

A DETECTION METHOD

The present invention relates to a method of detecting Hand-Foot-and-Mouth Disease (HFMD) in subjects. In particular, the method relates to detecting promising biomarkers in the saliva of these subjects.

HFMD is a widespread epidemic viral disease which afflicts millions of infants and children yearly in the Western Pacific region caused by the human enterovirus species A (HEV-A) from the genus *Enterovirus*. Till recent years, Coxsackievirus A16 (CA16) and Enterovirus 71 (EV71) were the principal etiological factors of HFMD (Wang and Liu, 2014). However, the surge in the number of cases caused by other HEV-A serotypes such as Coxsackievirus A6 (CA6) was also reported. HFMD is customarily a self-limiting disease characterized by fever and papulovesicular, sometimes maculopapular, rash on the palms, soles, elbows, and trunk as well as mouth ulcers (Wang and Liu, 2014). However, EV71-associated HFMD can quickly develop into severe neurological complications such as aseptic meningitis and acute flaccid meningitis in a modest proportion of cases. These neurological complications may in turn swiftly progress to cardiopulmonary failure and mortality (Solomon et al., 2010). Even though neurologic complications have been largely associated with EV71 (Lin et al., 2003), CA16 has also been reported to cause neurological complications (Li, 2010). The various complications and manifestations that could arise from enterovirus infections strongly necessitate a rapid and accurate identification of enterovirus so that efficient isolation of infected patients could be carried out to prevent further spreading.

HFMD is rapidly transmitted either via faecal-oral or droplet route and is currently diagnosed by physicians via clinical symptoms and manifestations. Additional laboratory testing is mostly deemed unnecessary for mild cases (Li et al., 2015). Nevertheless, the aforementioned can lead to misdiagnosis and could aggravate spreading of HFMD in atypical and mild cases. In addition, there is currently no cure for HFMD. Treatment options are confined to alleviating of physical symptoms. Therefore, rapid and accurate diagnosis spanning a range of etiological agents causing HFMD becomes critical when there is a risk of neurological complication leading to fatality (Chan et al., 2003).

The golden criterion of laboratory HFMD diagnosis is the identification of virus isolates from clinical samples such as throat or epidermal vesicle swab (Perez-Ruiz, 2003). The enterovirus could be isolated in human muscle rhabdomyosarcoma (RD) cells and African green monkey kidney (Vero) cells and subsequently could be subjected to reverse-transcription polymerase chain reaction (PCR) of viral RNA, indirect immunofluorescence and viral microneutralization assays (Perez-Ruiz, 2003). However, the abovementioned approaches are rather lengthy and time-consuming (Ooi et al., 2010). Although rapid diagnostic methods utilizing modern molecular routines such as quantitative real-time PCR (qRT-PCR) were recently developed to address those issues (Lin et al., 2003), the sensitivity of such assay needs significant improvement due to diverse genetic differences between serotypes of enteroviruses (Liang et al., 2012).

Therefore, there is a need for an improved method for detecting the viruses that cause HFMD.

The listing or discussion of an apparently prior-published document in this specification should not necessarily be taken as an acknowledgement that the document is part of the state of the art or is common general knowledge.

Any document referred to herein is hereby incorporated by reference in its entirety.

MicroRNAs (miRNA) are single-stranded RNA molecules of approximately 22 nucleotides that negatively regulate gene expression by degradation of its target mRNA through various mechanism such as Dicer cleavage or repressing translation machinery (Weber et al., 2010). Having a partial complementarity to its target mRNA, miRNA uniquely regulate hundreds of cellular gene expression making it a conceivable indicator of the state of a cell. A number of miRNA based diagnosis test utilizing serum of infected patients for HFMD were recently developed (Cookson et al., 2012b) (Cui et al.). However, miRNA is known to be readily isolated from exosomes (cell-secreted vesicles) in human saliva. As collection of saliva is less invasive than epidermal vesicle, rectal swab and phlebotomy, saliva based diagnostic test could be especially beneficial and convenient for HFMD that chiefly affects children. In addition, salivary miRNA has remarkable stability and resistance to cellular and physical degradation

(Arroyo et al., 2011b) thereby conferring it as a potential clinical biomarker. Here, we described a salivary miRNA qPCR analysis which could identify HFMD patients with nearly 90% accuracy.

- 5 In an aspect of the present invention, there is provided a method for detecting HFMD, the method comprising determining the presence of a miRNA in a saliva sample, wherein the presence of the miRNA in the saliva sample is indicative of HFMD.

Advantageously, the method of detection is carried out by machine learning algorithm known
10 as support vector machine using radial function which finds a pattern in the miRNA expression of several miRNAs in order to determine the amount of miRNA present in a sample.

By “miRNA”, it is meant to include any small non-coding RNA molecule that may function in RNA silencing and/or post-transcriptional regulation of gene expression. Several hundreds of
15 miRNAs have been identified in plants and animals - including humans - which do not appear to have endogenous siRNAs. Thus, while similar to siRNAs, miRNAs are nonetheless distinct. miRNAs thus far observed have been approximately 21-22 nucleotides in length and they arise from longer precursors, which are transcribed from non-protein-encoding genes. The precursors form structures that fold back on each other in self-complementary regions; they
20 are then processed by the nuclease Dicer in animals or DCL1 in plants. miRNA molecules interrupt translation through precise or imprecise base-pairing with their targets. miRNAs are transcribed by RNA polymerase II and can be derived from individual miRNA genes, from introns of protein coding genes, or from poly-cistronic transcripts that often encode multiple, closely related miRNAs. Pre-miRNAs, generally several thousand bases long are processed in
25 the nucleus by the RNase Drosha into 70- to 100-nt hairpin-shaped precursors. Following transport to the cytoplasm, the hairpin is further processed by Dicer to produce a double-stranded miRNA. The mature miRNA strand is then incorporated into the RNA-induced silencing complex (RISC), where it associates with its target mRNAs by base-pair complementarity. In the relatively rare cases in which a miRNA base pairs perfectly with an
30 mRNA target, it promotes mRNA degradation. More commonly, miRNAs form imperfect heteroduplexes with target mRNAs, affecting either mRNA stability or inhibiting mRNA translation.

In various embodiments, method for detecting HFMD comprises determining or detecting the expression pattern, presence or an increased level of one or more miRNA, relative to a predetermined criterion, and is indicative of a diagnosis of HFMD, and such one, two, three, 5 four, five, six, seven, eight or more microRNAs comprises a nucleotide sequence selected from the group consisting of miR-18b, miR-125a, miR-145, miR-155, miR-221, miR-324, miR-335, miR-23a and miR-142, or fully complementary nucleotide sequences thereto and combinations thereof, miRNAs that comprise at least 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more consecutive bases of any of these sequences or fully complementary nucleotide sequences thereto, or combinations thereof; microRNAs that are at least 80% or 85% or 90% 10 or 95% or more identical to the nucleotide sequence of any of these sequences or fully complementary nucleotide sequences thereto, or combinations thereof; or a nucleotide sequence that hybridizes to any of these sequences or the full complement thereof, or combinations thereof.

15 By “predetermined criterion”, it is meant to include any predetermined value or threshold that determines whether or not the amount of miRNA present in a sample indicates a HFMD infection in a patient from which the sample was taken from. For example, expression levels and patterns.

20 In other embodiments, it may include any statistical model that can weigh each miRNA expression with a value which can be inserted into a regression equation that is developed from the model. The data collected in the present invention is compared used SPSS statistic software. Typically, the comparison can be of any statistical value representing size, including 25 a ranking of each of the individual sizes and a statistical analysis of the individual rankings for the sequences of each miRNA. In the present invention, a regression analysis was performed with the statistical program for the determination of associations between the miRNAs determined in a sample and HFMD infections. Each miRNA has a specific weight / value (the amount that they contribute to the finale decision). Normalised amount will be subjected to 30 predetermined regression equation which produce a number on a linear scale which ultimately determine a “yes” or a “no” infection status. For example, a weight 10 is obtained for a particular miRNA after combining all signatures and subjecting it to a regression

equation. This means that if weight of 8 is obtained, then the sample came from a HFMD infected person. A weight less than 8 means the sample came from a non-infected patient. A receiver operating characteristic curve is used to determine our optimal specific and sensitive cut off points as shown in Figures 4 and 5 and Table 4. Table 5 below shows an example of a regression equation used in an embodiment of the present invention.

In various embodiments, the presence of the miRNA in the saliva sample is determined with at least one oligonucleotide primer or probe that is substantially complementary to a part of said miRNA. The miRNA is any one selected from the group comprising of miR-18b, miR-125a, miR-145, miR-155, miR-221, miR-324, miR-335, miR-23a and miR-142. In an embodiment, the method comprises determining the presence of at least two of the miRNA sequences in the group. In an embodiment, the method comprises determining the presence of miR-18b, miR-142, miR-155 and miR-324.

In other embodiments, the present invention also provides a method for detecting the HFMD in a subject wherein a differential expression level (increased or decreased/absence) or differential saliva level of the one or more microRNA relative to a predetermined criterion or range is indicative of a diagnosis of HFMD. For example, the level of the miRNA may be increased or decreased (or absence) relative to the level in samples of patients without HFMD. The method optionally further comprises the step of comparing the level of the miRNA (preferably a normalised level of miRNA) to a predetermined criterion or range. Alternatively, the detection of a level outside of a predetermined range, correlated to patients without HFMD, is indicative of a diagnosis of HFMD.

The term "differential expression" as used herein refers to both quantitative as well as qualitative differences in the expression patterns of one or more miRNA in a saliva sample versus the expression patterns of the one or more miRNAs in a saliva sample from a healthy subject. For example, a differentially expressed miRNA may either be present or absent in normal versus disease conditions, or may be increased or decreased in a disease condition versus a normal condition. Such a qualitatively regulated miRNA may exhibit an expression pattern within a saliva sample that is detectable in either control or disease conditions, but is not detectable in both. In other words, a miRNA is differentially expressed when expression

of the microRNA occurs at a different level (higher or lower, presence or absence) in the saliva sample of a subject with HFMD relative to the level of its expression in the blood sample from a disease-free subject without HFMD. The level of a differentially expressed miRNA may refer to either the uncorrected (raw) or normalized abundance of a miRNA in a sample.

- 5 Comparisons of miRNA levels may consider the uncorrected quantified abundance of a given miRNA relative to an uncorrected reference value. Alternatively, the abundance of a given miRNA may be expressed as a ratio relative to one or more additional miRNA (or other internal controls) in that sample. In such a case, this "normalized" ratio would be compared relative to a similar "normalized" reference value from a sample of healthy patients (or
- 10 patients without HFMD).

For example, the CT value of the household miRNA detected must be less than 35 while any other signatures the CT value must be less than 40.

- 15 The amount of the saliva sample required for detection is 50 μ l.

In various embodiments, the presence of the miRNA in the saliva sample is determined by: (a) centrifuging the saliva sample to get the supernatant, and filtering the supernatant; (b) extracting RNAs from the filtered supernatant obtained in step (a); (c) performing a reverse

20 transcription reaction using the RNAs extracted in step (b) to obtain a cDNA solution; (d) performing a PCR amplification reaction using the cDNA solution obtained in step (c); and (e) detecting the PCR products with a fluorescence quantitative PCR instrument so as to obtain the expression level of miRNAs in the medium.

- 25 In another aspect of the present invention, there is provided a method for aiding in categorizing, diagnosing or determining prognosis in a patient with HFMD, the method comprising determining the presence of a miRNA in a saliva sample. The miRNA is any one selected from the group comprising of miR-18b, miR-125a, miR-145, miR-155, miR-221, miR-324 and miR-335, miR-23a and miR-142.

30

In yet another aspect of the present invention, there is provided a kit for diagnosing HFMD in a human, the kit comprising an assay for determining the presence of a miRNA in a saliva

sample. The kit further comprising at least one oligonucleotide primer or probe that is substantially complementary, or specifically hybridise to, or primers that specifically amplify, to a part of a miRNA, wherein the miRNA is any one selected from the group comprising of miR-18b, miR-125a, miR-145, miR-155, miR-221, miR-324, miR-335, miR-23a and miR-142.

5

In another aspect of the present invention, there is provided a method of treating a subject diagnosed with HFMD by administering a therapeutic agent to the subject.

The therapeutic agent may comprise any molecule that modulates the activity or expression of any of the miRNA selected from the group comprising of miR-18b, miR-125a, miR-145, miR-155, miR-221, miR-324 and miR-335, miR-23a and miR-142.

In an aspect of the present invention, there is provided a method of treating a subject having HFMD, for example, by administering to the subject an effective amount of an agent which modulates the level of at least one miRNA in a target cell. In some embodiments, the agent increases or stimulates the expression or activity of a miRNA in a mammalian subject (i.e., a miRNA enhancer). In other embodiments, the agent decreases or inhibits the expression or activity of a miRNA in a mammalian subject (i.e., an miRNA or miRNA inhibitor).

By "an agent which modulates the level of miRNA" indicates that the agent, when administered to a sample or subject increases or a decreases in the measured value of at least one miRNA. In some embodiments the miRNA is increased or decreased by an amount between 1-fold and 20-fold, or more than 20-fold. In some particular embodiments the miRNA is increased or decreased by 1-fold, 2-fold, 3-fold, 4-fold, 5-fold, 7-fold, 9-fold, 10-fold, 12-fold, or 15-fold, or more. In other embodiments the miRNA is increased or decreased by 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200%, 300%, or more.

miRNA enhancers are molecules, e.g., nucleic acid molecules, which act to increase the level of a miRNA gene product in a cell. In one variation, a miRNA enhancer comprises a sequence of a miRNA, or a variant thereof. In another variation, the miRNA molecule is a synthetic molecule. In another variation, the miRNA molecule comprises one or more stabilizing mutations. The miRNA sequence may be 12-100 nucleotides in length. For example,

the miRNA sequence may comprise 20-80, 20-70, 20-60, 20-50, 20-40, 21-23, 21-25 12-33, 18-24, 18-26, or 21-23 nucleotides. In some embodiments, the miRNA sequence may comprise 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides. The sequence of the miRNA may be the first 13-33, or 21-25 nucleotides of the pre- miRNA. In
5 some embodiments, the sequence of the miRNA may be the last 13-33 or 21- 25 nucleotides of the pre- miRNA.

In another variation, the miRNA enhancer comprises a sequence of a pri- miRNA or a variant thereof. The pri- miRNA sequence may comprise from 30-300, 35-375, 45-250, 55-200, 70-
10 150 or 80-100 nucleotides. The pri- miRNA may also comprise a miRNA and the complement thereof, and variants thereof. The pri- miRNA may form a hairpin structure. The hairpin may comprise a first and second nucleic acid sequence that are substantially complimentary. The first and second nucleic acid sequence may be from 37-50 nucleotides. The first and second nucleic acid sequence may be separated by a third sequence of from 8- 12 nucleotides. The
15 hairpin structure may have a free energy less than -25 Kcal/mole as calculated by the Vienna algorithm with default parameters, as described in Hofacker et al., Monatshefte f. Chemie 125: 167-188 (1994), the contents of which are incorporated herein. The hairpin may comprise a terminal loop of 4, 5, 6, 7, 8, 9, 10, 11. 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides.

20 In contrast, miRNA inhibitors decrease or inhibit the expression or activity of a miRNA in a mammalian subject. In some embodiments, the miRNA inhibitor is antagomir. As used herein, the term "antagomir" is an anti- miRNA molecule that is capable of blocking the activity of a miRNA. The antagomir may comprise a total of 12-50 or 8-50, or 8-40, or 5- 40 nucleotides
25 in length. In some embodiments, the antagomir comprises a total of at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55 or 60 nucleotides. The sequence of an antagomir may comprise the complement of a sequence of a miRNA such that, e.g., the anti- miRNA binds to the miRNA to block its activity.

30 The kit may further include water and hybridization buffer to facilitate hybridisation of the oligonucleotide primer or probe with the miRNAs that may be present in a sample.

The components of the kits may be packaged either in aqueous media or in lyophilized form. The container means of the kits will generally include at least one vial, test tube, flask, bottle, syringe or other container means, into which a component may be placed, and preferably, suitably aliquoted. Where there is more than one component in the kit (labeling reagent and
5 label may be packaged together), the kit also will generally contain a second, third or other additional container into which the additional components may be separately placed. However, various combinations of components may be comprised in a vial. The kits of the present invention also will typically include a means for containing the nucleic acids, and any other reagent containers in close confinement for commercial sale. Such containers may
10 include injection or blow-molded plastic containers into which the desired vials are retained. When the components of the kit are provided in one and/or more liquid solutions, the liquid solution is an aqueous solution, with a sterile aqueous solution being particularly preferred.

However, the components of the kit may be provided as dried powder(s). When reagents
15 and/or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent. It is envisioned that the solvent may also be provided in another container means.

The container means will generally include at least one vial, test tube, flask, bottle, syringe
20 and/or other container means, into which the nucleic acid formulations are placed, preferably, suitably allocated. The kits may also comprise a second container means for containing a sterile, pharmaceutically acceptable buffer and/or other diluent.

The kits of the present invention will also typically include a means for containing the vials in
25 close confinement for commercial sale, such as, e.g., injection and/or blow- molded plastic containers into which the desired vials are retained.

Such kits may also include components that preserve or maintain the oligonucleotides or that protect against its degradation. Such components may be RNase-free or protect against
30 RNases. Such kits generally will comprise, in suitable means, distinct containers for each individual reagent or solution. A kit will also include instructions for employing the kit

components as well the use of any other reagent not included in the kit. Instructions may include variations that can be implemented.

It is contemplated that such reagents are embodiments of kits of the invention. Such kits, however, are not limited to the particular items identified above and may include any reagent used for the manipulation or characterization of the miRNAs.

The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

It is contemplated that any embodiment discussed herein can be implemented with respect to any method or composition of the invention, and vice versa. Furthermore, compositions and kits of the invention can be used to achieve methods of the invention.

In another aspect of the present invention, there is provided a diagnostic test system that is adapted for performing any of the methods as claimed in any aspect the present invention. Such a diagnostic test system can comprise means for obtaining test results comprising the activity or level of one or more miRNA correlated with a diagnosis of HFMD in a sample of the subject (for example, saliva in the case of the present invention); means for collecting and tracking test results for one or more individual sample; means for comparing the activity or level of one or more miRNA to a predetermined criterion; and means for reporting whether the activity or level of the one or more miRNA meets or exceeds the predetermined criterion.

In yet another aspect of the present invention, there is provided a computer programme comprising computer-executable instructions embodied in a computer-readable medium for performing the steps of any of the method steps embodiment in the any aspect of the present invention.

Throughout this application, the term "about" is used to indicate that a value includes the standard deviation of error for the device or method being employed to determine the value.

The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or."

- 5 As used in this specification and claim(s), the words "comprising" (and any form of comprising, such as "comprise" and "comprises"), "having" (and any form of having, such as "have" and "has"), "including" (and any form of including, such as "includes" and "include") or "containing" (and any form of containing, such as "contains" and "contain") are inclusive or open-ended and do not exclude additional, unrecited elements or method steps.

10

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit
15 and scope of the invention will become apparent to those skilled in the art from this detailed description.

In order that the present invention may be fully understood and readily put into practical effect, there shall now be described by way of non-limitative examples only preferred
20 embodiments of the present invention, the description being with reference to the accompanying illustrative figures.

In the Figures:

- 25 **Figure 1. Flowchart showing different patient cohorts utilized.** Screening of potential miRNA biomarkers for HFMD infection were performed on Singapore cohort 1 which consists of 3 EV71, 3 CA6 and 3 healthy pooled saliva. The 8 putative miRNA predictors from primary screen was subsequently validated on cohort-pooled patients in "Validation Set 1 & 2". Secondary model on 4 best-performing miRNAs was also constructed. UTI infection cohort here
30 comprises of 22 patients.

Figure 2. Expression analysis of Singapore HFMD Cohort 1 screen (A) PCA plot based on miRNA expression values across sample groups. (B) Heat map showing differentially expressed miRNA gene expression pattern across different samples.

- 5 **Figure 3: Volcano plot showing distribution of significant and non-significant miRNA in Singapore HFMD Cohort 1 screen.** A. Healthy Vs EV71 B. Healthy Vs CA6. Student T-test (non-parametric) is performed and HITs were determined using absolute fold change of more than 4 fold and *p-value* of (0.05).
- 10 **Figure 4. Receiver operating characteristic curve (ROC) of predictive models of “Validation Set 1” with different numbers of miRNA signatures.** Legend corresponds with color code. VS1.10, Validation set one with 10 signatures, VS1.4 Validation set one with 4 miRNA predictor.
- 15 **Figure 5. Receiver operating characteristic curve (ROC) of predictive models of “Validation Set 2” with different numbers of miRNA signatures.** Legend corresponds with color code. VS1.10, Validation set one with 10 signatures, VS1.4 Validation set one with 4 miRNA predictor.
- 20 **Figure 6. Risk of HFMD calculated with A. 8 miRNA and B. 4 miRNA model on validation set 2.** Significance is computed using Graphpad Prism 6.0 with non-parametric Mann-Whitney test, two-tailed. Data points represent mean readings of triplicate experiments. *** represent *p-value* less than 0.0001.
- 25 **Figure 7. Risk index in HFMD patient during infection and after recovery.** Circles denote data points with triplicate readings. Triangle and blue line denote trend-line calculated using ggplot2 library in R by linear regression. Significance is computed using Graphpad Prism 6.0 with paired t test of repeated measures. * represent *p-value* less than 0.05.
- 30 **Figure 8. Venn diagram shows HITs overlap between sample groups**

Figure 9: Composition of miRNA utilized in the two models according to an embodiment of the present invention.

Figure 10: Differential expression of validated miRNA in Singapore and Taiwan cohorts.

- 5 Boxplots of miRNA expressions in HFMD and healthy populations. Stars indicate significance between HFMD and healthy. ($***P < 10^{-3}$, $**P < 10^{-2}$, FDR-adjusted ANOVA)

Figure 11: Overall study design and patient cohorts involved in model development and

- validations.** Screening of potential miRNA biomarkers for HFMD infection was performed on
10 3 EV71, 3 CA6 and 3 healthy pooled saliva samples. The 8 putative miRNA predictors from primary screen was subsequently used to form diagnostic models using the training set which includes 75% of the "Singapore Cohort" with support vector machines. Cross validation was carried out using k-fold validation method for 10 folds and respective performances of the two models were determined. Blinded validation of the two models was carried out using the
15 test set which includes 25% of the "Singapore Cohort" and the whole "Taiwan Cohort".

Figure 12: Salivary miRNA expression of HFMD in the salivary miRnome screen. (A) PCA plot based on miRNA expression values across pools (n=3 each). (B) Heat map showing differentially expressed miRNA gene expression pattern across different samples (n=3).

- 20 Volcano plot was used to illustrate distribution of significant and non-significant miRNA in Singapore HFMD Cohort 1 screen in C. Healthy Vs EV71 (n=6) and D. Healthy Vs CA6 (n=6). Red color represent population with absolute 4-fold change while significant miRNAs were represented in yellow or green while red is non-significant. Student t-test (non-parametric) was performed and HITs were determined using absolute fold change of more than 4-fold and
25 p-value less than 0.05.

Figure 13: miRNA selection and performance tuning. A. Logistic regression of hit miRNAs. ROC curve was used to display sensitivity and specificity of individual miRNA in HFMD diagnosis with the entire "Singapore Cohort". B. Overall accuracy of the diagnosis model
30 resolved with increasing number of miRNA classifiers using support vector machine model in the training set of the "Singapore Cohort". C. Importance of individual miRNA in the 6-miRNA

model. Accuracy for each model was calculated using “caret” package in R software. “ggplot2” library was used for illustration using R software.

Figure 14: Risk score of healthy and HFMD patients in blinded validation. Risk index of HFMD was obtained for (A) the 6-miRNA model and (B) the 4-miRNA model in testing set of “Singapore Cohort”. The two models were also validated in the “Taiwan Cohort” using (C) the 6-miRNA model and (D) the 4-miRNA model. Circles denote data points with triplicate readings. Box plot was constructed using ggplot2 library in R software.

Figure 15: Differential expression of hit miRNAs in the Singapore and Taiwan cohorts. Boxplots of miRNA expressions in healthy (orange), “Singapore HFMD” (green) and “Taiwan HFMD” (blue) cohorts. Stars indicate significance between HFMD and healthy. (*** $P < 10^{-3}$, ** $P < 10^{-2}$, * $P < 0.05$, FDR-adjusted non-parametric ANOVA with Dunnett’s multiple comparison test using 95% CI).

Hand Food Mouth Disease or HFMD causes self-limiting fever and rashes in children. However, in rare isolated events, it can lead to serious neurological complications and fatality. In Singapore, diagnosis is done primarily via assessment of clinical symptom manifestations and polymerase chain reaction (PCR) analysis of viral genomic content.

This invention employed real-time quantitative polymerase chain reaction (qPCR) based detection for changes in circulating miRNA profile in the saliva sample of the HFMD infected patients. Initially, we screened patient saliva for changes in miRNA level and later significantly regulated miRNA were selected to validate further in multiple patient cohorts. miRNA expression value in patients were combined with multifactorial regression method and risk scores are determined for individual patients. Our scoring model with 8 miRNA predicted the disease state of HFMD with an overall accuracy of 88.3% with the area under the curve (AUC) of 93.6%. In addition, we observed that by decreasing the number of predictors to 4 miRNAs, a fairly accurate prediction could still be made. It could be observed that 4-miRNA model still exhibited 81.7% overall accuracy with AUC of 88.7%. This is the first study to prove that an infection state in human could be diagnosed by circulating miRNA in saliva. The study serves

as a stepping stone for the future development of many other infectious disease diagnosis workflows and will be extremely useful as a routine non-invasive surveillance platform.

Example 1

5

1. Materials and Method

(a). Patient Samples and Infection

10 Total of 35 number of HFMD suspected throat swab and saliva clinical samples were obtained from Kandang Kerbau (KK) Women's and Children's Hospital from August 2012 to February 2016. The collection was under the approval of centralized institutional review board (CIRB) of Singhealth under CIRB number 2012/448/E. Healthy saliva samples were collected from various child care centers which participated in saliva collection drive under National
15 University of Singapore Ethical review board approval number B-14-273. Presence of enteroviruses in patient samples were confirmed using previously established pan-entero PCR reactions in saliva samples.

(b). miRNA Extraction and Reverse Transcription

20

miRNA is extracted from 50ul of saliva using biofluid extraction kit (Exiqon, Inc.) with 1 µg of MS2 carrier RNA (Roche, Ltd.) and eluted in 30 µl of water. Slightly modified protocol from manufactures' was used to reverse transcribed the RNA. 7ul of RNA was used instead of 2ul to reverse transcribed previously extracted miRNA using universal cDNA synthesis kit (Exiqon,
25 Inc.). The synthesized cDNA was diluted 20 fold using RNase free water before PCR as opposed to 40 fold dilution in the manufacturer protocol.

(c). Primary Screen using miRNA qPCR Panel

30 Pools of saliva (described in the result section) were screened for dysregulated miRNA primarily using serum plasma focused miRCURY LNA™ microRNA PCR (Exiqon, Inc.). The panel is chosen as miRnome of saliva were found to be significantly overlapping with those from

serum/plasma. cDNA synthesized were diluted 40 folds in water and 2 µl is added to each well of qPCR plates. Quantitative real-time PCR reactions were carried out according to manufacture protocol using ExiLENT SYBR® Green master mix (Exiqon, Inc.).

5 (d). *Individual qPCR Assays for Validation*

Validation is carried out using individual qRT-PCR assays using selected 8 miRNA along with 1 normalizer RNA. Saliva were extracted, reverse transcribed and amplified as described above. Melting curve of each reaction is analyzed to ensure specific amplification of targets and only
10 samples with normalizer having CT value of less than 30 were taken into account for further analysis.

(e). *Statistical Analysis*

15 During the primary screening, data normalization is done in two steps. Firstly, inter-plate calibration was carried out across all plates using inter-plate calibrator present in each panel across all plates to minimize run to run variations. Second step of normalization involved determining reference gene. Raw CT values were analyzed for the most stably present miRNA with CT value below 30 using NormFinder algorithm ([http://www.moma.dk/normfinder-](http://www.moma.dk/normfinder-software)
20 [software](http://www.moma.dk/normfinder-software)). Significantly deregulated miRNA were determined using GenEx qPCR analysis software (<http://genex.gene-quantification.info>).

Raw CT values from validation studies were normalized using previously selected normalizer and normalized CT values were further analyzed with SPSS (IBM, Inc.) to determine potential
25 biomarkers. Multinomial regression analysis is performed using full factorial model to build predictive models. To ensure optimal model performance, lesser miRNA of 4 is used to construct another models. Although performance of models from both validation sets were not affected greatly (see results section), the best performing model was attained when maximal number of predictors were used.

30

Statistical significances of risk score differences between HFMD and Healthy groups are calculated using non-parametric Mann-Whitney test using Prism (GraphPad Software, Inc.).

Statistical differences of risk score between during HFMD infection and after recovery were calculated using paired t test of repeated measures with Prism (GraphPad Software, Inc.).

2. Results Set 1

(a). *Salivary miRNA expression in HFMD-infected patients and healthy individuals*

Saliva from multiple patient cohorts (Figure 1) were collected in which the Singapore cohort consists of 34 patients and 22 patients from Taiwan cohort both of whom were hospitalized for HFMD at the time of sample collection. Saliva samples from all patients were positively diagnosed as HFMD by using adapted pan-entero PCR protocol (Cardosa et al., 2003). We also collected healthy samples (considering confounding factors such as age, gender and race) from 25 children whose saliva were tested negative for HFMD (Figure 1). Primary screening was carried out by comparing pooled samples (n=3) for their miRNA expression levels. EV71, CA6 infected and healthy patient pools were spiked with synthetic miRNA, uniSP6 which was later used to detect PCR inhibition in each pools. miRNA expression levels were evaluated using Exiqon's serum plasma miRNA profiling qPCR array. Inter-plate calibration is done using pre-defined inter plate calibrators from Exiqon. After CT values were normalized, we applied student t-test to determine significantly ($p < 0.05$) dysregulated miRNA across pools.

A total of 179 miRNAs were analyzed for their abundance in previously-mentioned patient pools. Our primary screen classified a subset of miRNA to be significantly regulated in HFMD saliva respect to the healthy pool (Figure 3). One-way hierarchical clustering and principle component analysis exhibited two clear cluster with differential pattern of molecular miRNA signature between healthy and enterovirus infection (Figure 2). As expected, EV71 and CA6 pools showed rather similar expression profile relative to healthy pool (Figure 2).

For further validation, we included miRNA signatures that significantly differentiate HFMD apart from healthy from the screen by student t-test. 8 miRNA signatures with 1 normalizer from the primary screen were further validated in Singapore HFMD Cohort 2. Firstly, raw CT value of different pools were inter-plate calibrated. We performed outlier detection in different pools using Gurbb's test and no outliers were detected. Spike-in level, UniSp6 of

different samples were determined across different plates to access significant inhibition by PCR contaminants and so significant inhibition is detected. Missing values were computed by adding 1 to maximum CT value detected across different runs. We used Genorm's Norm Finder Algorithm to predict 2 best performing normalizers. Two normalizers were then validated independently in Healthy Cohort (n=25) and it's observed that one of the normalizer didn't perform well as expression of the particular miRNA was not detected in a number of samples (data not shown). Therefore, hsa-miR-23a-3p is selected as the normalizer and used to normalized CT values of all miRNA signatures. Ct values were log transformed and student-t test is used to determine significantly regulated miRNA in different samples. HITs were sorted according to significance level and top 2 HITs from EV71 VS Healthy, 2 HITs from CA6 Vs Healthy and 2 HITS from overlap between EV71 and CA6 were selected (see Table 3 below and Figure 8).

ID	Name	Predictor
1	hsa-miR-18b-5p	CA6
2	hsa-miR-23a-3p	Normalizer
3	hsa-miR-125a-5p	EV71
5	hsa-miR-145-5p	EV71
6	hsa-miR-155-5p	EV71
7	hsa-miR-221-3p	EV71+CA6
8	hsa-miR-324-3p	CA6
9	hsa-miR-335-5p	CA6

Table 3: List of significant miRNA selected for validation.

(b). *Multivariate model to identify HFMD infection*

Previously selected 9 miRNA expression values were employed to develop an HFMD prediction model in patients of Singapore HFMD Cohort 2 using multinomial regression

method. We took into consideration that independent of the causative agent, certain primary immune responses triggered upon infection could be similar. Therefore, 9 miRNA expression values were also profiled in urinary tract infection (UTI) patients caused by bacteria and factored in while developing the model to avoid potential misdiagnoses. Our refined algorithm predicted the disease state of HFMD with an overall accuracy of 88.3% with the area under the curve (AUC) of 93.6% (see Table 1 below; Figure 2).

Set	AUC	ACC %	SEN %	SPE %
Validation set 1 (8 miRNA predictors)	93.6	88.3	90.6	90.5
Validation set 1 (4 miRNA predictors)	88.7	81.7	87.5	85.7
Validation set 2 (8 miRNA predictors)	94.9	88.3	95.7	83.3
Validation set 2 (4 miRNA predictors)	94.2	81.7	91.3	83.3

Table 1. Predictive models' performances on HFMD discrimination. 4 different models were constructed with 2 validation sets and 2 set of miRNA predictors (Figure 1). The area under curve (AUC, from receiver operating characteristic curves (ROC), Figure 4,5) were shown together with overall accuracy (ACC), sensitivity (SEN, the probability to accurately predict HFMD patient as 'HFMD') and specificity (SPE, the probability to predict healthy individuals as 'Healthy'). Accuracy, sensitivity and specificity were calculated using multinomial regression in SPSS (IBM, Inc.). Bootstrapping is done 1, 000 times with 95% CI.

In addition, we observed that by decreasing the number of predictors to 4 miRNAs (see Table 4 and Figure 4), a fairly accurate prediction could still be made.

Term	P.Value
hsa04144:Endocytosis	0.006596686
hsa04810:Regulation of actin cytoskeleton	0.010425994
hsa04010:MAPK signaling pathway	0.011392528
hsa04514:Cell adhesion molecules (CAMs)	0.032187186

hsa00562:Inositol phosphate metabolism	0.035220961
hsa04620:Toll-like receptor signaling pathway	0.036366192
hsa04622:RIG-I-like receptor signaling pathway	0.042291808
hsa04666:Fc gamma R-mediated phagocytosis	0.042940302
hsa04070:Phosphatidylinositol signaling system	0.055516966
hsa00350:Tyrosine metabolism	0.061893175
hsa05120:Epithelial cell signaling in Helicobacter pylori infection	0.065976565
hsa00564:Glycerophospholipid metabolism	0.065976565
hsa04360:Axon guidance	0.079329426
hsa04210:Apoptosis	0.081450067
hsa04340:Hedgehog signaling pathway	0.09392249
hsa04912:GnRH signaling pathway	0.097040715

Table 4. Molecular pathways predicted to influence by significantly regulated miRNA from Singapore HFMD cohort 1. Target Scan 2.0 is used to predict gene target of validated miRNA. KEGG pathway analysis is used to enrich pathways involved.

5

It could be observed that 4-miRNA model still displayed 81.7% overall accuracy with AUC of 88.7% (see Table 2 below).

Categories	Validation Set 1	Validation
	(n=65)	Set 2 (n=22)
Age (< 1 year old)	8/65	7/22
Age (1- 5 years old)	23/65	11/22
Age (> 5 years)	3/65	4/22
Duration of hospitalization (< 3 days)	6/65	3/22
Duration of hospitalization (3 – 5 days)	23/65	16/22

Duration of hospitalization (> 5 days)	5/65	3/22
Fever	30/65	22/22
Breathlessness	1/65	0/22
Vomiting	15/65	3/22
Rash	24/65	15/65
Oral ulcer	5/65	19/65

Table 2. Pathological findings of patients in validation set 1 and 2.

(c). Validation with patient samples from different geographical origin

5

It is of note that our patient samples were only obtained solely from a hospital throughout three years of our study and hence it is questionable that our signatures previously validated were prone to bias from geographical, racial or many unknown commands. In order to avoid inherent study bias, we employed the former 9 miRNAs to the independent Taiwan HFMD Cohort (n=22) which was solicited in different time period. As Surprisingly, despite screening is performed on Singapore cohort, the model developed with Taiwan cohort utilizing the same 9 miRNA signatures was still remarkably accurate, 88.3% overall accuracy with AUC of 94.9 (Table 1 and Figure 3). Thereafter, we analyzed the risk score of HFMD in Validation set 2 using the 8 and 4 miRNA predictor models. As for healthy control, we made use of patients who returned for post-checkup after a week period of time. Indeed, the mean risk score of HFMD patients was observed to be significantly (p value) higher than healthy controls in both models (Figure 6).

10

15

	Weights			
	Singapore Cohort		Taiwan Cohort	
	10 miRNA model	4 miRNA model	10 miRNA model	4 miRNA model
Intercept	.270	1.062	13.699	.448
hsa-miR-18b-5p	.249	0.274	3.631	.734
hsa-miR-125a-5p	-.602		-1.574	
hsa-miR-142-3p	-1.843		.474	
hsa-miR-145-5p	1.945	-0.324	-1.631	-.325
hsa-miR-155-5p	-.166	-1.858	.202	.010
hsa-miR-221-3p	.156		-2.304	
hsa-miR-324-3p	.216	2.002	-.674	-.144
hsa-miR-335-5p	.207		.339	
Cut off point	0.4492	0.5425	0.6765	0.7380

Table 5

20 Table 5 above shows weight given for each miRNA that contributes to the decision models described above. Here, normalised CT values of signatures were subjected to equation: $V = 1 / (1 + \text{EXP}(\text{Intercept} + (\text{Weight}_b \times \text{Normalised CT}))$ where b is weight of a given miRNA from respective model. If V is less than pre-determined cut off point for a given model, patient was regarded as HFMD positive. Cut off points were determined using ROC analysis with the SPSS
 25 software.

Finally, we analyzed a group of saliva collected after which HFMD symptoms were subsided (i.e. enterovirus RNA was detected during the first onset of disease but unable to detect after the post checkup). In 3 of such cases, we had saliva samples on both during and after the
 30 onset of viral infection. It could be discerned that generally after clinical manifestations had dwindled, risk score of HFMD were significantly reduced. Although the number of samples tested was minimal (n=6), it could be recommending that the test could perhaps be used to

predict whether HFMD had subsided to level where the viral load is lowered adequately to deter further spreading which could be useful in patient isolation.

3. Results Set 2

5

(a) Patient information and study design

Saliva samples used in this study were collected from multiple patient cohorts (Figure 11). HFMD patients from both "Singapore Cohort" (n=35) and "Taiwan Cohort" (n=24) were hospitalized for symptomatic HFMD at the time of sample collection and collected saliva samples were diagnosed as enterovirus positive by using adapted pan-entero PCR protocol as described previously. We also collected healthy samples (considering confounding factors such as age, gender and race) from 23 children in Singapore whose saliva samples were tested negative for HFMD by pan-entero PCR. Details on patient characteristics are summarized in Table 6.

15

Categories	Singapore Cohort (n=35)	Taiwan Cohort (n=24)
Age (< 1 year old)	8/35	7/24
Age (1- 5 years old)	24/35	13/24
Age (> 5 years)	3/35	4/24
Duration of hospitalization (< 3 days)	6/35	3/24
Duration of hospitalization (3 - 5 days)	24/35	18/24
Duration of hospitalization (> 5 days)	5/35	3/24
Fever	30/35	22/24
Breathlessness	1/35	0/24
Vomiting	15/35	3/24
Rash	24/35	15/24
Oral ulcer	5/35	19/24

Table 6. Pathological findings of HFMD

Differential miRNA expression of HFMD patients in the screening population. Differential salivary miRNA expression between HFMD and healthy samples were profiled using Exiqon miRNA qPCR panel. The primary screen was carried out by identification of dysregulated miRNAs in pooled EV71 and CA6 patient saliva samples against the healthy group (n=3 each). Pooled samples were spiked with synthetic miRNA, uniSP6 which was later used to ensure absence of PCR inhibitors in each pool. To reduce plate to plate variation, inter-plate calibration was carried out using pre-defined inter-plate calibrators from the manufacturer. miRNA expressions normalization was carried out by selecting stably expressing miRNA with least variance and student t-test was used to determine significantly ($p < 0.05$) dysregulated miRNA across different pools.

A total of 179 miRNAs were analyzed and the primary screen classified a subset of miRNA to be significantly regulated in HFMD saliva respect to the healthy control pool. We found 23 significantly expressed miRNA between EV71 against the healthy controls pool and 10 between CA6 against healthy controls pool with overlap of 7 miRNAs using p-value of less than 0.05 and absolute 4-fold change difference (Figure 8). After dimension reduction with the principle component analysis, miRNA expression between independent repeats of screening arrays were found to be closely correlated (Figure 12A) and one-way hierarchical clustering analysis exhibited clear clusters with differential pattern of molecular miRNA signatures between healthy controls and enterovirus patients (Figure 12B). Gene set enrichment analysis was also carried out to classify over represented pathways using KEEG and identified a number of biological process involved in viral infection such as endocytosis, cytoskeleton regulation, and MAPK signaling pathways (Table 3). Later, significantly dysregulated 8 miRNA signatures with 1 normalizer (Table 4) from the primary screen were then selected to validate in "Singapore Cohort" and "Taiwan Cohort" in individual patients using qPCR with specific primers.

(b) *Diagnosis performance of individual miRNAs, feature selection and the model development*

After determining the expression levels of the 8-miRNA signatures in the “Singapore Cohort”, receiver operating characteristics analysis was employed to evaluate respective sensitivity and specificity of individual miRNAs at a given threshold by using “caret” and “ROC” package implemented in R software. While expression levels of the majority of miRNAs exhibited positive association with the disease status of HFMD (Figure 13A), hsa-miR-221-3p unveiled the highest accuracy in diagnosing HFMD with the positive predictive value of 83.90% and negative predictive value of 75.00%. hsa-miR-18b-5p, on the contrary, was the least effective in HFMD identification with the positive predictive value of 63.00% and negative predictive value of 50.00%.

Subsequently, to determine if the diagnosis accuracy could be further improved by integrating the expression of multiple miRNAs, the support vector machine algorithm was implemented for miRNA expression combination with the radial kernel in “caret” package in R software (R script for the model training and validations are shown in detail below). It is of note that expression levels of certain miRNA pairs were highly correlated to one other (data not shown) and therefore to reduce redundancy in the model, we determined the best performing miRNAs and combinations to be included in the final diagnosis model. For evaluation, the “Singapore Cohort” was randomly divided into the training set and the test set with the constraint such that both training and test set contained the same fraction of HFMD patients. The training set comprised 75% of the entire “Singapore Cohort” and was then applied to train the HFMD diagnosis model with all possible numbers of miRNAs and combinations. Finally, the model converged at peak accuracy of 85.00% with 6 miRNAs (Figure 13B). Although the diagnosis model with 6-miRNA resulted with the highest diagnosis accuracy of 85.00%, 4-miRNA model also rendered an acceptable accuracy of 80.00% and was therefore selected for further validations (Figure 13B).

To perform feature selection, two miRNAs expressions in the training data set was paired up in all possible combination to produce predictive models; the performance of respective models were then determined in the test set by comparing against the actual infection status. After obtaining the best pair, the process was repeated using one additional miRNAs into the pair in all combination and the models were again blindly validated in the test set to obtain

the best performing model with 3 miRNAs. The iteration was repeated until the predictive performance of resulted model did not improve predicative performance significantly.

(c) *Performance evaluation of the diagnosis model with the 10-fold cross-validation in the training set and blinded assessment in the test set*

Since the training dataset was relatively limited in size, in order to avoid overfitting, we leveraged the 10-fold cross-validation method to fairly evaluate the performance of the former 6-miRNA and 4-miRNA diagnosis models. Instead of including a separate validation set, 10-fold cross-validation method equally divided the training dataset into 10 parts; trained the diagnosis model with 9 parts of the dataset, tested the model performance on the remaining one part of the dataset and repeated the process for 10 iterations. The final performance of the model in the training set was determined by averaging the accuracy, sensitivity and specificity from each round (Table 7).

Set	ACC %	SEN %	SPE %
"Singapore Cohort" (6 miRNA predictors with the training set)	85.11	88.89	82.78
"Singapore Cohort" (6 miRNA predictors with the test set)	92.86	100.00	88.89
"Singapore Cohort" (4 miRNA predictors with the training set)	80.85	83.33	79.31
"Singapore Cohort" (4 miRNA predictors with the test set)	91.67	100.00	87.50
"Taiwan Cohort" (6 miRNA predictors)	77.08	78.26	76.00
"Taiwan Cohort" (4 miRNA predictors)	68.75	68.00	69.57

Table 7. Performance of predictive models in HFMD discrimination. 4 and 6 miRNA predictor models were constructed with miRNA expressions on the training set of the "Singapore Cohort". The two models were evaluated on the test set of the "Singapore Cohort" and the "Taiwan Cohort". The overall accuracy (ACC) is shown together with sensitivity (SPE, the probability to predict healthy individuals as "Healthy"). Respective accuracy, sensitivity and specificity were calculated using package "crossval" implemented in R. k-fold cross-validation was carried out using package "caret" and was repeated for 10 folds.

As expected, the 6-miRNA model was slightly more effective in the diagnosis of HFMD than the 4-miRNA counterpart with about 4% increase in accuracy.

Additionally, the fit of the model was also assessed in blinded fashion for the difference between estimated and true infection status with the test dataset which constituted 25% of the "Singapore Cohort". Surprisingly, both 6 and 4 miRNAs models performed excellently with the test set data in classifying blinded HFMD cases with respective accuracy of 92.86% and 91.67% (Table 7). Such high accuracies were a result of well separated risk scores in blinded test set data (Figures 14A and 14B) which reflected the appropriate complexity in our models, avoiding overfitting with a fine balance between variance and bias.

(d) Blinded model evaluation with HFMD patients from different geographical origin

It is of note that our patient samples were obtained solely from a single hospital throughout three years of our study and hence it is possible that our miRNA signatures previously validated were prone to bias from geographical, racial or other unknown factors. In order to avoid inherent study bias, we assessed the performance of the HFMD diagnosis model to the independent Taiwan HFMD Cohort which included 24 HFMD patients from Taiwan and 23 healthy individuals. HFMD patient samples from Taiwan were obtained in a different time period than the samples from Singapore. Interestingly, despite the initial model development was performed using the "Singapore Cohort", the HFMD diagnosis model was still remarkably accurate in discriminating HFMD in Taiwan, having 77.08% overall accuracy with the 6-miRNA model and 68.75% in the 4-miRNA model (Table 7). We also analyzed the risk score of HFMD in "Taiwan Cohort" and found that the mean risk score of HFMD patients was observed to be significantly higher than healthy controls in both 8 (Figure 14C) and 4 miRNA models (Figure 14D) with p-value less than 0.0001.

4. Discussion

Developing a robust HFMD testing kit is hampered by many factors. Firstly, HFMD is known to be caused by a wide range of enteroviruses such as EV71, CA16, CA6 and in some cases, also by Echoviruses. Moreover, EV71 itself have many strains presenting widely varying

genome and capsid structure. Prior reasons effectively render developing an assay which will cross-react all strains of HFMD causing enteroviruses while sustaining a good specificity and sensitivity greatly challenging. Our test attempted to address those issues by identifying general miRNA signature responses uniquely caused by HFMD. The test is more desirable in clinical context such that (1) it require modest starting material of 50ul saliva (2) collection of saliva is non-invasive (3) complete profiling of miRNA from extraction to data analysis could be done in mere 4 hours which is considerably shorter than widely accepted current methods of traditional pan-entero PCR which requires gel electrophoresis or virus isolation which entails weeks to complete (4) We also observed significance separation of signature miRNA levels in individuals correlating with viral load in throat swab samples in the span of 7 days (Figure 7). It is an indication that the model could be useful in the isolation of infected individuals until signatures return to normal to control the spread of the disease in a population.

A number of miRNAs purposed as HFMD diagnostic markers were reported recently (Cui et al.; Jia et al., 2014). Many of these tests utilized serum miRNA in symptomatic HFMD patients and notably those infected with a specific strain, EV71. Although the miRNA present in serum and saliva are known to be highly similar (Weber et al., 2010), we did not observe a significant overlap of signatures between our studies and other previous reports profiling serum of HFMD patients. To our knowledge, this study is the first to attempt saliva-based miRNA analysis in HFMD patients whereas the previous studies were focused on serum markers (Cui et al.; Wang et al., 2016). Out of the validated 6 and 4 miRNAs from our study, hsa-miR-221-3p was found to be the most important miRNA in both panels. In addition, *Wang et al.* reported that, hsa-miR-221-3p was also significantly downregulated in severe EV71 cases (Wang et al., 2016). Although all the rest of the miRNAs identified in our study were different from those previously published, such findings were expected since our study utilized saliva instead of serum.

A salivary miRNA-based HFMD diagnostic test is more desirable in the clinical context for the following reasons: (1) it requires only 50ul of saliva, (2) collection of saliva is non-invasive and (3) complete profiling of miRNA from extraction to data analysis can be performed within 4 hours which is considerably faster than current methods of traditional pan-entero PCR which

requires gel electrophoresis or virus isolation which requires days to complete. A rather less important yet interesting question is whether our signature miRNA studied were released from the infected tissues or from the surrounding uninfected ones as an elicited immune response against the viral infection. Entire miRNA panel identified in this study is known to be present in the serum and plasma of human blood samples (Arroyo et al., 2011a; Cookson et al., 2012a). hsa-miR-221-3p is known to be upregulated in many types of cancer (Calin et al., 2005; Szafranska et al., 2007) and the function of the miRNA is regarded to be positive regulator of apoptosis through inhibiting ARF4 protein (Wu et al., 2017). It is possible that significant downregulation of hsa-miR-221-3p in both of our HFMD cohorts seems to suggest an indication of the specific viral pathogenesis mechanism which inhibits the apoptosis of infected cell which might allow further replication of the enterovirus (**Figure 15**) (James and Green, 2002). Interestingly, we found that most of our miRNA predictors were generally upregulated during HFMD. In this study, hsa-miR-324-3p was found to be significantly upregulated in saliva samples (**Figure 15**). hsa-miR-324-3p expression levels were not known to alter in many dysregulated cellular processes such as infections and cancers. However, interestingly, we also found that EV71, CA16 and CA6 contains has-miR-324-3p target sites in their respective genomes predicted by ViTa software (<http://vita.mbc.nctu.edu.tw>). Therefore, we postulated that increase in hsa-miR-324-3p expression level was a possible large spectrum specific antiviral response against enteroviruses, although further *in vitro* studies are warranted to cement the phenomena.

The following shows the hit miRNAs target site predictions using ViTA, including the R Script used for the Model Training and Blinded Evaluations.

Hit miRNAs Target Site Predictions using ViTA

Human coxsackievirus A16

Coxsackievirus A16 polyprotein gene, complete cds.

Accession – AF177911

Rank	Target location	Virus gene	Mfe	Score	Hybridization	miRanda	TargetScan
1	1106 -- 1132	unknown	-15.4	140	GGUCCGUCGUGGAC--C-CCGUGAAG CCTACAGGAGCTGAGCTGTGAGTGA	Yes	NO
2	1973 -- 2006	unknown	-20.6	142	ggucgucguggacc-----ccgucagg agaccagagccctggagagagatgctccgtggcagttcc	Yes	NO
3	3383 -- 3388	unknown	-15.2	144	ggucgucgugg--accccgucagg atgaggcgaactatagggtagtgga	Yes	NO
4	1086 -- 1103	unknown	-14.8	145	ggucgucguggagcccgucagg acagatgcac--ggcagttgg	Yes	NO
5	1655 -- 1709	unknown	-17.7	148	ggucgucguggac--c-ccgucagg ttggggcgcctcgccagccagttga	Yes	NO
6	344 -- 385	unknown	-22.4	147	ggucgucguggagcccgucagg ccacggggcagccgtgacagttgg	Yes	NO
7	958 -- 975	unknown	-20.7	153	GGUCCGUCGUGGACCCCGU-CAAC CGA--AGGG-CTGAGGCATGTGG	Yes	NO
8	1306 -- 1321	unknown	-23	158	GGUCGUCGUG--GACC-CCGUCAGc CCAC-GGCCGTTTACTGGTGGCTGTGc	Yes	NO

Human coxsackievirus A6

Human coxsackievirus A6 strain Gdula, complete genome.

Accession - AY421764

Rank	Target location	Gene name	Size	Score	Hybridization	miRank	TargetRank
1	4030 - 4051	unknown	-21.9	140	GGU--CGUCGUGGACCCGUCACC 	Yes	NO
2	2117 - 2138	unknown	-16.9	144	ggugguuGUGACCCGUCACC 	Yes	NO
3	2229 - 2250	unknown	-17.4	145	gGUCGUUGUG---GA-CCCCGUCACC 	Yes	NO
4	3860 - 3881	unknown	-25.3	137	ggUGUGUGUGGACCCGUCACC 	Yes	NO
5	495 - 516	unknown	-21.5	158	CCGCGA-AACGAGGCGGCGGTGt gGUCGUUGUGGACCCGUCACC	Yes	NO
5	343 - 364	unknown	-23.4	177	ICACGCGCGACCGTGGGAGTGG 	Yes	Yes

Human enterovirus 71

Human enterovirus 71 genomic RNA, complete genome, sub_strain:BrCr-TR.

Accession - AB204852

Rank	Target location	Gene name	Size	Score	Hybridization	miRank	TargetRank
1	4004 - 4025	unknown	-25.3	140	GGUGUGUGU--GGACCCCGUCACC 	Yes	NO
2	3838 - 3857	unknown	-21.12	142	GGU-CGUCGUGGACC---CGGUCACC 	Yes	NO
3	1563 - 1587	unknown	-14.9	143	ggUGUGUGUG-GAGG--CGGUCACC 	Yes	NO
4	3834 - 3855	unknown	-23.3	137	ggUGUGUGUGGACCCGUCACC 	Yes	NO

5	493 - 514	unknown	-15.1	159	GGUCGUCGUGGACCCGUCACC	Yes	Yes
					CCCTTAATCCAAAGGCGAGTGT		
6	5552 - 5553	unknown	-24.6	161	G3UCGUC---GUGGACCCCG-UCACC	Yes	NO
					CCAGAAAGTGTACACC-GGGGCGAGTGTa		
7	241 - 262	unknown	-22.6	167	GGUCGUCGUGGACCCGUCACC	Yes	NO
					CCACGGGCGACCGTGGCGGTGG		

68

69 **Human enterovirus 71**

70 Enterovirus 71 polyprotein gene, complete cds.

71 Accession - AF176044

72

Rank	Target location	virus gene	ME	Score	Hybridization	inPanda	TargetScan
1	5517 - 5536	unknown	-17.5	141	ggUCGUCGUG-GACCCCGUCACC	Yes	NO
					aaAGCAGGACAGTGTGTGTGTGT		
2	3514 - 3539	unknown	-29	144	GGUC--GUCGUGGAC-CCGUCACC	Yes	NO
					CCAGTCACATCTCATGCTTGCAGTGG		
3	265 - 288	unknown	-14.4	145	GGUCGU-GUGGACCCGUCACC	Yes	NO
					CCACCATGAAC-GAGGCAGGGT		
4	957 - 975	unknown	-17.5	148	GGUCGUCGUGSACCCCGU-CACC	Yes	NO
					CCATCTG---CTGAGGCATGTGG		
5	1335 - 1359	unknown	-25.4	152	GGUCGUCGU--G-GACCCCGUCACC	Yes	NO
					CCAG-AGTATGTGATTGGGACAGTGG		
6	342 - 353	unknown	-22.1	174	GGUCGUCGUGGACCCGUCACC	Yes	Yes
					CCATGGGCGACCATGGCAAGTGG		

73

74

Individual Performances of hit miRNAs

hsa-miR-18b-5p

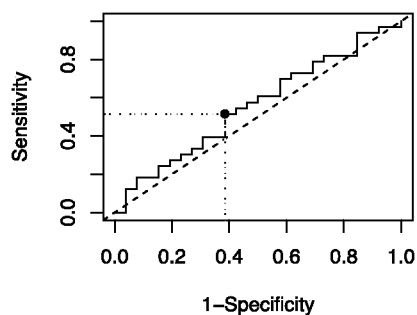
Cut-off Results

Optimal cut-off method	: Youden
Optimal cut-off point	: 12.51103
Optimal criterion	: 0.1305361

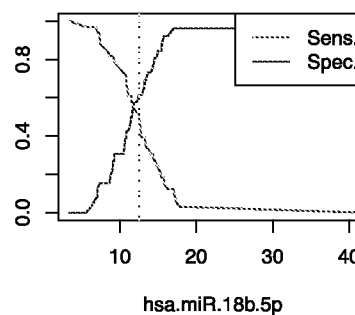
Performance Measures

	Value	Lower Limit	Upper Limit
Sensitivity	: 0.515	0.335	0.692
Specificity	: 0.615	0.406	0.798
Positive Predictive Value	: 0.630	0.420	0.782
Negative Predictive Value	: 0.500	0.322	0.711
Positive Likelihood Ratio	: 1.339	0.744	2.412
Negative Likelihood Ratio	: 0.788	0.495	1.254

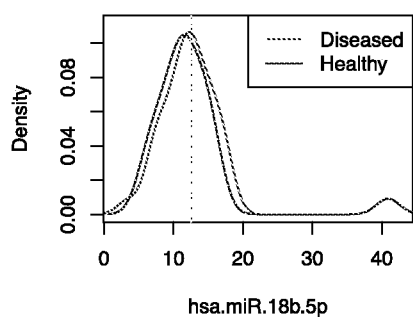
ROC Curve



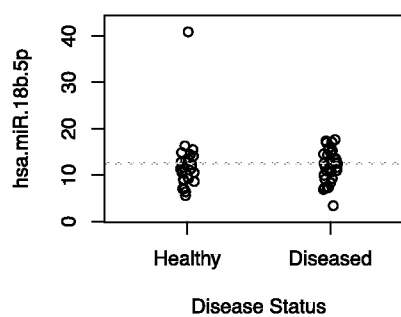
Sens. & Spec. Curves



Distribution of hsa.miR.18b.5p



Distribution of hsa.miR.18b.5p



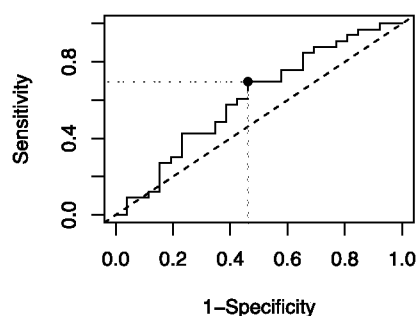
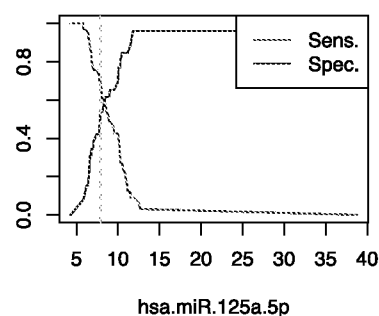
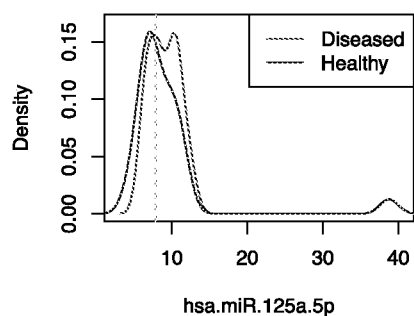
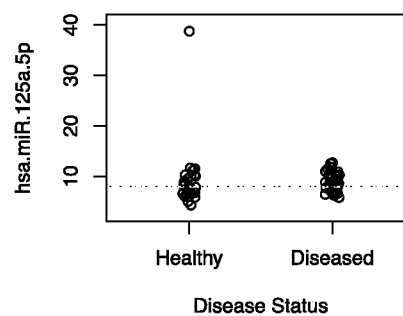
hsa-miR-125a-5p**Cut-off Results**

Optimal cut-off method	: Youden
Optimal cut-off point	: 7.86
Optimal criterion	: 0.2354312

Performance Measures

	Value	Lower Limit	Upper Limit

Sensitivity	: 0.697	0.513	0.844
Specificity	: 0.538	0.334	0.734
Positive Predictive Value	: 0.657	0.451	0.819
Negative Predictive Value	: 0.583	0.391	0.768
Positive Likelihood Ratio	: 1.510	0.942	2.422
Negative Likelihood Ratio	: 0.563	0.300	1.055

ROC Curve**Sens. & Spec. Curves****Distribution of hsa.miR.125a.5p****Distribution of hsa.miR.125a.5p**

hsa-miR-142-3p

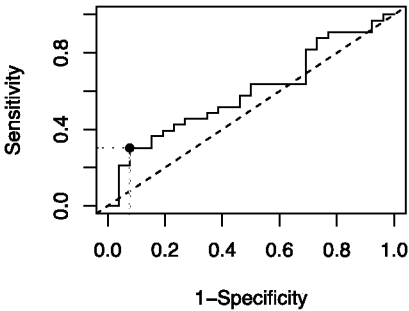
Cut-off Results

Optimal cut-off method : Youden
Optimal cut-off point : 14.51
Optimal criterion : 0.2261072

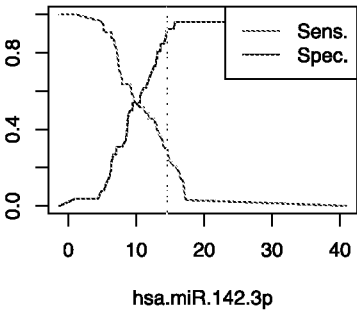
Performance Measures

	Value	Lower Limit	Upper Limit
Sensitivity	0.303	0.156	0.487
Specificity	0.923	0.749	0.991
Positive Predictive Value	0.833	0.554	0.916
Negative Predictive Value	0.511	0.307	0.901
Positive Likelihood Ratio	3.939	0.944	16.437
Negative Likelihood Ratio	0.755	0.588	0.970

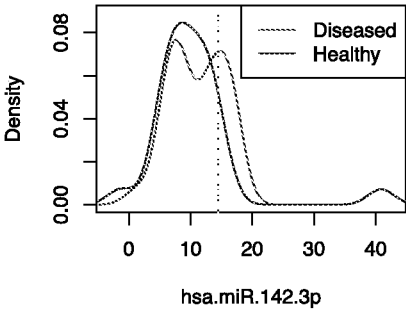
ROC Curve



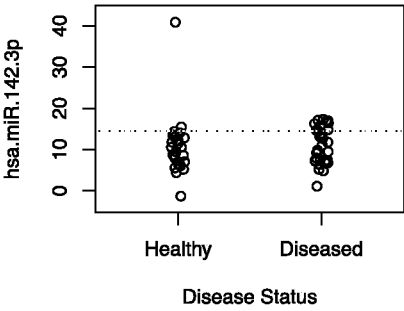
Sens. & Spec. Curves



Distribution of hsa.miR.142.3p



Distribution of hsa.miR.142.3p



hsa-miR-145-5p

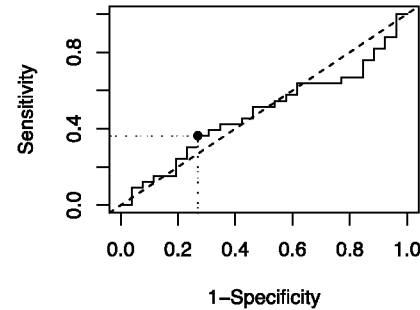
Cut-off Results

Optimal cut-off method : Youden
Optimal cut-off point : 9.23456
Optimal criterion : 0.09440559

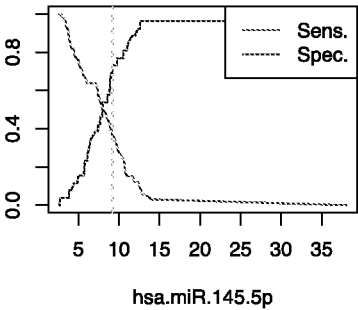
Performance Measures

	Value	Lower Limit	Upper Limit
Sensitivity	0.364	0.204	0.549
Specificity	0.731	0.522	0.884
Positive Predictive Value	0.632	0.408	0.785
Negative Predictive Value	0.475	0.289	0.718
Positive Likelihood Ratio	1.351	0.621	2.939
Negative Likelihood Ratio	0.871	0.615	1.233

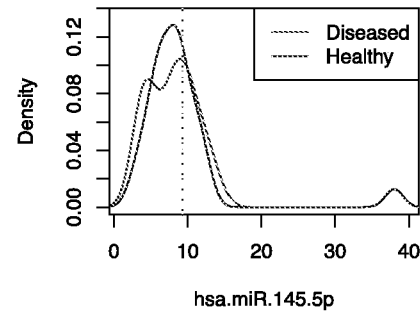
ROC Curve



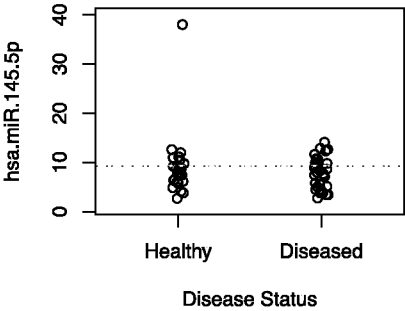
Sens. & Spec. Curves



Distribution of hsa.miR.145.5p



Distribution of hsa.miR.145.5p



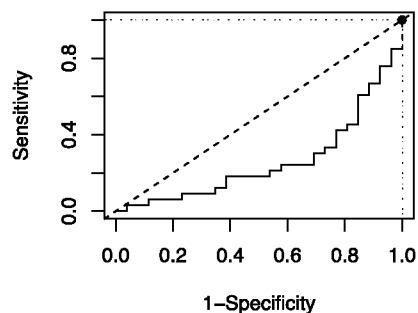
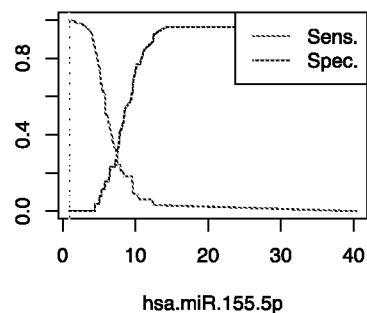
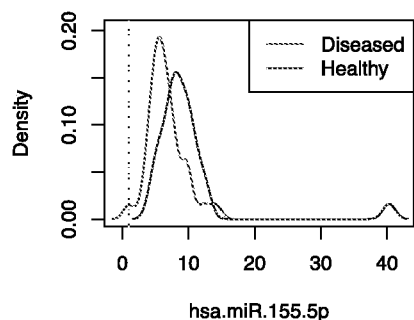
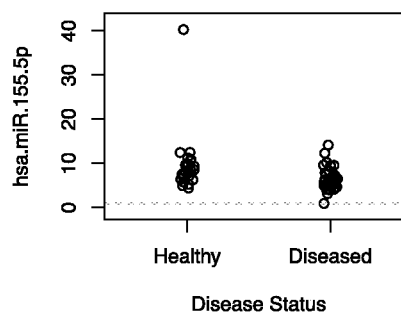
hsa-miR-155-5p

Cut-off Results

Optimal cut-off method : Youden
 Optimal cut-off point : 0.9508257
 Optimal criterion : 0

Performance Measures

	Value	Lower Limit	Upper Limit
Sensitivity	1.000	0.894	NaN
Specificity	0.000	NaN	0.132
Positive Predictive Value	0.559	NaN	NaN
Negative Predictive Value	NaN	0.000	1.000
Positive Likelihood Ratio	1.000	1.000	1.000
Negative Likelihood Ratio	NaN	NaN	NaN

ROC Curve**Sens. & Spec. Curves****Distribution of hsa.miR.155.5p****Distribution of hsa.miR.155.5p**

hsa-miR-221-3p

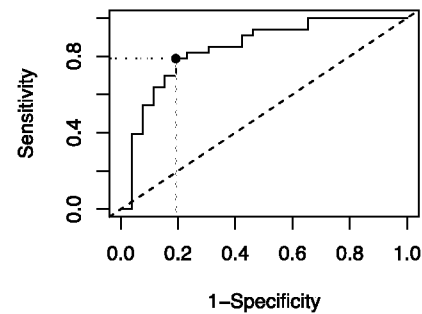
Cut-off Results

Optimal cut-off method : Youden
Optimal cut-off point : 9.028622
Optimal criterion : 0.5955711

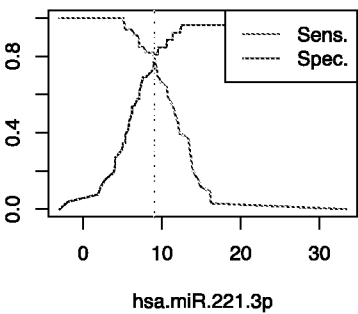
Performance Measures

	Value	Lower Limit	Upper Limit
Sensitivity	: 0.788	0.611	0.910
Specificity	: 0.808	0.606	0.934
Positive Predictive Value	: 0.839	0.656	0.934
Negative Predictive Value	: 0.750	0.559	0.911
Positive Likelihood Ratio	: 4.097	1.827	9.186
Negative Likelihood Ratio	: 0.263	0.133	0.520

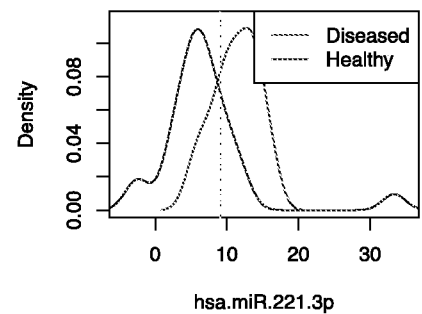
ROC Curve



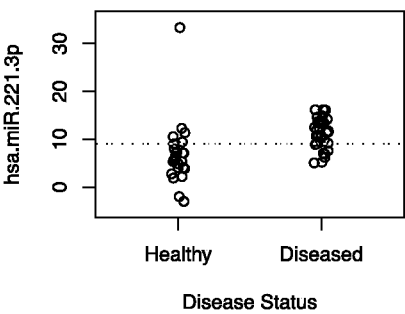
Sens. & Spec. Curves



Distribution of hsa.miR.221.3p



Distribution of hsa.miR.221.3p

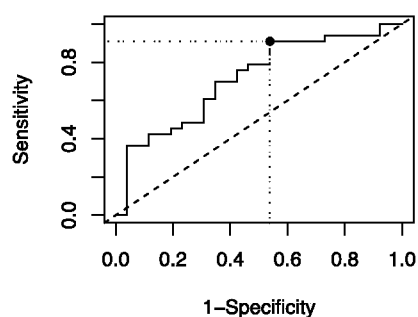
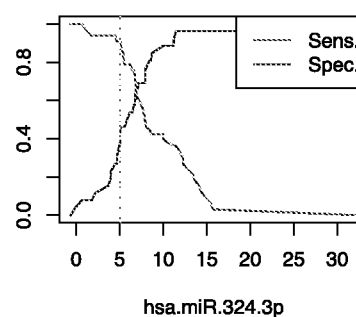
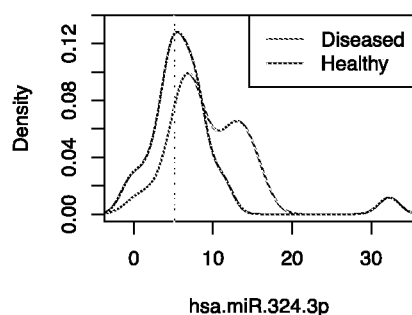
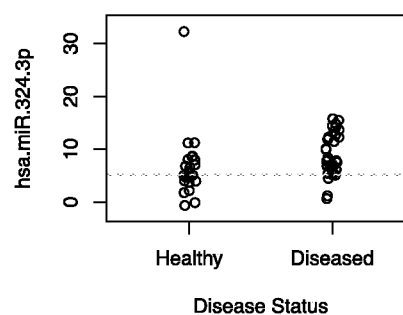


hsa-miR-324-3p**Cut-off Results**

Optimal cut-off method : Youden
 Optimal cut-off point : 5.09
 Optimal criterion : 0.3706294

Performance Measures

	Value	Lower Limit	Upper Limit
Sensitivity	: 0.909	0.757	0.981
Specificity	: 0.462	0.266	0.666
Positive Predictive Value	: 0.682	0.475	0.916
Negative Predictive Value	: 0.800	0.554	0.903
Positive Likelihood Ratio	: 1.688	1.164	2.449
Negative Likelihood Ratio	: 0.197	0.062	0.626

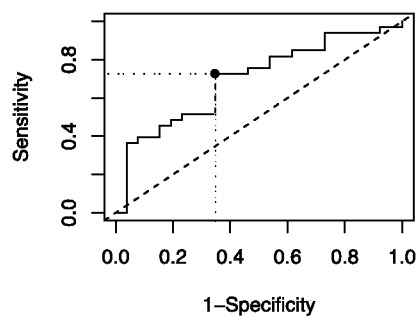
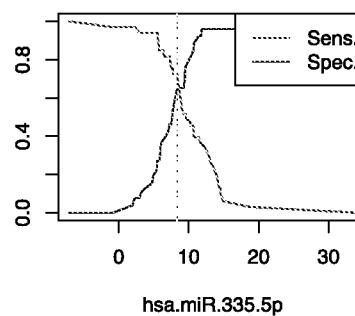
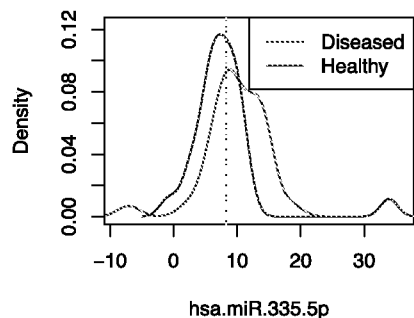
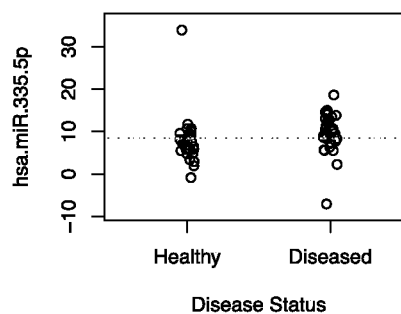
ROC Curve**Sens. & Spec. Curves****Distribution of hsa.miR.324.3p****Distribution of hsa.miR.324.3p**

hsa-miR-335-5p**Cut-off Results**

Optimal cut-off method : Youden
 Optimal cut-off point : 8.31
 Optimal criterion : 0.3811189

Performance Measures

	Value	Lower Limit	Upper Limit
Sensitivity	0.727	0.545	0.867
Specificity	0.654	0.443	0.828
Positive Predictive Value	0.727	0.529	0.867
Negative Predictive Value	0.654	0.459	0.828
Positive Likelihood Ratio	2.101	1.190	3.708
Negative Likelihood Ratio	0.417	0.224	0.778

ROC Curve**Sens. & Spec. Curves****Distribution of hsa.miR.335.5p****Distribution of hsa.miR.335.5p**

263 R Script for the Model Training and Blinded Evaluations

264

265 # Importing the dataset

266 dataset = SG

267 dataset = dataset[,c(2,8,3,4,5,7,10)]

268

269 # Encoding the target feature as factor

270 dataset\$Exited= factor(dataset\$Exited, levels = c(0, 1))

271

272 # Splitting the dataset into the Training set and Test set

273 # install.packages('caTools')

274 library(caTools)

275 set.seed(123)

276 split = sample.split(dataset\$Exited, SplitRatio = 0.80)

277 training_set = subset(dataset, split == TRUE)

278 test_set = subset(dataset, split == FALSE)

279

280 # Feature Scaling

281 training_set[-7] = scale(training_set[-7])

282 test_set[-7] = scale(test_set[-7])

283

284 # Fitting Kernel SVM to the Training set

285 # install.packages('e1071')

286 library(e1071)

287 classifier = svm(formula = Exited ~ .,

288 data = training_set,

289 type = 'C-classification',

290 kernel = 'radial', probability=TRUE)

291

292 # Predicting the Test set results

293 y_pred = predict(classifier, newdata = test_set[-7],probability = TRUE)

294

295 # Making the Confusion Matrix

```
296 cm = table(unlist(test_set[, 7]), unlist(y_pred))
297 #cm=confusionMatrix(unlist(test_set[, 7]), unlist(y_pred),negative="0")
298 #diagnosticErrors(cm)
299 # Applying k-Fold Cross Validation
300 # install.packages('caret')
301 library(caret)
302 library(crossval)
303 folds = createFolds(training_set$Exited, k = 10)
304 cv = lapply(folds, function(x) {
305   training_fold = training_set[-x, ]
306   test_fold = training_set[x, ]
307   classifier = svm(formula = Exited ~ .,
308     data = training_fold,
309     type = 'C-classification',
310     kernel = 'radial',probability=TRUE)
311   y_pred = predict(classifier, newdata = test_fold[-7],probability = TRUE)
312   cm = table(unlist(test_fold[, 7]), unlist(y_pred))
313   #cm=confusionMatrix(unlist(test_fold[, 7]), unlist(y_pred),negative="0")
314   #diagnosticErrors(cm)
315   #accuracy = (cm[1,1] + cm[2,2]) / (cm[1,1] + cm[2,2] + cm[1,2] + cm[2,1])
316   #sensitivity = (cm[1,1]) / (cm[1,1] + cm[2,1])
317   #specificity = (cm[2,2]) / (cm[2,2] + cm[1,2])
318   #return(accuracy)
319   #return(sensitivity)
320   #return(specificity)
321 })
322 #accuracy = mean(as.numeric(cv))
323 #sensitivity = mean(as.numeric(cv))
324 #specificity = mean(as.numeric(cv))
```

Although diagnosis accuracy of the 6-miRNA model in blinded evaluation of the “Taiwan Cohort” was significantly a notch under at 77.08% compared to the 92.86% of the “Singapore Cohort”; it is understandable that miRNA responses between different geographical region could slightly differ due to genetics and epigenetics differences. We envisioned that a systematic comparison of viral titer presents in HFMD infected patients and our miRNA predictor level in a larger cohort will be needed in the future to fine tune our detection algorithm. Nevertheless, by given the cross-validated and blinded evaluations data, it can be safely assumed that the 6-miRNA composition is unlikely to be altered. We believe our saliva test have the potential to further develop into a point of care device where general public and school could use to routinely monitor HFMD to prevent disease transmission.

4. Conclusion

The present invention provides for a reliable diagnostic platform for HFMD infection where saliva could be used and a panel of circulating miRNA could serve as a potential biomarker in perspective discrimination HFMD from healthy patients.

Whilst there has been described in the foregoing description preferred embodiments of the present invention, it will be understood by those skilled in the technology concerned that many variations or modifications in details of design or construction may be made without departing from the present invention.

References

- 5 Arroyo, J.D., Chevillet Jr Fau - Kroh, E.M., Kroh Em Fau - Ruf, I.K., Ruf Ik Fau - Pritchard, C.C., Pritchard Cc Fau - Gibson, D.F., Gibson Df Fau - Mitchell, P.S., Mitchell Ps Fau - Bennett, C.F., Bennett Cf Fau - Pogosova-Agadjanyan, E.L., Pogosova-Agadjanyan El Fau - Stirewalt, D.L., Stirewalt Dl Fau - Tait, J.F., Tait Jf Fau - Tewari, M., Tewari, M., 2011a. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma.
- 10 Arroyo, J.D., Chevillet, J.R., Kroh, E.M., Ruf, I.K., Pritchard, C.C., Gibson, D.F., Mitchell, P.S., Bennett, C.F., Pogosova-Agadjanyan, E.L., Stirewalt, D.L., Tait, J.F., Tewari, M., 2011b. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc Natl Acad Sci U S A* 108, 5003-5008.
- 15 Calin, G.A., Ferracin, M., Cimmino, A., Di Leva, G., Shimizu, M., Wojcik, S.E., Iorio, M.V., Visone, R., Sever, N.I., Fabbri, M., Iuliano, R., Palumbo, T., Pichiorri, F., Roldo, C., Garzon, R., Sevignani, C., Rassenti, L., Alder, H., Volinia, S., Liu, C.G., Kipps, T.J., Negrini, M., Croce, C.M., 2005. A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *N Engl J Med* 353, 1793-1801.
- 20 Cardosa, M.J., Perera, D., Brown, B.A., Cheon, D., Chan, H.M., Chan, K.P., Cho, H., McMinn, P., 2003. Molecular Epidemiology of Human Enterovirus 71 Strains and Recent Outbreaks in the Asia-Pacific Region: Comparative Analysis of the VP1 and VP4 Genes. *Emerg. Infect. Dis.* 9, 461-468.
- 25 Chan, K.P., Goh, K.T., Chong, C.Y., Teo, E.S., Lau, G., Ling, A.E., 2003. Epidemic hand, foot and mouth disease caused by human enterovirus 71, Singapore. *Emerging infectious diseases* 9, 78-85.
- Cookson, V.J., Bentley Ma Fau - Hogan, B.V., Hogan Bv Fau - Horgan, K., Horgan K Fau - Hayward, B.E., Hayward Be Fau - Hazelwood, L.D., Hazelwood Ld Fau - Hughes, T.A., Hughes, T.A., 2012a. Circulating microRNA profiles reflect the presence of breast tumours but not the profiles of microRNAs within the tumours.
- 30 Cookson, V.J., Bentley, M.A., Hogan, B.V., Horgan, K., Hayward, B.E., Hazelwood, L.D., Hughes, T.A., 2012b. Circulating microRNA profiles reflect the presence of breast tumours but not the profiles of microRNAs within the tumours. *Cell Oncol (Dordr)* 35, 301-308.
- 35 Cui, L., Qi Y Fau - Li, H., Li H Fau - Ge, Y., Ge Y Fau - Zhao, K., Zhao K Fau - Qi, X., Qi X Fau - Guo, X., Guo X Fau - Shi, Z., Shi Z Fau - Zhou, M., Zhou M Fau - Zhu, B., Zhu B Fau - Guo, Y., Guo Y Fau - Li, J., Li J Fau - Stratton, C.W., Stratton Cw Fau - Tang, Y.-W., Tang Yw Fau - Wang, H., Wang, H., Serum microRNA expression profile distinguishes enterovirus 71 and coxsackievirus 16 infections in patients with hand-foot-and-mouth disease.
- James, E.R., Green, D.R., 2002. Infection and the origins of apoptosis. *Cell Death Differ* 9, 355-357.
- 40 Jia, H.-L., He, C.-H., Wang, Z.-Y., Xu, Y.-F., Yin, G.-Q., Mao, L.-J., Liu, C.-W., Deng, L., 2014. MicroRNA expression profile in exosome discriminates extremely severe infections from mild infections for hand, foot and mouth disease. *BMC Infectious Diseases* 14, 506.
- Li, L.-J., 2010. Review of hand, foot and mouth disease. *Frontiers of Medicine in China* 4, 139-146.
- 45

- Li, W., Zhang, X., Chen, X., Cheng, Y.P., Wu, Y.D., Shu, Q., Chen, X.J., Shang, S.Q., 2015. Epidemiology of childhood enterovirus infections in Hangzhou, China. *Virology journal* 12, 58.
- 5 Liang, Z., Mao, Q., Gao, F., Wang, J., 2012. Progress on the research and development of human enterovirus 71 (EV71) vaccines. *Front. Med.* 7, 111-121.
- Lin, T.-Y., Twu, S.-J., Ho, M.-S., Chang, L.-Y., Lee, C.-Y., 2003. Enterovirus 71 Outbreaks, Taiwan: Occurrence and Recognition. *Emerg. Infect. Dis.* 9, 291-293.
- Ooi, M.H., Wong, S.C., Lewthwaite, P., Cardoso, M.J., Solomon, T., 2010. Clinical features, diagnosis, and management of enterovirus 71. *The Lancet Neurology* 9, 1097-1105.
- 10 Perez-Ruiz, M., 2003. Human rhabdomyosarcoma cells for rapid detection of enteroviruses by shell-vial assay. *Journal of Medical Microbiology* 52, 789-791.
- Solomon, T., Lewthwaite, P., Perera, D., Cardoso, M.J., McMinn, P., Ooi, M.H., 2010. Virology, epidemiology, pathogenesis, and control of enterovirus 71. *The Lancet Infectious Diseases* 10, 778-790.
- 15 Szafranska, A.E., Davison, T.S., John, J., Cannon, T., Sipos, B., Maghnouj, A., Labourier, E., Hahn, S.A., 2007. MicroRNA expression alterations are linked to tumorigenesis and non-neoplastic processes in pancreatic ductal adenocarcinoma. *Oncogene* 26, 4442-4452.
- Wang, R.Y., Weng, K.F., Huang, Y.C., Chen, C.J., 2016. Elevated expression of circulating miR876-5p is a specific response to severe EV71 infections. *Scientific reports* 6, 24149.
- 20 Wang, S.-M., Liu, C.-C., 2014. Update of enterovirus 71 infection: epidemiology, pathogenesis and vaccine. *Expert review of anti-infective therapy* 12, 447-456.
- Weber, J.A., Baxter, D.H., Zhang, S., Huang, D.Y., How Huang, K., Jen Lee, M., Galas, D.J., Wang, K., 2010. The MicroRNA Spectrum in 12 Body Fluids. *Clinical Chemistry* 56, 1733.
- Wu, Q., Ren, X., Zhang, Y., Fu, X., Li, Y., Peng, Y., Xiao, Q., Li, T., Ouyang, C., Hu, Y., Zhang, Y., Zhou, W., Yan, W., Guo, K., Li, W., Hu, Y., Yang, X., Shu, G., Xue, H., Wei, Z., Luo, Y., Yin, G., 25 2017. MiR-221-3p targets ARF4 and inhibits the proliferation and migration of epithelial ovarian cancer cells. *Biochem Biophys Res Commun.*

Claims

- 5 1. A method for detecting hand-foot-and-mouth disease (HFMD), the method comprising determining the presence of miR-18b, miR-145, miR-155 and miR-324 in a saliva sample, wherein the presence of the 4 miRNAs in the saliva sample is indicative of HFMD.
2. The method according to claim 1, wherein the method further comprising determining
10 the presence of a miRNA selected from the group consisting of miR-125a, miR-221, and miR-335.
3. The method according to claim 2, wherein the group of miRNA further consists of miR-23a and miR-142.
- 15 4. The method according to any one of the preceding claims, wherein the amount of the saliva sample is about 50 μ l.
5. The method according to any one of the preceding claims, wherein the presence of
20 the miRNAs in the saliva sample is determined by:
- (a) centrifuging the saliva sample to get the supernatant, and filtering the supernatant;
 - (b) extracting RNAs from the filtered supernatant obtained in step (a);
 - (c) performing a reverse transcription reaction using the RNAs extracted in step
25 (b) to obtain a cDNA solution;
 - (d) performing a PCR amplification reaction using the cDNA solution obtained in step (c); and
 - (e) detecting the PCR products with a fluorescence quantitative PCR instrument so as to obtain the expression level of miRNAs in the medium.
- 30

6. A method for aiding in categorizing, diagnosing or determining prognosis in a patient with HFMD, the method comprising determining the presence of miR-18b, miR-145, miR-155 and miR-324 in a saliva sample.