**Reviews for** “Evidence of Increased Hemangioblastic and Early Hematopoietic Potential in Chronic Myeloid Leukemia (CML)-derived Induced Pluripotent Stem Cells (iPSC)”

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**Reviewer 1**

Has selected to remain anonymous.

Originality, novelty and significance of results: Adequate

Technical Quality of Work: Inadequate

Comprehensibility and Presentation of Paper: Adequate

What is the overall impression: Inadequate

**Reviewer Recommendation Term:** Accepted pending major revisions

**Narrative (as sent to corresponding author):**

The Manuscript 'Evidence of Increased Hemangioblastic and Early Hematopoietic Potential in Chronic Myeloid Leukemia (CML)-derived Induced Pluripotent Stem Cells (iPSC)' has a very promising abstract and introduction however, moving onto the Materials and Methods the paper suddenly becomes very difficult to follow and this impacts on the Results and Discussion sections. I believe that the authors were enthusiastic to publish the results and this resulted in a number of omissions.

Starting with the Materials and Methods section:

Have the iPSC and hESC been tested for mycoplasma and have they been authenticated by STR profiling?
Did you check the karyotype of the cells before you started the experiments?
TKI- please use full name before abbreviation.
When you are implying that pluripotent cells are undifferentiated, please can you say 'undifferentiated' pluripotent cells please.
Other lines UT7 and K562 provenance? What are they used for?
Can you provide some detail on the derivation of the cell line ie that it was made using Sendai.
Mention the PB33 line too.
Why did you use a polyclonal stock of iPSC rather than the clones of the PB32?
Please can you state the provenance of the H1 cell line.
Are you sure that the culture conditions for the hPSC cell lines is accurate? Please supply more detail on the procedure and reagents used? Did you really treat the cells with Collagenase for 2 hours?
For the cultures treated with Imatinib for 5 days . Please explain what you are doing with this reagent rather than add it in at the end of the sentence. Also mention the use of Dasatinub here.
A diagram with a timeline for the differentiation of cells would be very useful.

The EB assays and Blast colony forming assays lack detail..
For the Blast colony forming assay, how many cells did you use at the start of the experiment?
Please put more detail in the RNA and QRT PCR sections please.
What temperature are you warming the TrypLE to please?
Please add detail as to which cells and at what stages in differentiation were used for the PCR analysis.
Also provide detail on the treatment of samples for the QRT-PCR, conditions, cycle numbers, PCR machine used, software used for analysis
The section Phenotypic analysis of haematopoietic progenitors on the first line of page 7 should be 'to analyse' not to generate
The AHR experiments section needs more detail. The CFC assay should be described. Please explain the use of FICZ?
For the statistical analysis were all parts of each experiment performed three times?

Results:

The results section appears comprehensive and complete.
Can you comment on the high CFU-E numbers generated from the CML blasts?
Why do you not distinguish between the different types of haematopoietic CFCs in the CML EBs ?
An overview diagram capturing the stages of differentiation, pathways and inhibition would be useful to review the findings reported in the results and help with the discussion.

Discussion

The discussion is detailed and well thought out, however on page 17 please explain the significance of the finding that IM and Dasatinib inhibition was observed explain the significance of this in a pluripotent stem cell model.

Page 18 explain the significance on the expression of the Gata 3 and LMO2 expression and why this is interesting. Also expand on the potential of druggable targets.
Why do you think the the BL compartment is expanded in these iPSC cells?
Do you think that these cells would engraft an immunodeficient mouse please discuss this in the context of human iPSC engraftment studies. Could there be any homing problems? How does the integrin profile of these differentiated cells compare to their 'in vivo' counterparts?
Can you expand on the significance of the hemangioblast in this model? It will be interesting to see if these cells contribute to the vasculature in immunodefiecient mice

Figures:

Figures 4A and 7 is Mixe actually Mixed?
Figure 7, What does NS mean?
Supplementary Figures 1 and 2 Are these CD31 cells co-expressing CD45 high?

Conclusion:

This manuscript contains a wealth of data and provides an important contribution to the modeling of CML with pluripotent stem cells. If the Materials and methods section was more complete, this would facilitate the flow of the content.

**Reviewer 2**

Has selected to remain anonymous.

Originality, novelty and significance of results: Adequate

Technical Quality of Work: Inadequate

Comprehensibility and Presentation of Paper: Adequate

What is the overall impression: inadequate

**Reviewer Recommendation Term:** Accepted pending minor revisions

**Narrative (as sent to corresponding author):**

In this manuscript, the authors used hiPSC generated from CML patients to study the role of Bcr-Abl on hemangioblasts and early hematopoietic progenitors.
They demonstrated an increase in hemangioblastic and early hematopoietic potential in the CML iPS line compared to H1 (hESC line). They found that the BCR-ABL pathway was inhibited by Imatinib in the CML cells in vitro, although the patient was resistant to the drug. Last, they found a reduced expression of AHR in CML iPS line compared to control iPS line and tested AHR antagonist and agonist during hematopoietic differentiation. AHR agonist inhibited blast cell colonies expansion and therefore could be a potential novel drug targeting CML.

Comments:
-In their previous manuscript describing the derivation of the CML lines, the authors generated both polyclonal iPS cells and 3 clones from the CML patient. In this study, they chose to work with the polyclonal line only. Can the authors explain why? It was previously shown (Miyauchi et al, 2018) that iPS clones with and without the Philadelphia chromosome could be generated from CD34+ cells from CML patient. It was also demonstrated that some clones are resistant to Imatinib while some are not. The results would be more conclusive if the experiments were done on 2-3 iPS clones from the CML patient (instead of the polyclonal lines) and 2-3 control iPS lines.
-For the blast CFC assay: the CML PB32 iPS line showed a 4 fold increase in BL-CFC clonogenic potential compared to H1. PB33 iPS line failed to generate BL-CFC in most experiments. Additional control iPS lines should be tested to confirm the increased hemangioblast potential of the CML lines. In Supplemental data figure 1, the authors showed FACS analysis (including endothelial markers) for the CML iPS line derived BL-CFC. It would be interesting to know if these markers were differentially expressed in control derived BL-CFC.
-For the CFU assay: Hematopoietic differentiation was performed from BL-CFC and from EB. An increased number of hematopoietic colonies was observed in PB32 iPS lines compared to control. Was the PB33 iPS line tested for CFU assay from EB? In Figure 4A and figure 7, is the "Control EB" the PB33 iPS control or H1 control?
-The authors looked at the expression of genes involved in hematopoietic development by Western Blot in CML versus control cells at different stages of differentiation. They claimed that Lmo2 is only expressed in CML-IPSC derived Bl-CFC while CXCR4 expression is downregulated in CML Bl-CFC or EB. These conclusions are not accurate. LMO2 is also expressed in control-iPSC derived Bl-CFC (albeit at a lower level). CXCR4 expression is higher in CML-EB than in control EB.

-The authors claimed that AHR expression is reduced in CML cells. However, as shown in figure 9A, the level of expression is similar to the level in H1 cells. The AHR expression seemed to vary greatly between donors. Evaluation of AHR expression in more CML and control lines is needed to determine whether the level of AHR is reduced in CML cells. The authors suggested that the AHR signaling pathway could represent a novel druggable target of CML. The AHR antagonist and agonist should also be tested on control lines.

-The authors should provide more information on the PB33 iPS line. Was it generated from CD34+ cells? Which reprogramming method was used? Is there a link to a reference?
-What was the passage number used for the three PSC lines? Was their karyotype normal?
-I noticed few mistakes in the protocol section: "0.1mg/ml bFGF". The bFGF concentration commonly used is 10ng/ml.
"treated 2h with 1mg/ml of collagenase": The treatment with collagenase is most likely shorter than 2 hours.

**Author’s Response to Reviewers:**

Reviewers' comments:

Reviewer #1: The Manuscript 'Evidence of Increased Hemangioblastic and Early Hematopoietic Potential in Chronic Myeloid Leukemia (CML)-derived Induced Pluripotent Stem Cells (iPSC)' has a very promising abstract and introduction however, moving onto the Materials and Methods the paper suddenly becomes very difficult to follow and this impacts on the Results and Discussion sections. I believe that the authors were enthusiastic to publish the results and this resulted in a number of omissions.

Starting with the Materials and Methods section:

Have the iPSC and hESC been tested for mycoplasma and have they been authenticated by STR profiling?

Yes, both iPSC and hESC have been tested for mycoplasma infection (as it is done regularly in our lab) and were negative. Authenticity has also been checked for both using STR. These informations were added to the text.

Did you check the karyotype of the cells before you started the experiments?

Yes, karyotype analyses were done in both cell lines before the experiments.

TKI- please use full name before abbreviation.

This information was added to the paper.

When you are implying that pluripotent cells are undifferentiated, please can you say 'undifferentiated' pluripotent cells please.

This terminology was added to the paper.

Other lines UT7 and K562 provenance? What are they used for?

UT7 cells have indeed been used in our lab for CML modeling for several years. UT7 is a GM\_CSF-dependent megakaryoblastic cell line generated by Dr Komatsu’s lab in Japan (Reference added). They do not express BCR-ABL. We have generated BCR-ABL-expressing UT7 cells by BCR-ABL gene transfer in our work published in 2000 (Reference added). These have been proved to be very useful tool to analyze BCR-BL-induced signaling. UTT-11 cells that we have generated are used here as positive controls for BCR-ABL expression. K562 cell line used here is a CML blast crisis cell line, but it has no counterparts without BCR-ABL expression. In this paper, they were used as positive controls for BCR-ABL expression.

Can you provide some detail on the derivation of the cell line ie that it was made using Sendai.

The cell line was obtained by Sendaï-virus mediated pluripotency gene transfer. The description of the methodology was published previously (Telliam et al, Stem Cell Research 2016). We now added the summary of these experiments on the paper.

Mention the PB33 line too.

PB33 was generated using the same technology from a bone marrow donor.

Why did you use a polyclonal stock of iPSC rather than the clones of the PB32?

The use polyclonal stock avoids the potential interclonal differences which might be present leading to flawed results. We feel that this strategy permits the more accurate assessment of the effects induced by BCR-ABL as the leukemic gene is expressed in all the progeny in a more homogenous manner.

Please can you state the provenance of the H1 cell line.

H1 ES line was obtained from Wisconsin (WiCell) with the importation authorization obtained from Agence de Biomedicine in France. These informations were added to the text.

Are you sure that the culture conditions for the hPSC cell lines is accurate? Please supply more detail on the procedure and reagents used? Did you really treat the cells with Collagenase for 2 hours?

We have added some more informations in the text. Indeed, cell lines can be cultured either on Mitomycin-C-treated MEF layers of in feeder-free conditions. We have added this information in the text. In terms of collagenase treatment, the accurate timing in the article was dependent on the experiments; we agree that in some cell lines cells were detached as quickly as in 20 minutes, we have let this information in the text. We thank the reviewer for this comment.

For the cultures treated with Imatinib for 5 days . Please explain what you are doing with this reagent rather than add it in at the end of the sentence. Also mention the use of Dasatinib here.

iPSC were cultured in the presence of Imatinib to determine the effects of the drug on the expression of BCR-ABL. Dasatinib and Imatinib were both added in methycellulose cultures, to determine the inhibition of the clonogenic activity of CML-iPSC-derived hematopoietic progenitors.

A diagram with a timeline for the differentiation of cells would be very useful.

We think this would overcrowd the text.

The EB assays and Blast colony forming assays lack detail..
These details have no been added to the text.

For the Blast colony forming assay, how many cells did you use at the start of the experiment?

Blast colony assays were performed 3.5 days after bFGF deprivation of iPSC or hESC detached from cultures. They were suspended at ta concentration of 30-60. 103cells in 60-100 microliter of Stemline medium containing VEGF (50 ng/ml) TPO, Flt3-L and BMP4 (2à ng/ml each)) and EPO (5 U/ml) and mixed with 600- 1000 microliter of methycellulaorz H4436 (Serum free Methocult, StemCell Technologies).
Blast cell colonies were coiunted at day 6-8.

104 cells / dish were used for blast colony assays.

Please put more detail in the RNA and QRT PCR sections please.

Please see next paragraphs.

What temperature are you warming the TrypLE to please?

Frozen TrypLE is slowly warmed at 4°C overnight and then kept at 4°C for regular use.

Please add detail as to which cells and at what stages in differentiation were used for the PCR analysis.

For PCR analyses different types of cells were used:
-iPSC at undifferentiated stage
-Embryoid bodies derived from iPSC
-Blast cell colonies
-Hematopoietic progenitors

Also provide detail on the treatment of samples for the QRT-PCR, conditions, cycle numbers, PCR machine used, software used for analysis

RTqPCR amplification was conducted with TaqMan Universal PCR Master Mix No AmpErase UNG (Applied Biosystems). PCR conditions were the following : PCR analyses were performed in the following conditions:
Fast Start Mix 2.5 μl, MgCl2 2.5 μl,Forward Primer 0.5 μl,Reverse Primer 0.5 μl, dNPTs 0.5, Taq 0.1 μl, cDNA 2 μl+ qsp H20 to 20 μl.
Amplification protocol included a denaturation step for 4 min 95°C and PCR was performed using 30 sec 95°C, 30 sec 65°C, 2 min 72°C followed by an elongation step of 10 minutes 72°C.
(Agilent Technologies, Stratagene Mx3005P).PCR machine used : Agilent Technologies, Stratagene Mx3005P.
This information was added to the text.

The section Phenotypic analysis of haematopoietic progenitors on the first line of page 7 should be 'to analyse' not to generate

We changed the sentence to “To analyze the hematopoietic potential of Bl-CFC…”

The AHR experiments section needs more detail. The CFC assay should be described. Please explain the use of FICZ?

For the statistical analysis were all parts of each experiment performed three times?

Yes, this was the case.

Results:

The results section appears comprehensive and complete.

Can you comment on the high CFU-E numbers generated from the CML blasts?

One of the phenotypic features of human CML is the establishment of the proliferation myeloid progenitors with sometimes EPO-independent growth of CFU-E. We think the high numbers of CFU-E could be part of this phenomenon.

Why do you not distinguish between the different types of haematopoietic CFCs in the CML EBs ?

Figure 7 details different types of CML-EB-derived hematopoietic progenitors.

An overview diagram capturing the stages of differentiation, pathways and inhibition would be useful to review the findings reported in the results and help with the discussion.

We think this would make the text overcrowded.

Discussion

The discussion is detailed and well thought out, however on page 17 please explain the significance of the finding that IM and Dasatinib inhibition was observed explain the significance of this in a pluripotent stem cell model.

These experiments validate the use of iPSC-CML-derived progenitors as a screen for tyrosine kinase inhibitors. We have now added this information in the discussion.

Page 18 explain the significance on the expression of the Gata 3 and LMO2 expression and why this is interesting. Also expand on the potential of druggable targets.

These observations are difficult to translate to clinical significance and we have preferred not to speculate on these expressions, whivh was not the scope of this paper.

Why do you think the the BL compartment is expanded in these iPSC cells?
Do you think that these cells would engraft an immunodeficient mouse please discuss this in the context of human iPSC engraftment studies. Could there be any homing problems? How does the integrin profile of these differentiated cells compare to their 'in vivo' counterparts?
Can you expand on the significance of the hemangioblast in this model? It will be interesting to see if these cells contribute to the vasculature in immunodeficient mice.

There has been a controversy about the involvement of endothelial cells in CML, the first paper being published more than 20 years ago. (Gunsilius E, Duba HC, Petzer AL, Kähler CM, Grünewald K, Stockhammer G, Gabl C, Dirnhofer S, Clausen J, Gastl G. Evidence from a leukaemia model formaintenance of vascular endothelium by bone-marrow-derived endothelial cells.Lancet. 2000 May 13;355(9216):1688-91. PMID:10905245)
Obviously the use of CML iPSC can not resolve this controversy (as all derivative cells including Bl-CFC harbor Ph1) but it is possible that by some paracrine effect induced by BCR-ABL we have observed increased hemangioblast formation.
We have not performed in vivo experiments using blast-CFC.

Figures:

Figures 4A and 7 is Mixe actually Mixed?

The Figures have been corrected (CFU-Mix)

Figure 7, What does NS mean?

NS means “not significant”; this has been clarified in the Figure Legend.

Supplementary Figures 1 and 2 Are these CD31 cells co-expressing CD45 high?

Yes, this could correspond to primitive cells able to differentiate towards erythroid cells as described (Stem Cell Research & Therapy 2021 12:236)

Conclusion:

This manuscript contains a wealth of data and provides an important contribution to the modeling of CML with pluripotent stem cells. If the Materials and methods section was more complete, this would facilitate the flow of the content.

Thanks to the Reviewer for these comments and suggestions for corrections which are now added to the text and improved the paper.

**After major revision:**

**REVIEWER 1:**

Originality, novelty and significance of results: Good

Technical Quality of Work:

Comprehensibility and Presentation of Paper: Good

What is the overall impression: Good

This is a revision of the manuscript 'Evidence of Increased Hemangioblastic and Early Hematopoietic Potential in Chronic Myeloid Leukemia (CML)-derived Induced Pluripotent Stem Cells (iPSC)'. The authors have addressed most of the reviewer's comments but still need to put the UT7 and K562 information into the materials and methods rather than in the results and include the growth conditions for these cells. Also as requested previously, please explain the use of FICZ in the materials and methods section.
Can I also please ask you to have the English checked for errors in language and syntax. Once these points are addressed I believe that it should be ready to be accepted for publication.

**REVIEWER 2:**

Originality, novelty and significance of results: Good

Technical Quality of Work: Adequate

Comprehensibility and Presentation of Paper: Adequate

What is the overall impression: Adequate

The authors have satisfactorily addressed most of my concerns. I still have few comments:

-Differentiation of polyclonal iPS lines versus multiple iPS clones: I understand the authors' rationale for choosing to work on polyclonal iPS lines, however clonal drift has been well documented and since the PB32 line is over passage 20, there is a concern that the PB32 line represents a unique dominant clone. Can the authors comment on this?
-Regarding the FACS data (including endothelial markers), was the analysis performed on H1 derived Blast-CFC as well? If so, it should be added in the supplemental data.
-LMO2 expression: The authors modified the sentence in the results section but maintained it in the discussion: "LMO2 expression was detectable only in CML Bl-CFC". Please change.
Moreover, the LMO2 western blot picture is not very convincing. There are 2 bands for CML BL-CFC. Is it two isoforms of LMO2?

-For the AHR pathway study. The authors agreed that there is variability in AHR expression between donors however the following sentence was maintained in the abstract: "In CML iPSC, we have also found a significant reduction of Aryl Hydrocarbon Receptor (AHR) expression which is involved in hematopoietic quiescence". I don't think the authors can reach this conclusion based on the comparison of PB32 and PB33 only.
It is not clear what was done with the AHR agonist FICZ. In figure 9B, the schematic does not show the use of FICZ but a significant reduction of BL-CFC is shown after FICZ treatment in the graph below. This was not mention in the result section and briefly cited in the discussion. No data is shown on the effect of FICZ treatment during the subsequent hematopoietic differentiation.

Minor edits:
Sendai virus reprogramming: The MOI recommendation from the manufacturer is 5-5-3. MOI 15 seems very high. Is this a mistake? Was the SeV RNA elimination tested in PB32 and PB33 pools?

There are multiple typo/mistakes in the added texts:

"Briefly, CD34+ cells were cultured for 4 days in the presence of growth factors including hSCF (100 ng/ml), hFLT-3 (100 ng/ml), hIL-6 (20ng/ml) , hIL-3 (20 ng/ml), and hIL7 (20 ng/ml) (all from Peprotech) for 4 days". For 4 days is included twice in this sentence.

"5life Technologies)"

"for Western blots, the analyze the expression of BCR-ABL". To analyze instead of the analyze.

"were treated treated for 1h with 1mg/ml Collagenase type IV for 20 minutes (EB assays)." Remove treated and for 1h

"PCR conditions were the following : PCR analyses were performed in the following conditions: " (redundant)
Qsp?
"PCR was performed using 30 sec 95°C, 30 sec 65°C, 2 min 72°C" Number of cycles?

**AUTHOR’S RESPONSE TO THE REVIEWERS ROUND 2**

Reviewer #1: This is a revision of the manuscript 'Evidence of Increased Hemangioblastic and Early Hematopoietic Potential in Chronic Myeloid Leukemia (CML)-derived Induced Pluripotent Stem Cells (iPSC)
The authors have addressed most of the reviewer's comments  but still need to put the UT7 and K562 information into the materials and methods rather than in the results and include the growth conditions for these cells.
Also as requested previously, please explain the use of FICZ in the materials and methods section. Can I also please ask you to have the English checked for errors in language and syntax. Once these points are addressed I believe that it should be ready to be accepted for publication.
Culture conditions for K562 and UT7 cell lines have now been explained in the Materials and Methods section. The use of FICZ has also now been explained in the same section. The English of the paper has been checked with regard to language errors and syntax.
All changes have now been indicated in blue letters.

Reviewer #2: The authors have satisfactorily addressed most of my concerns. I still have few comments:  -Differentiation of polyclonal iPS lines versus multiple iPS clones: I understand the authors' rationale for choosing to work on polyclonal iPS lines, however clonal drift has been well documented and since the PB32 line is over passage 20, there is a concern that the PB32 line represents a unique dominant clone. Can the authors comment on this?

The PB32 line is polyclonal stock, we have obviously no genetic marking to evaluate a clonal drift, because all cells have the Ph1 chromosome. But there is ample evidence showing that iPSC lines remain stable for several dozens of passages, we therefore feel that the polyclonal stock keeps its polyclonal nature in the CML PB32 cell line.
 -Regarding the FACS data (including endothelial markers), was the analysis performed on H1 derived Blast-CFC as well? If so, it should be added in the supplemental data.

No, this analysis was not performed.
 -LMO2 expression: The authors modified the sentence in the results section but maintained it in the discussion: "LMO2 expression was detectable only in CML Bl-CFC". Please change. Moreover, the LMO2 western blot picture is not very convincing. There are 2 bands for CML BL-CFC. Is it two isoforms of LMO2?

We apologize for this mistake, the sentence was now corrected in both Results and Discussion sections :

“LMO2 was expressed both in CML and control Bl-CFC albeit at lower levels in the latter”

We think the upper band in CML Bl-CFC, not seen in control Bl-CFC is an artefact.
  -For the AHR pathway study. The authors agreed that there is variability in AHR expression between donors however the following sentence was maintained in the abstract: "In CML iPSC, we have also found a significant reduction of Aryl Hydrocarbon Receptor (AHR) expression which is involved in hematopoietic quiescence". I don't think the authors can reach this conclusion based on the comparison of PB32 and PB33 only.

The involvement of AHR in hematopoietic quiescence is a well-established fact (Gasiewicz TA, Singh KP, Bennett JA. The Ah receptor in stem cell cycling, regulation, and quiescence. Ann N Y Acad Sci. 2014 Mar;1310:44-50. Epub 2014 Feb 3. PMID: 24495120).

However, we agree with the reviewer the we cannot reach this conclusion here using the comparison of the expression in these two IPSC lines. However, the differential expression by PCR is real, we have removed from the abstract the sentence about the hematopoietic quiescence.
 It is not clear what was done with the AHR agonist FICZ. In figure 9B, the schematic does not show the use of FICZ but a significant reduction of BL-CFC is shown after FICZ treatment in the graph below. This was not mention in the result section and briefly cited in the discussion. No data is shown on the effect of FICZ treatment during the subsequent hematopoietic differentiation.
FICZ which is the agonist of AHR inhibits Bl-CFC formation. This was not indicated in the scheme of the Figure 9B but the experiments conducted have now been explained in the Figure Legend 9B.
The effect of FICZ was tested only in the Bl-CFC generation and not on hematopoietic differentiation, this was briefly discussed in page 19 (Discussion).
 Minor edits:
 Sendai virus reprogramming: The MOI recommendation from the manufacturer is 5-5-3. MOI 15 seems very high. Is this a mistake? Was the SeV RNA elimination tested in PB32 and PB33 pools?
No, we have used MOI 15.
Yes, SeV elimination was checked using PCR.
 There are multiple typo/mistakes in the added texts:   "Briefly, CD34+ cells were cultured for 4 days in the presence of growth factors including hSCF (100 ng/ml), hFLT-3 (100 ng/ml), hIL-6 (20ng/ml) , hIL-3 (20 ng/ml), and hIL7 (20 ng/ml) (all from Peprotech) for 4 days". For 4 days is included twice in this sentence.
This has now been corrected.
 "5life Technologies)"

This has now been corrected.
  "for Western blots, the analyze the expression of BCR-ABL". To analyze instead of the analyze.

This has now been corrected.
 "were treated treated for 1h with 1mg/ml Collagenase type IV for 20 minutes (EB assays)." Remove treated and for 1h

This has now been corrected.
 "PCR conditions were the following : PCR analyses were performed in the following conditions: " (redundant) Qsp? "PCR was performed using 30 sec 95°C, 30 sec 65°C, 2 min 72°C" Number of cycles?

This information was provided on our responses but we omitted to include them in the Manuscript. This is now done.

**AFTER THE SECOND REVISION ROUND THE ASSOCIATE EDITOR DECIDED TO ACCEPT.**