# ASSOCIATION BETWEEN A FAVOURABLE CLINICAL RESPONSE TO ANTI-TUBERCULOSIS TREATMENT AND A POSITIVE PCR TEST FOR MYCOBACTERIUM TUBERCULOSIS IN HISTOLOGICALLY PROVEN ERYTHEMA INDURATUM / NODULAR VASCULITIS

**by**

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## ABSTRACT

**Background:** Polymerase chain reaction (PCR) has been used for many years to detect *Mycobacterium tuberculosis (M.tb)* DNA in biopsy tissue of erythema induratum (EI)/nodular vasculitis. Studies to ascertain the association between a positive PCR and clinical response to anti-tuberculosis (TB) therapy are lacking.

**Objectives:** Our aim was to determine the association between a favourable clinical response to anti-TB treatment and a positive PCR for *M.tb* in histologically proven EI.

**Methods:** Twenty-four cases of histologically proven EI were identified that had been biopsied in our department between 1 January 2009 and 31 December 2014. The response to anti-TB therapy was then determined retrospectively, establishing in which patients the subcutaneous nodules of EI had resolved on treatment. Thereafter the formalin-fixed paraffin-embedded tissue sections were sent for PCR.

**Results:** All patients included in our study received anti-TB treatment. The clinical response was favourable in eighteen patients (75%), no response to treatment was observed in five (20,8%) and in one patient (4,1%), who was lost to follow-up, the response could not be determined.
The PCR for *M.tb* was positive in only one sample (4,1%) whereas the other twenty-three samples (95,8%) had a negative PCR for *M.tb.*

**Conclusion:** The PCR technique on formalin-fixed tissue remains subject to multiple technical pitfalls. Thus only positive results are meaningful, whilst negative results are inconclusive.

## INTRODUCTION

Erythema induratum/nodular vasculitis is a panniculitis that usually presents clinically as tender, red to violaceous subcutaneous nodules, mostly on the posterior lower legs, which recur in crops, may ulcerate and can heal with scarring.

Histopathological features are classically those of a lobular or septolobular granulomatous panniculitis, a vasculitis most commonly of the small lobular venules of the subcutaneous fat, and coagulation-type fat necrosis.

The pathogenesis of erythema induratum (EI) is best discussed in tandem with what is known as -Id reactions.

EI represents an immunologic response of the host to certain antigens.

In cases of EI caused by tuberculosis (TB), the antigens consist of fragments of *Mycobacterium tuberculosis (M.tb)* bacilli which are spread via the bloodstream from either an identified source, for instance TB of the lungs, or more often from a hidden focus of TB.

A delayed or type IV hypersensitivity reaction in EI develops when abovementioned fragments of *M.tb* are engulfed and processed by antigen presenting cells, which present the mycobacterial antigens to T helper 1 memory cells. On re-exposure to the antigens, activation of macrophages occurs, with several cytokines being secreted.

This delayed cell-mediated reaction results in granulomatous inflammation in areas of slow circulation.

The vasculitic changes of small vessels in EI can be attributed to a type III hypersensitivity reaction, as mycobacterial antigens form complexes with antibodies. These complexes are deposited in vessel walls causing inflammation and fibrinoid necrosis of the vessels.

An -Id reaction (or autoeczematisation) represents a systemic reaction to antigens of infectious origin, causing skin lesions at sites distant from the initial antigenic stimuli.

No viable organisms are present in the cutaneous lesions of an -Id reaction. Once the cause has been successfully treated, the -Id reaction resolves.

EI, the most commonly encountered tuberculid, represents an -Id reaction to *M.tb* infection distant from the subcutaneous nodules of EI seen mostly on the lower legs.

Viable bacilli are not found in the lesions of EI and they resolve on antituberculosis treatment.

Other tuberculids, which form a spectrum together with EI – their size depending on the size of the blood vessels affected – include:

Lichen scrofulosorum,
Papulonecrotic tuberculid,
Nodular tuberculid and
Phlebitic tuberculid.

In 1855 Ernest Bazin first described EI. In later years a causal association between EI and TB was noticed, confirmed by studies in recent years, even though Mycobacterial bacilli have never been found histopathologically, nor have tissue cultures of biopsy specimens of EI lesions produced *Mycobacterium tuberculosis* (*M.tb*). Multiple other infectious and non-infectious disorders have also been associated with EI [1,2].

However, since the polymerase chain reaction (PCR) technique has been developed, minute quantities of *M.tb* DNA have been detected in lesional biopsies.
In countries with a high prevalence of TB, a greater percentage of the cases of EI would be associated with TB. These cases of EI resolve with combined anti-TB treatment.

In the literature, considerable differences exist between various studies regarding the percentage of EI biopsy specimens that had a positive PCR test to *M.tb* [3,4,5]. See Table 1.

| Authors of study | Year of publication | Number of EI cases | Number of positive PCR tests | % positive PCR for *M.tb* |
|---|---|---|---|---|
| Schneider *et al.* | 1995 | 20 | 5 | 25% |
| Baselga *et al.* | 1997 | 52 | 40 | 77% |
| Tan *et al.* | 1999 | 20 | 0 | 0 % |
| Tan *et al.* | 2001 | 26 | 14 | 53% |

Table 1: PCR positivity for *M.tb* in EI

Very limited data has been published thus far on the association between a favourable clinical response to anti-tuberculosis treatment and a positive PCR test for *M.tb* in histologically confirmed EI. Only one study was found describing this association. Tan *et al*. had 26 cases of EI in a study done in Singapore. Just over half had a positive PCR, and of the five cases which had a documented favourable clinical response to anti-TB treatment, four cases (80%) were PCR-positive [4].

Our aim was to do a retrospective 6 year study investigating the association of the abovementioned.

## AIM OF RESEARCH

Our aim was to determine the association between a favourable clinical response to anti-tuberculosis treatment and a positive polymerase chain reaction (PCR) test for *Mycobacterium tuberculosis* in histologically proven erythema induratum/nodular vasculitis, for the period from January 2009 to December 2014 at the Dermatology Clinic, Universitas Academic Hospital, University of the Free State (UFS).

## METHODOLOGY

### Study design

The study was a cross sectional analytical descriptive study.

### Sample

Included in the study were all patients with histologically proven erythema induratum/nodular vasculitis diagnosed in the period from January 2009 to December 2014 at the Dermatology Clinic, Universitas Academic Hospital, UFS, who did receive anti-tuberculosis treatment. A total of 24 patients met the inclusion criteria.

Above information was obtained by searching the patient database of histological diagnoses of the Department of Dermatology, UFS.

Excluded from the study were those cases of EI that had been diagnosed elsewhere and of which no histology sample was available, and any patient with EI that went for follow-up and treatment elsewhere – even if the initial diagnosis was made in our department – and for whom critical data was lacking.

### Measurement

The five steps that were followed to obtain the required data were:

Step 1: All histologically proven cases of erythema induratum/nodular vasculitis diagnosed between 1 January 2009 and 31 December 2014 by the Dermatology Department, UFS, were identified by searching the database of histological diagnoses of the Department of Dermatology, UFS.

Step 2: The histopathological features of each case of erythema induratum/nodular vasculitis were checked to confirm that the initial diagnosis in each case was correct

and whether the key findings (including vasculitis and necrosis) were present on the histology slides available to us. These were documented on the data form.

Step 3: The clinical notes from the files of the patients mentioned in step 1 were obtained from the Dermatology Clinic, Universitas Academic Hospital, and it was determined to which cases anti-tuberculosis treatment was given. The patients who did not receive any anti-tuberculosis treatment were not included in the study.

Then it was established and documented on the data form which of those that were treated had a favourable clinical response to the anti-tuberculosis therapy and which did not (in other words in which patients the subcutaneous nodules of EI did resolve on treatment, and in which they did not).

Step 4: All the formalin-fixed paraffin-embedded tissue sections of the above patients who did receive anti-tuberculosis treatment, were sent to the National Health Laboratory Service (NHLS) laboratory at the University of the Witwatersrand in Johannesburg, where the nested reamplification polymerase chain reaction (PCR) for *M.tuberculosis* was performed as follows:

DNA extraction: Sections (10µm) were prepared from each formalin-fixed paraffin-embedded sample. These sections were deparaffinised using 1ml xylene, and subsequently treated with 1ml ethanol. After centrifugation, DNA was then extracted from the samples using the DNA Micro QIA amp kit (Qiagen, Whitehead Scientific), according to manufacturer's instructions.

Control of contamination: Tissue blocks were sectioned using new blades for each sample to prevent cross contamination. Work area and work tools were cleaned with 3% Virkon between each block handled. Work areas were decontaminated with ultra violet light between subsequent procedures.

The extraction procedure was assessed by PCR amplification of the internal ß-globin control and DNA was quantified using the Nanodrop 1000 Spectrophotometer.

*Mycobacterium* nested PCR:

Nested PCR was done using primers designed to amplify a region of the gene encoding the 65-kDa mycobacterial antigen.

For the first round of the nested reaction, the reaction mix contained 5µl of template DNA, 200µM dNTP's (Roche) , 0.38 µM of each primer viz T1U1, T1U2 and T1D (Whitehead Scientific), 1.0 U Taq DNA polymerase (Roche), 10x Reaction Buffer (with $MgCl_2$, 15mM) in a total volume of 50µl.

The thermal conditions of amplification were as follows:

Initial denaturation to 94ºC for 4 minutes; subsequent 35 cycles consisting of 94 ºC for 1 minute (denaturation), 57 ºC for 2 minutes (annealing) and 72 ºC for 2 minutes (extension). A final extension at 72 ºC for 7 minutes completed the PCR run. PCR was carried out in the 9700 Gene Amp PCR System (Life Technologies).
The second round of the nested reaction was performed with internal primers T2U and T2D resulting in a 133 base-pair product. For nested reamplification, 5 µl of first round PCR product was transferred into a 45 µl of master mix solution containing second primers T2U and T2D. dNTP's, primers and Taq DNA polymerase were maintained at the concentrations of the first round master mix. PCR was repeated as above except that an annealing temperature of 52 ºC was used.

Gel electrophoresis:

Amplified PCR products were examined by agarose gel electrophoresis. Samples were electrophoresed at 100 volts using a 3 % agarose gel (Celtic Diagnostics), stained with

ethidium bromide (Merck). The gel was visualised under UV light. Positive samples appeared as a visible band with a molecular size of 150bp.

Controls: The PCR procedure was controlled with the use of both positive and negative controls. The positive control included paraffin embedded samples that had previously tested positive with Ziehl-Neelsen histology staining. The negative control used included a no-template control in which nuclease-free water was substituted as a template.

Primers (Table 2), PC04/ GH20 (Whitehead Scientific) targeting the ß-globin housekeeping gene served as a control for efficacy of extraction and amplification of DNA from paraffin embedded tissue material.

| **Primer Name** | **Sequence** |
|---|---|
| T1U1 | 5'-AAG GAG ATC GAG CTG GAG GA –3' |
| T1U2 | 5'-AGG CGT TGG TTC GCG AGG G –3' |
| T1D | 5'-TGA TGA CGC CCT CGT TGC C –3' |
| T2U | 5'-GTC TCA AAC GCG GCA TCG –3' |
| T2D | 5'-GTC ACC GAT GGA CTG GTC –3' |
| PCO4 | 5'-CAA CTT CAT CCA CGT TCA CC-3' |
| GH20 | 5'-GAA GAG CCA AGG ACA GGT AC-3' |

Table 2: *Mycobacterium* PCR primers and ß-globin PCR primers

Real-time amplification of the ß-globin gene:

This assay was performed using the Corbett Research RotorGene 6000 (Whitehead Scientific) RT-PCR machine using the Bioline SensiMix™SYBR No-Rox kit (Celtic Diagnostics). A final volume of 20 µl reaction mix was made using 0.2 mM of each primer, 10 µl 2x SensiMix™SYBR No-Rox Master Mix(with $MgCl_2$, 50mM) and 2µl of template DNA. The thermal cycling profile of this assay consisted of an initial denaturation step at 95ºC for 10 minutes, followed by 50 cycles consistent of 95ºC for 10 seconds (denaturation), 55ºC for 10 seconds (annealing) and 72 ºC for 15 seconds (elongation). After amplification, melt curve analysis was carried out at 95ºC

with a ramp rate 1ºC/5seconds. The average melting temperature ($T_{m)}$ of the β-globin amplicon was 85.5 +/-1.0°C.

Gel electrophoresis was performed on samples with doubtful $T_m$ values to confirm the presence of the 268 bp PCR fragment [6,7].

The results were sent to us and transferred to the data forms. See examples of PCR results in Appendix A and of internal control results in Appendix B.

Step 5: Using the data gathered, with the help of the Department of Biostatistics, we determined what the association between a favourable clinical response to anti-tuberculosis treatment and a positive PCR test for *Mycobacterium tuberculosis* in our patient sample was.

## PILOT STUDY

No pilot study was done.

## ANALYSIS

Numerical variables were summarised by means, standard deviations of medians and percentiles. Categorical variables were summarised by frequencies and percentages. Differences between groups were evaluated using appropriate statistical tests and confidence intervals for unpaired data.

These were done by the Department of Biostatistics of the Faculty of Health Sciences, UFS.

## ETHICAL CONSIDERATIONS

Permission to collect the applicable patient information was obtained from the Research Committee of the Free State Department of Health.

Personal information of all patients, whose clinical data was collected, was kept strictly confidential and not recorded on the data form.

All the formalin-fixed paraffin-embedded biopsy specimens which were sent to the laboratory in Johannesburg remained with the NHLS at all time.

## RESULTS

In our study all 24 patients received anti-tuberculosis (anti-TB) treatment for 6 weeks or longer. Of these, two patients received a trial of 6 weeks only, three patients had more than 6 weeks' treatment, 14 received anti-TB medication for 6 months or longer and for the remaining five the duration was unknown.
See Table 3.

| Duration of anti-TB treatment | Amount of patients (Total=24) |
|---|---|
| Trial of 6 weeks only | 2 |
| > 6 weeks | 3 |
| ≥ 6 months | 14 |
| Unknown | 5 |

Table 3: Anti-TB treatment

A favourable clinical response to anti-TB treatment was observed in 18 patients (75,0%), no response to treatment in five patients (20,8%) and in one patient (4,1%) the response could not be determined because the patient was lost to follow-up. See Figure 1.

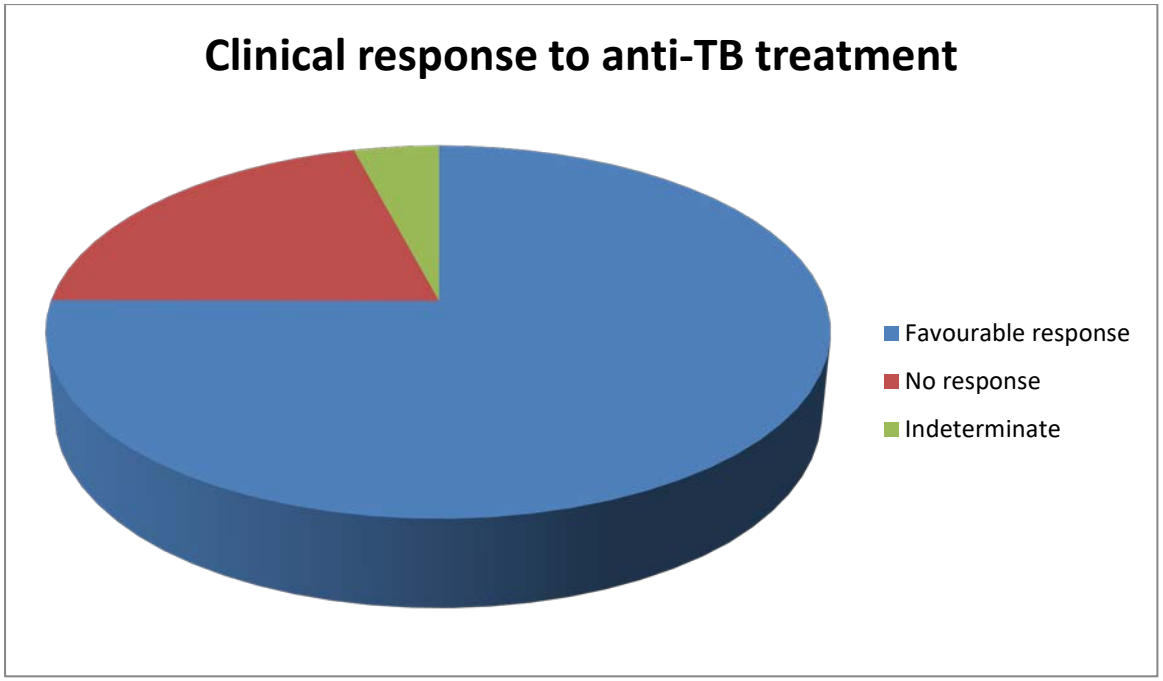


Figure 1: Clinical response to anti-TB treatment

The gender distribution was as follows: 22 were female and two were male. Seven patients had a positive test for the human immunodeficiency virus (HIV), 11 a negative test and in the remaining six patients the HIV status was unknown.

Histopathologic features of EI were present in the hematoxyllin and eosin slides of all the biopsy samples including a vasculitis in all 24 and necrosis of the fat in 16 of the samples.

The polymerase chain reaction (PCR) for *Mycobacterium tuberculosis (M.tb)* was positive in only one sample (4.1%) whereas the other 23 samples (95,8%) had a negative PCR for *M.tb*.

## DISCUSSION

Three quarters of the histologically proven cases of EI in our study had a favourable clinical response to anti-TB treatment, yet the PCR for *M.tb* was positive in only one case (4,1%). This was contrary to our expectation.

The first question that needs to be answered is whether these 18 patients did indeed have TB. The causal association between EI and TB has been well established for many years[1,2], and South Africa is a country with a very high prevalence of tuberculosis (estimated at 993/100 000 of the population in 2014). The fact that the skin lesions resolved on anti-TB treatment favours the likelihood that these 18 cases of EI represented tuberculids.

The five patients who showed no response to anti-TB treatment almost certainly had EI from causes other than TB.

The samples and method used to perform the PCR for *M.tb* can in various ways lead to false negative results.

Naturally the question would then arise as to whether a fresh biopsy specimen is needed or whether archival formalin-fixed paraffin-embedded samples are adequate.

In two studies done by Tan *et al.* the authors confirmed the effectiveness of the PCR method in archival specimens, some dating back a decade and a half. In one of the two studies the authors describe 26 cases of erythema induratum/nodular vasculitis, of which 14 (53%) had a positive PCR for *M.tb*. Five of the cases had a documented favourable clinical response to anti-TB treatment, and of these five, four cases (80%) were PCR-positive.

Abovementioned outcome is the reason why we strongly suspect that the results of our study include many false negative PCR tests.

There are multiple factors that each play an important role in the sensitivity of PCR for *M.tb* : This is underlined by the observation that 2 years prior to aforementioned study by Tan *et al.,* a report was published form the same centre using PCR to detect *M.tb* DNA in cutaneous tuberculosis and tuberculids. In the 20 specimens of erythema induratum/nodular vasculitis, the PCR then was negative in all [5].

Regarding archival samples, some authors again have described a reduced PCR sensitivity in formalin-fixed paraffin-embedded tissue [8].

Schneider *et al.* have furthermore experienced that PCR will not necessarily detect mycobacterial DNA in all the serial sections taken from the same *M.tb*-containing paraffin-embedded specimen [2]. They found 25% (or 5 of 20 cases) PCR positivity for *M.tb* in EI in a study in the Western Cape (with a very high prevalence of TB), and mentioned that factors like sampling errors or complete destruction of DNA by the inflammation present in EI, could also be causes for absent *M.tb* DNA.

In another study by Baselga *et al.* 74 paraffin-embedded biopsy specimens of erythema induratum/nodular vasculitis yielded 40 (54%) positive PCR tests for *M.tb*. DNA degradation was however thought to be the reason why in 22 of the specimens the results could not be interpreted due to negative or weakly reactive internal controls.

When the unreliable results of the PCR amplification were excluded, the rate of positivity for *M.tb* DNA increased to 77% [3].

The authors also found that the type of fixative influenced the results of the PCR amplification, with formalin (the fixative also used exclusively in our study) being far superior to Bouin solution [3].

The next possible cause for a false negative result can be unsuccessful DNA extraction form the paraffin-embedded specimens due to errors in the methodology.

Other causes might lie with the primers used to amplify a specific fragment of the *M.tb* DNA (as the detection limits of different primers vary) [9], or a lack of targeted sequences, or an unlikely problem with the DNA polymerase.

Lastly, false negative results can arise from inadequate DNA amplification or reamplification (if a nested PCR procedure is used, which has been shown to increase PCR sensitivity [4]).

When all of the mentioned possibilities for false negative results are taken into account, it becomes clear why there is such variability in the outcomes of different studies measuring the PCR-positivity for *M.tb* in EI.

## CONCLUSION

A positive PCR test for *Mycobacterium tuberculosis* in erythema induration/nodular vasculitis can be very helpful in clinical dermatology as the result can be available within 5 days or less, and will lead to prompt anti-TB treatment.

In our study approximately 75% of the patients did have TB, based on the response to treatment.

False negative PCR is a potentially significant practical problem when formalin-fixed tissue is being used.

The PCR technique on tissue remains subject to multiple technical pitfalls. Therefore only positive results are meaningful, whilst negative results are inconclusive.

Our recommendations:

Every case in which EI is clinically suspected, should be evaluated for TB by PCR.

In conjunction with the initial biopsy, PCR should be requested on fresh tissue, preferably on frozen or saline samples.

A prospective study is urgently needed, in which immediate fresh tissue PCR is being correlated with response to anti-TB therapy.

## ACKNOWLEDGEMENTS

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We would like to thank the Department of Biostatistics, University of the Free State, for their help with the statistical analysis.

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primer pairs to differentiate Bacille Calmette-Guerin (BCG) from virulent strains of *Mycobacterium tuberculosis* complex. J of Dermatol 2000; **27**: 717-23.

## APPENDIX A

### Mycobacteria Nested PCR

Name: DR ANTON BOTHA Date: 14 AUGUST 2015

| Reagent: | Lot no: | Exp Date: | Date Received: | Date In Use: |
|---|---|---|---|---|
| Roche Taq | 13647830 | 06-2015 | 23-7-15 | 5-10-15 |
| dNTPs | 14406000 | 12-2014 | 4-3-15 | 4-3-15 |
| T1U1 | 69393535 | Not Stated | 23-07-2015 | 23-07-15 |
| T1U2 | 69393535 | Not Stated | 23-05-2015 | 23-07-15 |
| T1D | 69393535 | Not Stated | 23-05-2015 | 23-07-15 |
| T2U | 69393535 | Not Stated | 23-05-2015 | 23-07-15 |
| T2D | 69393535 | Not Stated | 23-05-2015 | 23-07-15 |
| $ddH_2O$ | SW122B | 10-2015 | 15-4-14 | 15-4-14 |

FIRST ROUND

| PCR reaction | 1X | X30 |
|---|---|---|
| $ddH_2O$ | 32.75 µl | 982.50µl |
| 10X Reaction Buffer ($MgCl_2$, 15mM) | 5.0 µl | 150 µl |
| dNTPs (10 mM) | 1.0 µl | 30 µl |
| T1U1 (10 µM) | 2.0 µl | 60 µl |
| T1U2 (10 µM) | 2.0 µl | 60 µl |
| T1D (10 µM) | 2.0 µl | 60 µl |
| Roche Taq (5U/µl) | 0.25 µl | 7.5 µl |
| **Total** | **45.0 µl** | |
| **Sample** | **5.0 µl** | |

SECOND ROUND

| PCR reaction | 1X | X30 |
|---|---|---|
| $ddH_2O$ | 34.8 µl | 1044 µl |
| 10X Reaction Buffer ($MgCl_2$, 15mM) | 5.0 µl | 150 µl |
| dNTPs (10 mM) | 1.0 µl | 30 µl |
| T2U (10 µM) | 2.0 µl | 60 µl |
| T2D (10 µM) | 2.0 µl | 60 µl |
| Roche Taq (5U/µl) | 0.2 µl | 6.0 µl |
| **Total** | **45.0 µl** | |
| **Sample** | **5.0 µl** | |

## Mycobacteria Nested PCR

| Lane | Sample | Result | Comment |
|---|---|---|---|
| 1 | FUA 0902752 | NEGATIVE | |
| 2 | FUA 0910725 | NEGATIVE | |
| 3 | FUA 0915727 | NEGATIVE | |
| 4 | FUA 1001658 | NEGATIVE | |
| 5 | FUA 1010838 | NEGATIVE | |
| 6 | FUA 1013811 | NEGATIVE | |
| 7 | FUA 1107615 | NEGATIVE | |
| 8 | FUA 1107665 | NEGATIVE | |
| 9 | FUA 1114569 | NEGATIVE | |
| 10 | FUA 1118486 | NEGATIVE | |
| 11 | FUA 1122563 | NEGATIVE | |
| 12 | FUA 1223615 | NEGATIVE | |
| 13 | FUA 1311112 | NEGATIVE | |
| 14 | FUA 1403591 | NEGATIVE | |
| 15 | FUA 1404251 | NEGATIVE | |
| 16 | FUA 1408816 | NEGATIVE | |
| 17 | FUA 1006522 | NEGATIVE | |
| 18 | FUA 1409966 | NEGATIVE | |
| 19 | FUA 1410921 | NEGATIVE | |
| 20 | FUA 1411568 | NEGATIVE | |
| 21 | FUA 1411678 | NEGATIVE | |
| 22 | POS | POSITIVE | |
| 23 | BLANK | NEGATIVE | |
| 24 | NEGATIVE | NEGATIVE | |
| 25 | | | |

**APPENDIX B**

# Melt Report

## Experiment Information

| | |
|---|---|
| Run Name | INTERNAL CONTROL 2015-08-12 (1) |
| Run Start | 12/08/2015 06:25:49 |
| Run Finish | 12/08/2015 07:51:35 |
| Operator | |
| Notes | |
| Run On Software Version | Rotor-Gene Q Software 2.3.1.49 |
| Run Signature | The Run Signature is valid. |
| Gain Green | 2.67 |
| Machine Serial No. | 070846 |

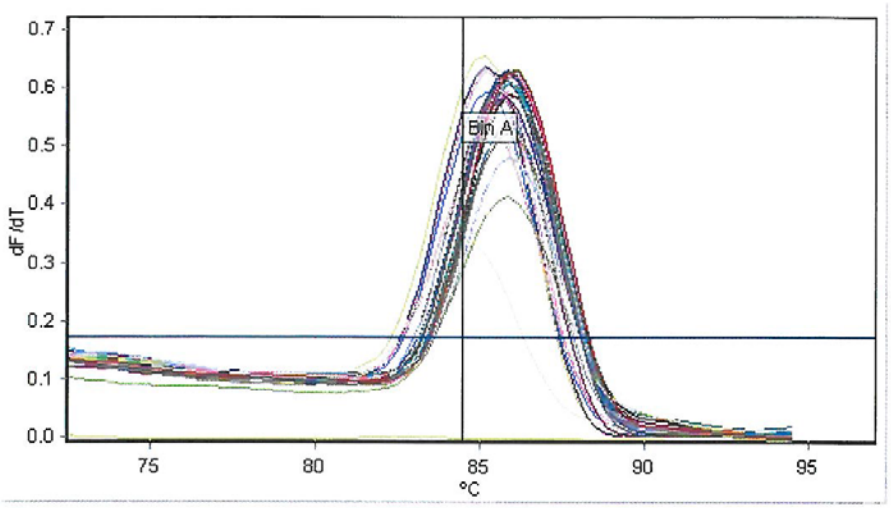
## Melt Information

| | |
|---|---|
| Digital Filter | Light |
| Imported Analysis Settings | |
| Sample Page | Page 1 |
| Temp. Threshold | 0°C |
| Threshold | 0.17144 |

## Profile

| Cycle | Cycle Point |
|---|---|
| Hold | Hold @ 95°C, 10min 0s |
| Cycling (50 repeats) | Step 1: Hold @ 95°C, 10s |
| | Step 2: Hold @ 60°C, 10s |
| | Step 3: Hold @ 72°C, 15s, acquiring to Cycling A([Green][1][1]) |
| Melt | Ramp from 72°C to 95°C |
| | Hold for 90s on the 1st step |
| | Hold for 5s on next steps,Melt A([Green][1][1]) |

## Melt data for Melt A.Green




| No. | Color | Name | Genotype | Peak 1 |
|---|---|---|---|---|
| 1 | | FUA 0902752 | POSITIVE | 86.0 (Bin A) |
| 2 | | FUA0910725 | POSITIVE | 85.0 (Bin A) |
| 3 | | FUA0915727 | POSITIVE | 85.8 (Bin A) |
| 4 | | FUA1001658 | POSITIVE | 85.2 (Bin A) |
| 5 | | FUA1010838 | POSITIVE | 85.2 (Bin A) |
| 6 | | FUA1013811 | POSITIVE | 85.2 (Bin A) |
| 7 | | FUA1107615 | POSITIVE | 85.8 (Bin A) |
| 8 | | FUA1107665 | POSITIVE | 85.3 (Bin A) |
| 9 | | FUA1114569 | POSITIVE | 86.0 (Bin A) |
| 10 | | FUA1118486 | POSITIVE | 85.8 (Bin A) |
| 11 | | FUA1122563 | POSITIVE | 86.0 (Bin A) |
| 12 | | FUA1223615 | POSITIVE | 85.8 (Bin A) |
| 13 | | FUA1311112 | POSITIVE | 84.5 (Bin A) |
| 14 | | FUA1403591 | POSITIVE | 85.8 (Bin A) |
| 15 | | FUA1404251 | POSITIVE | 85.5 (Bin A) |
| 16 | | FUA1408816 | POSITIVE | 86.0 (Bin A) |

| No. | Color | Name | Genotype | Peak 1 |
|---|---|---|---|---|
| 17 | | FUA1006522 | POSITIVE | 86.0 (Bin A) |
| 18 | | FUA1409966 | POSITIVE | 86.0 (Bin A) |
| 19 | | FUA1410921 | POSITIVE | 85.8 (Bin A) |
| 20 | | FUA1411568 | POSITIVE | 86.0 (Bin A) |
| 21 | | FUA1411678 | POSITIVE | 85.8 (Bin A) |
| 22 | | T5806 | POSITIVE | 85.8 (Bin A) |
| 23 | | T5807 | POSITIVE | 86.0 (Bin A) |
| 24 | | T5808 | POSITIVE | 85.8 (Bin A) |
| 25 | | T5809 | POSITIVE | 85.7 (Bin A) |
| 26 | | T5810 | POSITIVE | 85.7 (Bin A) |
| 27 | | T5812 | POSITIVE | 85.5 (Bin A) |
| 28 | | MSI63 | POSITIVE | 85.7 (Bin A) |
| 29 | | T5811 | POSITIVE | 85.5 (Bin A) |
| 30 | | KS/14-21242 | POSITIVE | 85.5 (Bin A) |
| 31 | | KS/14-42882 | POSITIVE | 85.7 (Bin A) |
| 32 | | KS/14-42467 | POSITIVE | 85.8 (Bin A) |
| 33 | | KS/14-34009 | POSITIVE | 86.0 (Bin A) |
| 34 | | NEGATIVE | | |

| Bin Name | Temperature | Sample No. | Sample Name | Peak |
|---|---|---|---|---|
| Bin A | 84.46 | 1 | FUA 0902752 | 86.0 |
| | | 2 | FUA0910725 | 85.0 |
| | | 3 | FUA0915727 | 85.8 |
| | | 4 | FUA1001658 | 85.2 |
| | | 5 | FUA1010838 | 85.2 |
| | | 6 | FUA1013811 | 85.2 |
| | | 7 | FUA1107615 | 85.8 |
| | | 8 | FUA1107665 | 85.3 |
| | | 9 | FUA1114569 | 86.0 |
| | | 10 | FUA1118486 | 85.8 |
| | | 11 | FUA1122563 | 86.0 |

| Bin Name | Temperature | Sample No. | Sample Name | Peak |
|---|---|---|---|---|
| | | 12 | FUA1223615 | 85.8 |
| | | 13 | FUA1311112 | 84.5 |
| | | 14 | FUA1403591 | 85.8 |
| | | 15 | FUA1404251 | 85.5 |
| | | 16 | FUA1408816 | 86.0 |
| | | 17 | FUA1006522 | 86.0 |
| | | 18 | FUA1409966 | 86.0 |
| | | 19 | FUA1410921 | 85.8 |
| | | 20 | FUA1411568 | 86.0 |
| | | 21 | FUA1411678 | 85.8 |
| | | 22 | T5806 | 85.8 |
| | | 23 | T5807 | 86.0 |
| | | 24 | T5808 | 85.8 |
| | | 25 | T5809 | 85.7 |
| | | 26 | T5810 | 85.7 |
| | | 27 | T5812 | 85.5 |
| | | 28 | MSI63 | 85.7 |
| | | 29 | T5811 | 85.5 |
| | | 30 | KS/14-21242 | 85.5 |
| | | 31 | KS/14-42882 | 85.7 |
| | | 32 | KS/14-42467 | 85.8 |
| | | 33 | KS/14-34009 | 86.0 |
| | | | Mean | 85.67 |
| | | | Std. Dev. | 0.34 |