**Editorial comments:**  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

>>This was completed.

3. Please define all abbreviations before use.

>>This was completed.

4. Please use SI units, e.g. please use “µL” instead of “µl”, “mL” instead of “ml”. Please leave a white space between the values and the units.

>>The changes were made.  
  
5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: “TaqMan”, “StepOnePlus”, “NanoDrop”, etc.

>>Commercial language was removed.  
  
6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

>>This was completed.

7. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. The Protocol steps should contain only 2-3 actions per step and a maximum of 4 sentences per step.

>>This was completed.  
  
8. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

>>This was completed.

9. For steps that involve software or analyzing tools, please make sure to provide all the details such as “click this”, “select that”, “observe this”, etc. Please mention all the steps that are necessary to execute the action item. Please provide details so a reader may replicate your analysis including buttons clicked, inputs, screenshots, etc. This is the level of detail we’re looking for. Please keep in mind that software steps without a graphical user interface cannot be filmed.

>>This was completed.

10. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

>>An ethics statement was provided.

11. Please include the age, sex and strain of the animals.

>>>The information was added.  
  
12. Protocol: 1.1: Please include all the conditions for keeping the animals or refer to appropriate references. Please define all abbreviations before use.

>>ZT was defined and a reference was inserted describing basic animal husbandry.

13. Protocol: 2.2: How much of each solution should be prepared? Please include the containers.

>>The information was added

14. Protocol: 2.3: How the culture media is warmed? Using what? How much PBS is needed? What is the pH of the buffer?

>>The information was added  
  
15. Protocol: 2.4: Please include the pipette size.

>>The information was added

16. Protocol: 2.5: How much ethanol is needed?

>>The information was added  
  
17. Protocol: 3.1: Please clearly describe the euthanasia process or refer to appropriate references. Please use the imperative tense for all the sentences in the protocol step. Please attention that euthanasia process can not be filmed, so please do not highlight this steps.

>>The highlight was removed. The following sentence was also removed, “For this particular experiment, splenocytes were isolated from mice that were sacrificed at ZT13” as the timing of sampling is described in the discussion. Details regarding the euthanasia process was added as was a reference.

18. Protocol: 3.2: How to cut away the fur? Using what?

>>This information was added.  
  
19. Protocol: 3.3: Please use sub-steps for long protocol steps.

>>sub-steps were added  
  
20. Protocol: 3.5: Please use the imperative tense for all the sentences in the protocol steps.

>>The step was reworded

21. Protocol: 3.7: How to transfer the medium?

>>This information was added

22. Protocol: 3.8: What is the temperature of the centrifugation?

>>This information was added.  
  
23. Protocol: 4.1, 4.2: Please use the imperative tense for all the sentences in the protocol steps. Please avoid using any commercial language, please use a generic term instead.

>>The changes were made and a sub-step was added

24. Protocol: 4.3: The Protocol steps should contain only 2-3 actions per step and a maximum of 4 sentences per step. Please use sub-steps. Please clearly describe the actions in the imperative tense. Please include the pipette size and the containers.

>>The relevant information was included and the step was sectioned into sub-steps.

25. Protocol: 5.1, 5.2, 5.4: Please avoid using any commercial language (e.g., StepOnePlus, TaqMan, etc.), please use a generic term instead.

>>These terms were removed

26. Protocol: 6.1: Please use the imperative tense for all the sentences in the protocol steps. Please clearly describe the actions or refer to appropriate references.

>>The section was re-written

27. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file.

>>The table was removed

28. If you are reusing figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [AUTHOR] et al.[REFERENCE]”.

>>This does not apply

29. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please list all the materials, equipment, instrument, and software used in your work.

>>The table has been modified

**Reviewers' comments:**  
  
**Reviewer #1:**  
Manuscript Summary:  
very nice summary of the relevance of the clock and what this technique will provide. Very detailed protocol and nice discussion of how to interpret the data  
  
Major Concerns:  
None  
  
Minor Concerns:  
None  
  
  
**Reviewer #2:**  
Manuscript Summary:  
This paper describes a method to use mouse splenocytes to assess Pathogen-Associated Molecular Pattern Influence on clock gene expression. Over all, this paper is well written, and clearly stated.  
  
Major Concerns:  
1. In this paper, the author collected splenocytes from the tissue, and wanted to isolation the immune cells from the splenocytes. But in this method, the author did not detect the cell type he/she got, so it is not sure whether the cell in this paper is immune cells or not.

>>While I did not demonstrate the splenic cell composition in this method, the immune cell percentages present in the spleen have been previously determined and confirmed by my colleagues and I (unpublished data). The cell percentages are listed in the discussion. We used this splenocyte isolation protocol previously and subsequently worked with an adhere cell population: https://www.heliyon.com/article/e00579

2. In the paper, after separated the cell from the splenocytes, the author used RPMI 1640 with 10% FBS to incubate the cell. As we know, FBS can induce circadian gene expression in mammalian tissue culture cells (PMID:9635423), and the cultured cells. So, the dissecting time does not have much meaning.

>>I have previously tried to trigger the clock *ex vivo* in splenocytes using the above method. However, I was unable to do so (unpublished data) and that was using 50% serum as was used in the paper (Balsalobre, 1998) mentioned by reviewer #2. If a serum shock using 50% serum did not reset the clock in these cells, then I do not believe 10% would.

>>More importantly, I have assessed expression of *Per2* and *Rev-erba* in splenocytes isolated over a 24-h period using the same method I am describing in this protocol. Daily changes in gene expression were observed (<http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0189949#sec013>), which demonstrates that the serum in the RPMI is not resetting the clock. If the clock was reset, expression would not change over the 24-h period.

Minor Concerns:  
1. Line 44, it should make clear that the definition of the "master clock" in mammals or mice.

>>This information was added.

2. Line 82, "Animal Ethics Statement" should be stated.

>>An ethics statement was added

3. Line 82, Light intensity should give. Mouse age and gender should be made clear.

>>This information was added

4. Line 101, How long the time need for sterilize should give.

>>Scissors and forceps were originally autoclaved. The ethanol was used to prevent contamination and keep them ‘clean.’ This information was added to the protocol.

5. Line 105, "600 ml per sample", it is not clear the sample size, sample weight or cell number should give.

>>This information was added.

6. Line 157, it is better to give the RNA weight, not the volume.

>>In Protocol: 4.2 I added that up to 2 μg can be used. Since every well contains 106 cells, the amount of RNA that is extracted is very consistent and yields under 2 μg, therefore, I use 10 μl of RNA during the cDNA synthesis step.

7. Line 181 and 187, again, cDNA weight should also give.

>>In Protocol: 5.2, I added the range of cDNA that can be used for the reaction.

8. Figure 1, the SD or SEM should be given in the figure, not only the mean value.

>>The SEMs were added to the figure.

**Reviewer #3:**  
In this manuscript, Dr. Silver describes a protocol to measure the impact of PAMPs on clock gene expression in splenocytes. The protocol consists in harvesting spleens from mice, culturing the splenocytes in the presence or absence of LPS or other PAMPs, preparing RNA and using it for RT-qPCR for clock genes. Although the manuscript is generally clear and well written, and the protocol might be useful to some readers/viewers, it would benefit from the clarifications requested below.  
  
Main concerns:  
1) This protocol is extremely basic. Culture of primary splenocytes is routine use in numerous labs, as qPCR is too. I know that JoVE is fine with publishing even very basic approaches, so I guess that publishing this manuscript is relevant. I have not checked previous JoVE manuscripts/videos, but I would have assumed that there are already some that describe either splenocyte culture or qPCR, perhaps both together. The novelty here is to present a protocol that brings the two together, in the context of clock gene expression. In any case, this protocol and video might be of interest to JoVE readers, but I feel that the author should better explain the novelty of what is presented.

>>While addressing the editor’s and reviewers’ comments, I believe this was accomplished.

2) The manuscript (and presumably the video too) should better explain why it is important to study the effect of PAMPs and TLR activation on clock genes. Such an explanation is absent both from the introduction and the discussion. A sentence on line 77 says "since disruptions of circadian rhythms can lead to serious pathology" as a rationale for the proposed approach. However, the protocol only addresses the acute effects of PAMPs, not the (longer term) effect on clock function itself.

>>I believe the following will better explain the usefulness of this protocol.

>>The following sentence was added to the introduction: “The molecular clock has been shown to modulate various aspects of the immune response, therefore, disruption of the molecular clock would most likely impair the proper time-dependent variation of the immune response.”

>>>The following paragraph was added to the discussion: Moving forward, while this protocol only addresses the acute effects on clock gene expression after PAMP challenge, it could provide proof of principle for further investigation. For example, this assay could be used as a model to decipher the molecular mechanisms regarding TLR – PAMP interaction and how it influences the molecular clock. It could also be used to determine the length of time it takes for the molecular clock to recover after a PAMP challenge, which could be determined by conducting a time-course experiment (*i.e.*, assessing expression after varying times post challenge). As mentioned above, subsequent experiments could be performed to examine PAMP challenge on specific splenocyte cell populations. Since several pathogens stimulate multiple TLRs upon infection, it would be interesting to use this protocol to investigate if challenging with multiple PAMPs have a synergistic effect on clock gene expression.”

3) A general comment about the Protocol section of the manuscript is that although well written, I feel that it should be more detailed, and should describe which aspects are critical for it to work, including tips to optimize the procedure. Since this is a very basic protocol, it will be read (and watched) by people who presumably know little about splenocyte culture, qPCR or clock genes. Hence, no details should be omitted and no basic knowledge assumed as known by the readers.

>>While addressing the editor’s and reviewers’ comments, I believe this was accomplished.

4) The section on animals should be detailed. How are they entrained in the LD conditions, under which conditions exactly?

>>More details were provided.

It should also be explained that the procedure could be done under constant darkness if one would be interested in splenocytes under circadian free-running conditions.

>>The following was added to the discussion: “Diurnal rhythms are subjected to environmental stimuli (*e.g.*, light or food), which are termed zeitgebers. In the case of a 12-hr light / 12-hr dark cycle, the zeitgeber (*i.e.*, light) resets the clock to a 24-hr period. While most diurnal rhythms are circadian (*i.e.*, daily rhythms that occur in the absence of an external cue), they not true circadian rhythms until they have been shown to oscillate with an approximate 24-h period under constant environmental conditions. Therefore, this procedure could be performed using mice under constant conditions, which would entail entraining mice to the light-dark cycle as described above, but then the animals would be held in constant darkness for 3 days prior to sampling. This type of experiment is referred to as a dark-dark (DD) experiment and the time point of sampling would be referred to as CT (circadian time), not ZT.”

Even more important, the reason for the time of collection should be explained. Why harvesting the spleens at ZT13? This is not a time of day (evening) where most people would normally do their procedures, so explanations are needed.

>>The following was added to the first paragraph of the discussion: “Since *Dbp* and *Rev-erbα* have been shown to demonstrate significant expression peaks in splenocytes and splenic immune cells around the light-dark interphase (ZT12), in the current method, cells were isolated and challenged at ZT13 in order to have a greater chance at detecting a reduction in these genes.”

Actually, based on what is already known in the literature about the circadian control of response to PAMPs, couldn't one expect that the response be different if spleens/splenocytes are taken at different times over the 24 h cycle? This would be important to mention, as a possible factor to consider (and test) in such experiments. (Or maybe this is not relevant, if the medium treatment resets the clock? See point 5, below.)

>>Yes, and this was addressed in the discussion: “When examining the impact of PAMPs on the molecular clock, the time of day when mice are sacrificed and splenocytes are subsequently challenged must be taken into consideration. *Tlr* expression and responsiveness has previously been shown to demonstrate time-of-day dependent variation9,23, therefore, a time of day when TLR responsiveness is at its peak, could result in a greater influence on the clock. Furthermore, expression of molecular clock genes will also fluctuate throughout the day in splenocytes, therefore, a reduction of clock gene expression due to PAMP challenged would be most significant if examined during the time of peak expression10. Conversely, a PAMP that could increase clock expression, would most likely be observed if looking at a time of day when clock expression is at its lowest.”

5) I feel that the culture of the splenocytes and their stimulation is problematic when one considers that these cells have clocks which can be reset by serum treatment (like the one taking place when 3 ml of medium is added, line 134). Could this resetting by the serum mask the effect of PAMPs? For example, if a gene transcript is at high level in the cells, maybe one would not see a stimulation by a PAMP, but such an effect would be seen upon treatment at another clock time (time after synchronization of the cellular clocks). So maybe the protocol should include a wait of a few days after putting the splenocytes in culture (to let the clocks desynchronize) or conversely, take advantage of this clock synchronization and stimulate with PAMPs at different times after synchronization?

>>I have previously tried to trigger the clock *ex vivo* in splenocytes using the above method. However, I was unable to do so (unpublished data) and that was using 50% serum as was used in the paper (Balsalobre, 1998). If a serum shock using 50% serum did not reset the clock in these cells, then I do not believe 10% would.

>>More importantly, I have assessed expression of *Per2* and *Rev-erba* in splenocytes isolated over a 24-h period using the same method I am describing in this protocol. Daily changes in gene expression were observed (<http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0189949#sec013>), which demonstrates that the serum in the RPMI is not resetting the clock. If the clock was reset, expression would not change over the 24-h period.

6) Why harvest cells 3 h after PAMP stimulation (line 136)? Is the time course the same for all PAMPs? And for all clock (or other target) genes? A time course (e.g. 1-2-3-6-12h) would be more advisable.

>>While a 3 h timepoint was chosen for this protocol, a time course experiment would be very interesting as it would allow one to determine the length of time it takes the clock to recover from PAMP challenge. I added this potential experiment to the discussion.

7) Why isn't the qPCR part planned to be in the video? It is not less complex nor less tricky than splenocyte preparation and culture (in fact, it is probably more, for someone not experienced in qPCR).

>>I would leave that decision up to the editor. From what I have seen, protocols in JoVE frequently have links to previously established protocols. I assumed this protocol would have a link to qPCR set up. However, I would be fine with including it in the video.

8) A problem with this protocol is the complex cell population that is studied, which limits the interpretation of the data. This is somewhat addressed in the Discussion, but it should be expanded, for example to explain how to interpret the data despite this limitation (e.g. based on the known expression of the TLRs in the spleen cells?) and how it could be solved experimentally (e.g. purification of the cell subsets prior to stimulation). I feel that this limitation is more problematic than the advantages listed for the protocol (large cell numbers, little tissue manipulation).

>>The following was added to the discussion: “For example, *Tlr9* expression rhythms in the mouse spleen differ between splenocytes, macrophages, B cells, and DCs16. Additionally, *Tlr1*, *Tlr3*, *Tlr4*, *Tlr6*, *Tlr7*,and *Tlr8* displayed significant daily oscillations in an adherent splenocyte population but only *Tlr2* and *Tlr6* experience daily oscillations in enriched splenic macrophages25. Therefore, in order to investigation the outcome of a challenge on individual cell types, cells could be isolated via magnetic cell sorting, as previously described10,16 and then subsequently challenged.”

>>Additionally, this protocol can be used for proof of principle for more expanded studies. This is stated in the revised concluding paragraph in the discussion.

Actually, the list of cells (line 259) is not correct: among the splenocytes obtained by the technique as described, there would be no DCs or macrophages, which would require collagenase treatment to be released from the tissue; this needs to be corrected. Related to this is the point made by the author about the circadian rhythm of these cell types within the spleen: what could be the solution for this? Discuss.

>>I have previously used this isolation procedure and was able to obtain both macrophages/monocytes and dendritic cells: [https://doi.org/10.1016/j.heliyon.2018.e00579](https://doi.org//10.1016/j.heliyon.2018.e00579)

>>Keller et al. use a similar approach as to what I describe (i.e., no collagenase treatment) to isolate CD11b+ cells.

>>Also listed below is a reference for a protocol comparing DC isolation via cell strainer vs. collagenase treatment.

>>While DC and macrophage numbers are lower without collagenase, it is incorrect to say that DCs and macrophages would not be present.

A circadian clock in macrophages controls inflammatory immune responses

Maren Keller, Jeannine Mazuch, Ute Abraham, Gina D. Eom, Erik D. Herzog, Hans-Dieter Volk, Achim Kramer, Bert Maier

Proceedings of the National Academy of Sciences Dec 2009, 106 (50) 21407-21412; DOI: 10.1073/pnas.0906361106

**Isolation of Mouse Spleen Dendritic Cells Andrew J. Stagg, Fiona Burke, Suzanne Hill, and Stella C. Knight** From: Methods in Molecular Medicine, vol. 64: Dendritic Cell Protocols Edited by: S. P. Robinson and A. J. Stagg © 2001 Humana Press Inc., Totowa, NJ

More specific comments:  
- Line 3-4: The title should be revised to: "The use of mouse splenocytes to assess pathogen-associated molecular pattern influence on clock gene expression". (first few words revised to read better; and "molecular clock expression" — somewhat meaningless — revised to "clock gene expression")

>>The change was made

- Line 54: "Functional molecular clocks have been described..." In 2 of the 3 references cited here (Keller et al being the exception), only clock gene expression has been described. This is not sufficient to conclude that a "functional clock" is present in those cells. Assays to assess the cell-autonomous rhythmic gene expression or circadian function must be performed (e.g. rhythms in ex vivo culture) to conclude that there is a functional clock. Revise the sentence or change the references.

>>I removed one of the references. However, the Silver et al reference assessed clock gene expression under constant conditions in ex vivo splenic immune cells, which is what the reviewer is requesting (i.e., “rhythmic gene expression in ex vivo culture”). Therefore, I am confused as to why this reference would not be appropriate. The authors of this paper state, “Our data demonstrate that macrophages, DCs, and B cells enriched form mouse spleen possess functional molecular clocks as demonstrated by the daily oscillations in clock gene expression.”

- Line 86: Define ZT at first occurrence.

>>The change was made.

- Lines, 97, 99, 118, and other occurrences: singular is "medium", not "media".

>>The changes were made.

- Line 105 (and 145): What is RLT buffer?

>>The following was added to the first place the buffer was used: “… Buffer RLT (a proprietary component of the RNA extraction kit that supports the binding of RNA to the silica membrane)… ”

- Line 115: Cut only the fur, not also the tissue under it? And "left side of the mouse" is unclear: from which standpoint?

>>The following was added: “Using forceps, grab the peritoneum and carefully make an incision as not to damage the spleen.”

>>“left side of the mouse” was changed to, “mouse’s left side”

- Line 122: "between two sterile frosted slides": Mention that the tissue is grinded between the frosted parts. This is unclear. Also mention that the tissue and cells should remain wet and in the buffer at all times.

>>This information was added.

- Line 150: Briefly describe those manufacturer's instructions? Are there aspects of this manufacturer's protocol that are particularly tricky or that should be performed in a certain way?

>>While initially writing this protocol I asked the science editor about this issue. She told me that writing “according to manufacturer’s instructions” would suffice.

- Line 154-155: Explain Nanodrop quantification and what the optimal range is. Also explain how to assess the purity of the RNA.

>>The following was added to the discussion: Within this protocol, a microvolume spectrophotometer can be used to quantify and assess the purity of the RNA being used in determining gene expression. Nucleic acids absorb UV light at 260 nm, proteins typically absorb light at 280 nm, while other potential contaminants used during an RNA extraction procedure (*e.g.* phenol) are detected at 230 nm. Therefore, by assessing the absorbance (A) ratio at 260/280 nm (RNA to protein) and A260/230 (RNA to non-protein contaminants) the quality of the RNA can be assessed. High quality RNA has an A260/280 ratio between 1.8 – 2.1, as lower ratios indicate protein contamination. A pure RNA sample will have an A260/230 ratio of 2.0.

>>The range of RNA used in the cDNA synthesis was added to the protocol.

- Line 191: Why use actin as the control? Explain that any gene used as control in the qPCRs must be verified to ensure that it does not vary with the treatment studied. Explain how.

>>The following paragraph was added: “When determining the relative expression of a target gene (*i.e., Per2, Clock, Rev-erbα,* and *Dbp*), an endogenous control gene (a gene in which expression levels do not differ between samples) must also be selected. Relative expression of the target gene is then normalized to the expression of the endogenous control gene. Differences in starting material (number of splenocytes), variation in reverse-transcriptase efficiency, varying rates of RNA degradation, etc., will be corrected for by the endogenous control gene (*β-actin* in this protocol). However, it is wise to verify that the treatment being examined does not alter expression of the endogenous control gene. This can be accomplished by assessing *β-actin* levels from several replicates of an equal amount of cells (treatment vs. non-treatment). In theory, their *β-actin* levels should be identical. Another approach to guard against endogenous control variation would be to use a panel of endogenous controls (*e.g., β-actin, Gapdh,* and 18S rRNA gene).”

- Lines 193-195: This data analysis point is very vague. A well validated approach such as the 2(-ddCt) must be used. Explain how. (See Livak KJ, Schmittgen TD. 2001. Methods 25:402-408.)

>>I have previously used this method:

Silver et al., The circadian clock controls toll-like receptor 9-mediated innate and adaptive immunity. *Immunity,* 2012

Silver et al., Circadian expression of clock genes in mouse macrophages, dendritic cells, and B cells. *Brain, Behavior, and Immunity*, 2012.

- Line 199: Use present tense as this is in the protocol ("is used").

>>This was changed.

- Line 263: Explain why it is essential to minimize animal and cell manipulations in clock studies.

>>The following was added: “because as mentioned above, these actions can disrupt the timing of the clock as well as clock controlled genes.”

- Lines 264-265: A n=3 is low, and could be insufficient depending on the stimulus used or the gene tested. Actually, the data provided in this manuscript suggest that there might be an effect of ODN1826 and HKLM on Reverba that does not reach significance due to a lack of power. If true, these data would argue for using a higher group size.

>>The following as added to the discussion: “It should be noted that increasing the number of animals per group might have revealed statistically significant differences between a challenge group and control (*e.g.,* ODN 1826 and *Rev-erbα*).”

- Lines 266-268: The sentence starts by "Moving forward" but the rest is just to state the primary objective of this manuscript. The manuscript would gain from ending on a description of other possible applications of this protocol, beyond PAMPs, beyond clock genes, and perhaps with more conditions and time points.

>>I added a paragraph in the discussion that addresses these points.

- Line 262: Reference 20 is incomplete.

>>The reference was fixed.

**Reviewer #4:**  
Manuscript Summary:  
This work introduced an method to analysis the molecular clock genes expression in mouse splenocytes after challenged ex vivo with the pathogen-associated molecular patterns (PAMPs), lipopolysaccharide (LPS), ODN1826, and heat-killed Listeria monocytogenes. Overall, from my impression, this work is well done indeed. It introduced detailed procedures about entrainment of animals, instruments and reagents preparation, mouse splenocyte isolation and challenge, RNA isolation and cDNA synthesis, Quantitative PCR, and Statistical analysis. People who are interested to investigate the pathogens influence on splenocyte molecular clock work can easily followed the protocols to achieve their research targets. Therefore, I recommend to publish this manuscript without any hesitate.  
  
Major Concerns:  
No  
  
Minor Concerns:  
No