each clone was digested with the restriction enzymes *Bam*HI and *Eco*RI. Clones 2, 3, 4, 6, 8, 13, 14, 15, 25, and 26 contained the 2-kb *Bam*HI insert (Fig. 2, see website).

Further analysis by *Eco*RI digestion showed that clones 13, 25, and 26 contained the NS-1 gene in one orientation, while clones 6, 14, and 15 contained the GOI in the opposite orientation. PCR using the NS-1 primers was also performed to confirm that each clone contained the NS-1 gene. Clone 6 was selected and the plasmid isolated was designated as pNS1His6R. The plasmid DNA was digested with *Bam*HI to excise the NS-1 gene and then ligated into a baculovirus transfer vector, pBlueBacHis2A. This resulting recombinant plasmid, denoted pBBHANS1, was used to transform *E. coli* and selected for LB plates containing 100 µg/mL ampicillin.

Out of 13 original positive colonies selected for analysis, three did not grow

during replating. Plasmid analysis of the remaining 10 clones indicated that only two, 4 and 7, pBBHANS1 clones had the correct size plasmid, 6.9 kb (Fig. 3). The plasmid DNA of these two clones was digested with both *Bam*HI and *Eco*RI to identify the GOI in the correct (forward) orientation. Only clone 4 had the correct band pattern (data not shown).

Static cultures of Sf9 insect cells were co-transfected with the pBBHNS1

plasmid (Clone #4) and linear non-infectious recombinant baculovirus DNA using the MaxBac transfection kit (Invitrogen). Recombination took place between the plasmid DNA containing the NS-1 GOI and homologous sequences of the baculoviral DNA resulting in the restoration and propagation of only recombinant virus. The cell cultures were incubated for six days at 27° C, harvested and pelleted by centrifugation. Expression of the recombinant NS-1 fusion protein was detected by western blot and NS-1 ELISA. Further verification that the NS-1 gene was inserted into the baculoviral DNA was done using PCR. Primers that flank the homologous recombination sites were used to identify the insert. A 2.3 kb fragment was amplified (identified on agarose gel electrophoresis) consisting of the 2 kb gene of interest and an expected extra 300 base pairs from the recombinant baculoviral DNA.

The original infected cell culture

supernatant (parent seed stock) was used in a plaque assay to check the baculoviral recombination. Individual plaques were assayed for the NS-1 insert by PCR and expression of the recombinant protein by western blot (data not shown). Two strong recombinant baculovirus subcloned plaques, 12 and 51, were used to infect cultures of Sf9 insect cells and the expression of NS-1 fusion protein in them was assayed and verified by western blot. The parent seed stock was further virus propagated to make master, working and production seed virus stocks (MSV, WSV and PSV) and frozen as aliquots (Fig. 4, see website).

Antigen Production

Sf9, Sf21, High Five (Invitrogen), and expresSF+ (Protein Sciences Corporation, Meridian, CT) insect cell lines were used for production of the NS-1 AG.

Static Cultures

Static insect cell cultures were maintained using TNM-FH medium (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS) at 27° C. For the production of NS-1 AG, 15–20 flasks of 90% confluent cells were each inoculated with 10 ml of NS-1 recombinant PSV. No consideration was given for multiplicity of infection (MOI) in static cultures. Cells were incubated at 27° C, harvested on day 8, then pelleted by centrifugation at 2,000 rpm for 10 minutes. The clarified supernatant was saved under sterile conditions (for virus seeds). The cell pellet was washed once with phosphate-buffered saline (PBS, pH 6.2) and re-centrifuged. The NS-1 AG was purified from the cell pellet.

Non-static Cultures (Shaker or Wave Bag Cultures)

Shaker cultures were grown at 27° C in Sf-900 II serum-free media (Gibco, Invitrogen) at a speed of 130–150 rpm or 28–30 waves/minute for Wave Bioreactors (Wave Biotech, Bridgewater, NJ). For AG production, cultures were infected at $\sim 2 \times 10^6$ cells/ml (>90% viable) with an MOI of 1.0, harvested on day 3 after infection, then pelleted by centrifugation at 2,000 rpm for 10 minutes. The clarified supernatant was

Quality control of the lysate (# 41-1-11)

| Serum | ID | Dilution | Lot # | Net scores |
|---------------------|-------|----------|--------|------------|
| Mouse anti-MPV | S-881 | High | 052799 | 16.5 |
| Mouse anti-MPV | S-724 | Low | 030899 | 6.0 |
| Rat anti-RPV | S-775 | High | 061099 | 14.7 |
| Rat anti-RPV | S-775 | Low | 061099 | 7.5 |
| Non-immune mouse | S-863 | 50 | - | 1.1 |
| Non-immune rat | S-753 | 50 | - | 1.0 |
| BLOTTO ^a | - | - | 071999 | 0.6 |

^aBLOTTO – Bovine Lacto Transfer Technique Optimizer (Diluent)

Figure 6. QC

Quality control of test-run purification (#41-1-15)

| Parameter | Specification | Results (#41-1-15) |
|---------------------------------------|----------------------------------|---|
| Bio-Rad Protein | | 11ml, 305ug/ml |
| Peak Standard ELISA Titer | ≥10 | ~100 |
| Antigen Potency ^A | ≥ 25% of Prior Lots | 333 (Prior lot value = 300) |
| Standard ELISA non-immune serum score | <3 at estimated working dilution | NMS=0.6, NRS=1.3 |
| Antigen Activity Yield ^B | ≥0.2 (or 20%) | 0.36 (36%) |
| Peak SDS-PAGE | >80% pure | 83KD band of NS-1 was >80% pure |
| Lysate and peak Western blot | Most antigen is intact. | Selective reactivity of MαMVM (S-536) with 83KD intact band |

^AAntigen Potency = Titer (standard ELISA)/ mg/mL Protein

^BAntigen Activity Yield = Peak (Total antigen activity)/ Lysate (Total antigen activity)
 Total antigen activity = mL of Fraction X Titer (Ni ELISA)

Figure 8. QC of test run

Quality control of the purified NS-1 AG (# 41-P)

| Parameter | Specification | Results (Lot # 41-P) |
|--|-------------------------|---|
| Bio-Rad Protein | -- | 416ml; 355ug/ml (Total protein ~148mg) |
| Peak Standard ELISA Titer | ≥10 | Working dilution =100 |
| Antigen Potency | ≥ 25% of Prior Lots | 282 (Prior lot value = 300) |
| Standard ELISA non-immune serum score | ≤2 at working dilution. | NMS=0.3, NRS=2.2 |
| Purity: Homologous/cross-reacting immune serum net score | ≥3 | See table for purity of NS-1 AG (#41-P) below |
| Purity: Heterologous-/non-immune serum net score | ≤2 | NMS=0.0, NRS=0.2 |
| Peak SDS-PAGE | >80% pure | 83KD band was >80% pure |
| Western blot: immune serum | Most antigen is intact. | Yes |
| Western blot: non-immune serum | Minimal reactivity. | No reaction with NMS |

Figure 11. QC of purified AG

saved under sterile conditions (for virus seeds). The cell pellet was washed once with PBS (pH 6.2) and re-centrifuged. The NS-1 AG was purified from the cell pellet (Fig. 5, see website).

Seven-liter (lot 41-1) and 8-liter (lot 41-2) production batches of NS-1 AG were produced in SF+ cells grown in 10-liter Wave bags using Sf-900 II media. Approximately 3.5 liters of SF+ cells (3.8×10^6 cells/ml, 96% viable) were diluted 1:1(v/v) with Sf-900 II media to a final cell count of $\sim 1.7 \times 10^6$ cells/ml. These cells were infected at a MOI of 1.0 plaque forming units (pfu)/cell with NS-1 PSV (lot #052799 for the 7-liter and PSV lot #040599 for the 8-liter batch) and incubated at 27° C with gentle rocking (28 waves/min). After 72 hours the cells of each lot (2×10^6 cells/ml, 92% viable) were harvested and pelleted by centrifugation at 2,000 rpm for 10 minutes. Cell pellets were

washed once with cold PBS (pH 6.2) and pelleted again as above.

Cell pellets were re-suspended in 2.8 liters of 1% CHAPS-PBS-imidazole loading/extraction buffer (5×10^6 cells/ml). Immediately, 28 ml of protease inhibitors was added and mixed for 30 minutes at 4° C. Additionally, 4 ml of 2-mercaptoethanol was added and the lysate was clarified by centrifugation at 7,000 rpm for 10 minutes. The clarified lysate was divided into aliquots for testing and purification of the NS-1 recombinant protein. The lysate aliquots were stored at -70° C and qualified by Ni-plate ELISA. The lysate was considered satisfactory if, at a 10-fold dilution or higher, it gave clear positive scores with immune sera and near negative scores with non-immune sera and diluent. The table below shows Ni-ELISA scores for various sera at a 300-fold dilution of the lysate #41-1-11 (Fig. 6).

Lysate Preparation

The harvested cells were re-suspended to 5×10^6 cells/ml with cold 1% CHAPS-PBS-5 mM imidazole extraction buffer to lyse the cells and release the AG. A protease inhibitor cocktail (1 ml/100 ml of lysate) was immediately added and gently stirred at 2-8° C for 30 minutes to prevent protein degradation. Additionally, 2-mercaptoethanol was added to a final concentration of 20 mM and the material clarified by centrifugation at 7,000 rpm for 10 minutes. The supernatant was removed, the pH checked and, if necessary, adjusted to 8.0 using 1N NaOH or 1N HCl for the final clarified lysate.

The clarified lysate was divided into two 1-ml aliquots (quality control), one 100-ml aliquot (test run), and ~ 500 ml/bottle (production run), then stored at -70° C until use. The lysate was tested by Ni-plate ELISA against high and

low immune-positive sera, non-immune sera, and diluent. The lysate was satisfactory for purification if a 10-fold dilution or higher gave a clear positive score with immune sera and near negative scores with non-immune sera and diluent.

AG Purification

Test-run: The 100-ml lysate was thawed and filtered through a 0.2- μ m filter. The His-tagged NS-1 recombinant protein AG (#41-1-15) was purified by Ni-NTA matrix chromatography on a Bio-Rad BioLogic LP system (Bio-Rad, Hercules, CA) and Foxy fraction collector (Isco, Lincoln, NE) as indicated in the table below. Purification of His-tagged NS-1 AG was monitored by recording the absorbance at 280 nm (Fig. 7, see website).

The His-tagged AG was eluted from the column and collected using the fraction collector. All the collected fractions were tested for their protein concentra-

tion by a Bio-Rad protein assay. Fractions containing significant protein were pooled (Fractions 2–10, 10 ml) and glycerol was added to make a final concentration of 10% v/v. Samples of lysate, void, and peak were saved for analysis and the remainder stored at -70° C in bulk until further use. The purified AG was frozen at -70° C in two 0.5-ml aliquots and one 10-ml aliquot.

QC Testing: Lysate, void, and peak were analyzed by Ni-plate ELISA, standard ELISA, SDS-PAGE, and Western blot. Wherever possible, a sample from a previously qualified lot was included for comparison. The results were evaluated to the quality control specifications listed in Figure 8 (Figs. 9 and 10, see website).

Production-run purification: After the test-run passed the QC test, production-run purification of 450-ml batches each of NS-1 cell lysates (lots 41-1 and 41-2) was done using 10 ml of Ni-NTA column. The production-run lysate was

pre-filtered using a 0.5- μ m cartridge prior to the final 0.2- μ m filtration for loading. Purification was performed as above and the eluted product compared with the purified batch 41-1-15 using standard ELISA (results not shown).

All purified and qualified NS-1 recombinant protein AG from lots 41-1 and 41-2 was pooled to give batch 41-P (416 ml, 355 μ g/ml, total protein \sim 148 mg) and tested as indicated below. The purified and pooled AG was divided into aliquots and frozen at -70° C (Fig. 11).

Results and Discussion

QC Testing of final AG: ELISA analysis of mouse and rat sera was done using Immulon-4 microplates (Dynex Technologies, Chantilly, VA). The plates were coated with 50 μ l/well of diluted NS-1 AG in carbonate buffer (CBB), kept overnight at 4° C, and washed to remove excess AG and buffer. Assays were performed using our standard procedure for ELISA. The plates were incubated with diluted serum samples, washed, and then incubated with horseradish peroxidase (HRP)-labeled secondary conjugate (50 μ l/well for 40 minutes each step). Finally, 100 μ l/well of ABTS substrate was added to develop the plates. The plates were read at 405 nm after a 40-minute incubation. Net scores were calculated from the absorbance values using the formula:

$$\text{Net Scores} = (\text{AbsAG} - \text{AbsTC}) / 0.13$$

Standard ELISA titration: Titration of pooled NS-1 AG (#41-P) was performed to check its potency against various mouse and rat parvovirus-positive sera and normal sera. False parvovirus-positive mouse and rat sera were included to check the specificity of the purified NS-1 AG. The AG was tested at a dilution of 10, 30, 100, 300, and 1000 in CBB and the scores for the 1/100 dilution are shown in Figure 12.

AG Potency: The results from the above titration experiment suggest that NS-1 AG should be used at dilution of 1/100 for coating ELISA plates. To confirm these results, Immulon-4 plates were coated with 1/80 and 1/100 dilu-

| Serum | ID | Lot # | Dilution | Net score |
|--------------------|--------|--------|----------|-----------|
| Mouse anti-MPV | S-724 | - | 50 | 9.6 |
| | | | 250 | 4.8 |
| | | | 1250 | 1.6 |
| Rat anti-RPV | S-775 | - | 50 | 14.6 |
| | | | 250 | 10.0 |
| | | | 1250 | 4.9 |
| Non-immune rat | S-753 | - | 50 | 3.9 |
| Rat false + pool | - | 050599 | 50 | 4.2 |
| Mouse false + pool | - | 111398 | 50 | 2.1 |
| Mouse anti-NS1 | S-881B | 092999 | High | 10.5 |
| | | | Low | 4.6 |
| Rat anti-NS1 | S-775 | 061099 | High | 11.9 |
| | | | Low | 6.2 |
| Non-immune mouse | S-863 | - | 50 | 0.7 |

Figure 12. AG titration

| Result | Number (%) | | | | Total |
|---------------------------------|--------------------|-------------------|--------------------|-------------------|--------------------|
| | Antigen Lot # 40-P | | Antigen Lot # 41-P | | |
| | Mouse | Rat | Mouse | Rat | |
| + | 24(2.2%) | 16(4.2%) | 34(2.1%) | 14(3.5%) | 88(2.5%) |
| - | 1078(96.8%) | 357(93.5%) | 1593(96.7%) | 373(92.5%) | 3401(95.9%) |
| TC | 1(0.1%) | 0(0.0%) | 8(0.5%) | 0(0.0%) | 9(0.3%) |
| +/- | 10(0.9%) | 9(2.3%) | 12(0.7%) | 16(4.0%) | 47(1.3%) |
| Not Positive^A | 1089(97.8%) | 366(95.8%) | 1613(97.9%) | 389(96.5%) | 3457(97.5%) |
| #Tested: | 1113 | 382 | 1647 | 403 | 3545 |

^A Not Positive = Sum of -, TC and +/-

Figure 18. Field sera

tions of the NS-1 AG (one plate each). Both microplates were coated using identical conditions, using CBB buffer (50 µl/well) overnight at 4° C, and were assayed with the sera mentioned in the first experiment. The scores for mouse and rat positive sera (high and low) confirmed that the ideal coating dilution for NS-1 AG is 1/100. The scores are given in Figure 13 (see website).

Purity assay: A purity assay was performed to test the cross-reactivity of the AG (#41-P) to various heterologous sera (data not shown). Additional testing was performed to test the capability of the AG to detect NS-1 antibodies in mouse and rat parvovirus-positive antisera. Results are shown in Figure 14 (see website). NS-1 AG showed titers only with sera positive for KRV, MPV, MVM, and RPV, and had no reaction with non-immune mouse and rat sera.

Normalization of ELISA assay conditions: For His-tagged NS-1 ELISA, the extracts from infected cells were initially attached to Ni-coated 96-well microtiter plates. This assay had low sensitivity and specificity. The antigen was purified using Sarkosyl and, later, CHAPS detergent. ELISA was modified for Immulon-4 plates and assays were initially performed using 100 µl each of antigen, serum sample, and conjugate. Once the purification and stability of NS-1 AG was improved, the assay was normalized to 50 µl/well incubations with primary serum and HRP-labeled secondary conjugate (40 minutes each) like all other standard ELISA assays. Experiments were conducted using NS-1 AG (#40-P) to compare different ELISA conditions. The amount of AG coated on the plates was maintained by compensating for the different coating volumes used in our standard NS-1 ELISA (100 µl/well and one hour incubations) versus the new conditions (50 µl/well and 40 minute incubations) by adjusting the AG dilution factor, 1:100 and 1:50 respectively and results are shown in Figures 15 and 16 (see website).

Different blockers/diluents were compared against our standard BLOTTO (Bovine Lacto Transfer Technique Optimizer). Addition of Tween-20 (T-20), PVP, or PVA at different concentra-

| Correspondence of His/NS-1 ELISA to IFA | | | | | | | |
|--|----------|------------------------|----------|--------------|----------|--------------|----------|
| | | MVM/KRV/MPV IFA | | | | | |
| | | Mouse | | Rat | | Total | |
| | | + | - | + | - | + | - |
| NS-1 ELISA | + | 50 | 10 | 22 | 8 | 72 | 18 |
| | - | 29 | 111 | 1 | 25 | 30 | 136 |
| Specificity | | 91.7% | | 75.8% | | 88.3% | |
| Sensitivity | | 63.3% | | 95.7% | | 70.6% | |
| PV+ | | 83.3% | | 73.3% | | 80.0% | |
| PV- | | 79.3% | | 96.2% | | 81.9% | |

Figure 19. ELISA vs. IFA

tions or combinations of T-20 with PVP or PVA added to BLOTTO did not improve the sensitivity and specificity of the assay (results not shown).

The following changes were made for the standard NS-1 ELISA:

- Coating of NS-1 AG was changed from 100 µl/well to 50 µl/well.
- The ELISA plates, after coating with the AG, were washed with 3 x 300 µl/well instead of 6 x 300 µl/well.
- Incubations with primary serum and HRP-conjugate were 50 µl/well for 40 minutes each instead of 100 µl/well for one hour each.

Field Trials: Field trials using mouse and rat sera samples from serology were conducted using the final ELISA conditions as given above. Figure 18 summarizes the results of NS-1 His-tagged ELISA using purified NS-1 antigen batches 40-P (Week 042100) and 41-P (Week 052600).

A comparison of the His/NS-1 ELISA to MVM/KRV/MPV IFA results was performed for mouse and rat sera. The tables below summarize data for mouse and rat sera. From the collected data the sensitivity, specificity, predictive value of a positive test result (PV+) and predictive value of a negative test result (PV-) were calculated according to the formulas given below (Fig. 19):

Sensitivity:
 $TP / (TP+FN)$

$PV(+) = TP / (TP+FP)$

Specificity:
 $TN / (TN+FP)$

$PV(-) = TN / (TN+FN)$

Conclusion

ELISA using purified His-tagged NS-1 antigen is highly sensitive and specific for detecting only NS-1 antibodies in mouse and rat sera. The results also show that although the NS-1 protein is highly conserved, NS-1 antibodies to parvovirus may not be present in all seropositive mouse and rat serum samples. Alternatively, the serum sample may have enough antibodies to structural proteins (e.g., VP-2) but a very low level of detectable NS-1 antibodies and hence produce false negative results from rNS-1 ELISA.

Therefore, rNS-1 ELISA results must be used in combination with results from other assays for detecting paroviruses, such as MVM ELISA for mice, KRV and H-1 ELISA for rats, and VP-2 ELISA for mice and rats.

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