IAI PadoTest 4·5®

1. Description and procedure

IAI PADOTEST 4·5® is a biological molecular test which allows the identification and the quantification of 4 bacteria best indicators of periodontitis and of the total number of bacteria present (TBL). The targeted bacteria are: *Actinobacillus actinomycetemcomitans (*Aa*)*, *Tannerella forsythia* (Tf, old name was *Bacteroides forsythus*), *Porphyromonas gingivalis* (Pg) and *Treponema denticola* (Td). These bacteria are regarded as important etiologic factors in periodontitis. The choice of these markers is discussed in sub-chapter 2 below.

The bacterial identification and quantification are done by direct hybridization (without genetic amplification of the original sample) of specific probes (labelled for chemiluminescence) on the ribosomal RNA of the bacteria. The result of hybridization is subsequently detected and measured by chemiluminescence. The probes are specific oligonucleotides for the 4 targeted bacteria and universal oligonucleotides for the calculation of the total bacteria load (TBL). The sequences of the specific probes were selected on standard alignments of bacteria, validated for hybridization on the program Primer Premier© and tested for specificity with BLAST on the entire bacteria data of GENBANK. According to these tests, these probes are perfectly specific. Each probe is modified at 3' and 5' to permit chemiluminescence measures, and then quantification. With this method, it is possible to highlight relatively small quantities of bacteria.

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Procedure

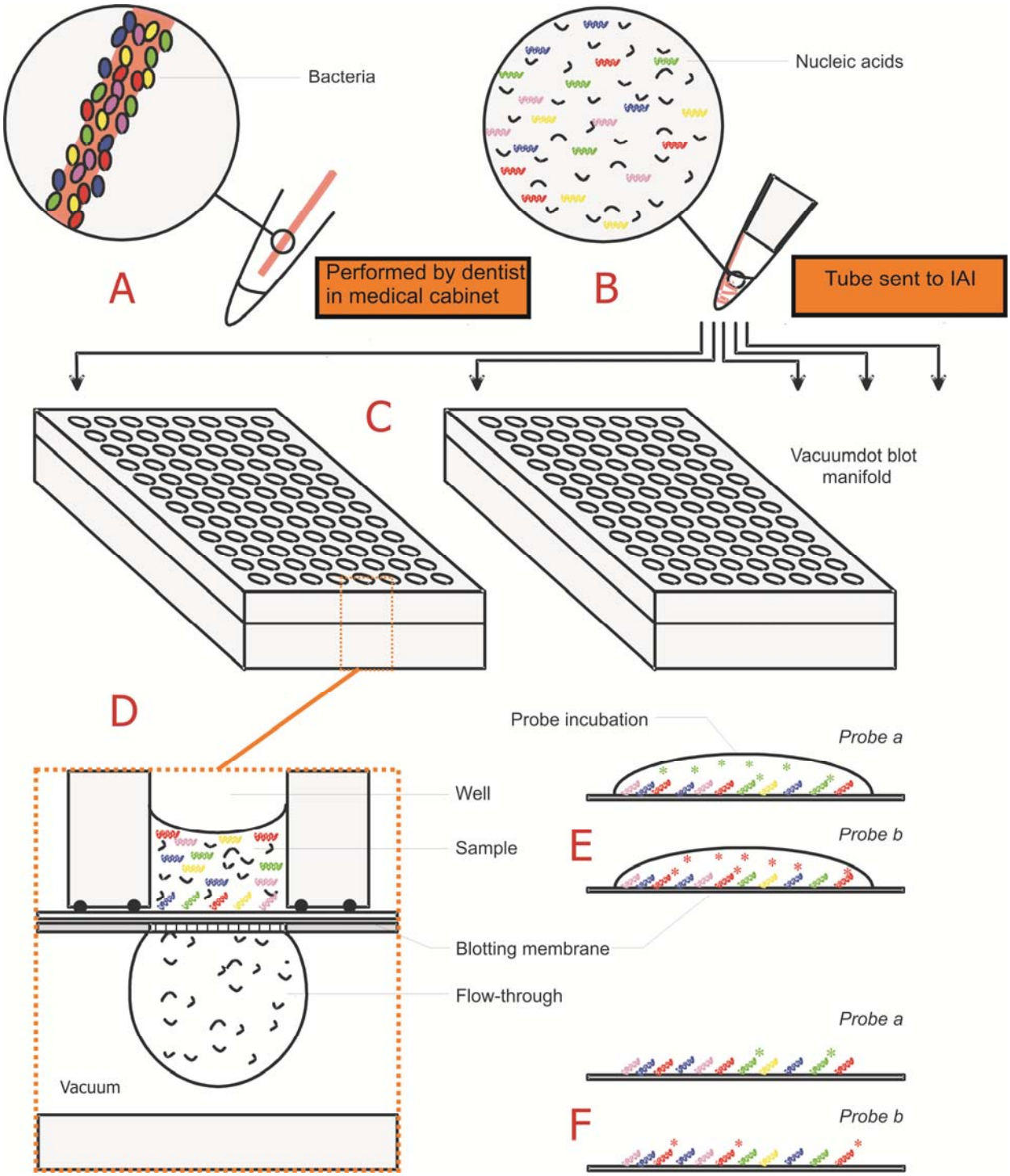

Figure IV.1: IAI PADOTEST 4·5® procedure

A *Sampling (carried out by the dentist in its cabinet):*
A paper point of cellulose is introduced into the gingival furrow or the periodontal pocket to be analyzed. After 10" the point is withdrawn and placed in a tube containing a lysis and RNA stabilizer buffer.

B *Preparation of the samples in the laboratory:*
The samples are treated so that the RNA can be coupled with charged membranes.
A standard of quantification is treated simultaneously as an additional tube (sample).

C *Deposit on membrane*
Five membranes are assembled on vacuum filtration apparatus.
Aliquots of each sample and standard are deposited on each membrane.

D After complete aspiration, the membranes are dismantled and exposed to UV to fix the nucleic acids on them. Each membrane is identified to be marked for one of the 4 required bacteria and the total bacteria.

E *Hybridization with specific labelled probes*
The membranes are placed in hybridization tubes and the corresponding probes are added.

F *Washings*
After hybridization, the membranes are washed such as only the perfectly homologous probe-targeted RNA hybridization are stable.

Thereafter a marking with a chemiluminescent system is carried out. The chemiluminescence is measured in a suitable apparatus. An image of each membrane is recorded and filed. The results for each tube are calculated by means of data-processing programs especially designed for this test. Each measured tubes is compared to the standard by means of a curve of calibration of the apparatus. All the values, rough, intermediate and final are recorded and filed. There is no intervention of the operator in calculations.

In addition to evaluating the population of pathogenic bacteria, the IAI PadoTest 4·5® allows a classification in 5 types of bacterial population within the periodontal pockets. This typology results from an important single study in periodontology. Thanks to this typology, the IAI PadoTest 4·5® is a therapeutic support measure, because its informative value goes beyond rough diagnosis of the periodontal pathogenic germs, by allotting to each type of pocket a suitable treatment. See chapter V.

The sensitivity of the PadoTest 4·5® for the inferior limit level of bacteria is 5000 individuals for *Actinobacillus actinomycetemcomitans*, and 10000 for the others. One must realise the as soon a bacteria is present and active, in few hours it becomes a colony that reaches several tens of thousands of individuals.

2. Significance and objective of a microbiological examination in periodontology

The evaluation of periodontal treatments must make it possible to know if the treatment led to a state that allows transferring the patient in a maintenance phase or if it is necessary to continue therapeutic measurements. In traditional periodontology, this evaluation is carried out by comparison of the level of adhesion, the depth of pockets and possibly by the osseous density observed on radiological image. These measurements being carried out before and after the treatment. The difficulties of interpretation lie in the fact of giving to the perceptible modifications appeared after the treatment, a value forecasting for the future evolution. "Can one tolerate a vestige of pocket?", "the result obtained is stable?" wonders the experts. "The pathogenic one was it eliminated? "or" were the pathogenic potentials sufficiently reduced so that the tissues of the host will not suffer any more any damage? ", such are the questions of microbiological nature (Mombelli A., SMZ, Vol. 102:2, 1992) to which a test must answer in order to help the experts.

Dr. Hubert Jotterand, Geneva (Switzerland) expert, underlines the roles which a microbiological examination must bring:

- Motivation of patient
  - at the beginning of treatment
  - in phase of maintenance, as a psychological support for patients with depressive tendency, as one often meets some in the repeating or "refractory" cases.
- Quality control after a periodontal treatment or a periimplantitis (these last treatments often have an important financial implication because of the prosthetic rehabilitations fixed on the implants).

- Long-term monitoring of the treated cases:
  - Indications for a new treatment, in phase of maintenance, particularly for strategic pillars in prosthetic rehabilitation paro/prosthetic.
  - Seek minimal treatment necessary, for example for old people not wishing more "invasive" treatment.
- Differential trauma-occlusal/periodontal infection or periimplantitis diagnosis.

The **IAI PADOTEST 4·5®** fulfils all these roles and bring answers to the experts not only concerning the presence and the quantity of pathogenic remaining, but also on the forecast and the treatment to be followed, thanks to the typology of the pockets.

Moreover, the **IAI PADOTEST 4·5®** allows:

- The fast and early detection of the patients at risk
- A reduction in the superfluous treatments (antibiotic)
- An antibiotic prescription directed and realized on the basis of indication.

3. The 4 selected markers

Actinobacillus (Aggregatibacter) actinomycetemcomitans (Aa)

Aa is a stick shape bacterium, gram-negative and anaerobic facultative. It was phylogenetically reclassified in the genus *Haemophilus*, but this name is only employed by few. It is known for a long time by its very important pathogenic role in periodontitis and other infections such as septicaemias, meningitides, endocarditis and pulmonary and cerebral abscess.

Aa cannot be eliminated in a sure way by curettage/radicular surfacing. A complementary antibiotherapy is usually necessary. The active antibiotics against Aa are amoxicillin, minocyclin, doxycyclin and ciprofloxacin. Aa can migrate in surrounding tissues and thus escape the diagnosis. Consequently, it is recommended to analyze the deepest pocket of each quadrant. With 4 analyses, the probability of highlighting Aa is sufficient. The most recent studies let suppose that Aa also migrates in the coronary vessels and can cause an endocarditis.

Tannerella forsythia (Tf; more known under its old name *Bacteroides forsythus*)

It is a gram-negative strict anaerobic which is very clearly in greater quantity in the active periodontal pockets than in the inactive ones. Tf is also present in the cases of repeating periodontitis. Tf can be usually eliminated by curettage/radicular surfacing. If the antibiotic use is necessary to eliminate Tf, the ones to be chosen are metronidazol and the clindamycin.

Porphyromonas gingivalis (Pg)

Gram-negative bacterium strictly anaerobic which one finds in the severe cases of periodontitis. One can usually eliminate Pg by curettage/radicular surfacing. If Pg is still present in spite of this treatment, a new intervention consisting of curettage or a surgical act is indicated. Antibiotics must be prescribed when Pg is present simultaneously with Aa in great quantity. The antibiotics to be used are metronidazol and the clindamycin + amoxicillin.

Treponema denticola (Td)

It is a small strictly anaerobic spirochete which plays a role in the periodontal destruction. Td is useful as marker in the evaluation of the therapeutic success of pockets resistant to the treatment. The active antibiotics against this bacterium are the metronidazol or clindamycin.

T. forsythia, P. gingivalis and *T. denticola* form the “red complex” of species associated with aggressive periodontal infections (Socransky and Al, 1998).

Notes on the choice of the marker bacteria for PadoTest 4·5®

The number of bacteria species present in the human subgingival flora can be estimated today at more than one thousand.

Among them, at least a hundred could be suspected of having a role in the aetiology of the periodontitis.

The goal of a diagnostic test is not to quantify the hundred bacteria **but to detect and permit to control** the periodontal lesions. The test must in addition remains in a **reasonable limit of cost**. Therefore the test has to fill its objectives while testing the smallest number of bacteria.

The panel of bacteria of PadoTest 4·5® has been chosen after consultation of the literature available and of specialist researchers in the field.

The capacity of the test to fill its objectives was tested on approximately 2000 samples. Simultaneously clinical indications were gathered and compared. We have reasons to believe that the determination of Aa, Tf, Pg and Td enables us to detect approximately 95% of the periodontal lesions and to follow their evolution.

To introduce a new bacterium in the test, we will have to be sure not only that the new bacterium plays a part in periodontitis but especially that **the test will be improved** in the direction, for example, that 98% of the lesions will then be detected.

However the only obviousness which we have in the majority of the cases (this is also valid for the other bacteria detected by the other tests) to introduce a new species is that it was associated to periodontal lesions by a small number of researchers.

This obviousness suffers from two major defects:

- The “new” species was determined in majority of the cases by culture. Culture can not certify on the real identity of the bacteria, that we must however know to develop a specific test.
- The quantitative estimates of this “new” are based on imprecise techniques, non-reproducible, and on a number of samples very reduced.

Let us take an example (real):

A researcher finds that fusobacteria are present in certain types of necrotizing lesions. It judges that it is *Fusobacterium necrophorum*. A study undertaken with imprecise techniques on a very reduced number of samples suggests that this bacterium is present in much greater number on the subjects reached than on the healthy subjects.

A test for detection/monitoring of these lesions could incorporate *Fusobacterium necrophorum*.

Actually determinations and quantifications by reliable techniques of molecular biology show that:

- It was not *Fusobacterium necrophorum* but *Fusobacterium nucleatum.*
- The prevalence of the bacterium is the same one in lesions that in the healthy sites.

Then, the inclusion of *Fusobacterium necrophorum* in the panel of bacteria detected would have been at best useless, at worst detrimental. Moreover, the price of the test should have increased.

The same applies to the other bacteria proposed. No real obviousness permits to ensure that they would increase our detection level significantly.

Thus, the longer lists of bacteria detected by other tests do not have a real value and meaning, because these bacteria do not bring profit to the detection of periodontitis and to the monitoring compared to our test. In other words, if for example *Eikenella corrodens* is present and that it really affects the periodontum, then our 4 germs say it already and we do not need it to detect/monitor periodontitis. This, again, makes it possible to promote a very precise, reliable, simple and cheap test.

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4. Nucleic acids: DNA & RNA

DeoxyriboNucleic Acids (**DNA**) is a molecule that is found in all cells of living organisms. The DNA is present in the nucleus of eukaryote cells, in the cytoplasm of the prokaryote cells, in the matrix of mitochondria like in chloroplasts. DNA constitutes the genome of organisms. It is the support of the hereditary genetic information used in the development and functioning of all living organisms. It is transmitted generation through generation during the processes of reproduction. It is one of the components of chromosomes. Genes are segments of DNA.

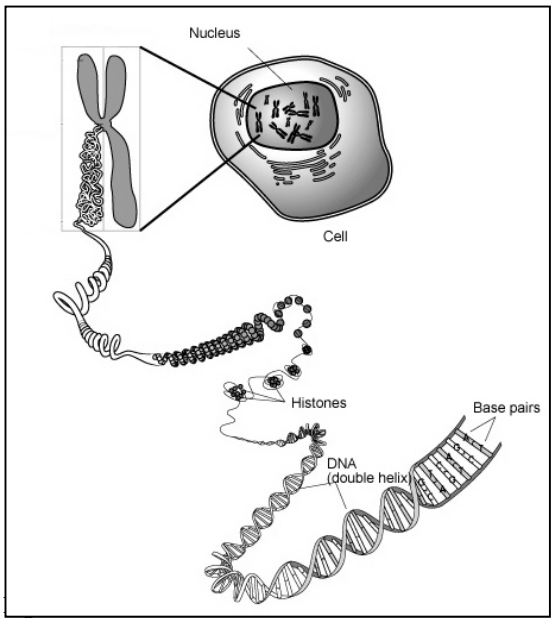

Figure IV. 2: DNA

The molecule of DNA consists of two polynucleotidic **antiparallel** strands, associated by hydrogen bonds between puric and pyrimidic bases around a common axis. It is famous the double helix of DNA. Unrolled, the long spiralling strands stretch in a very long sequence of basic units which are the nucleotides (Adenine "A", Thymine "T", Guanine "G" and Cytosine "C"). Specific pairing between nucleotides of each strands, induced a **complementarity.** The sequence (or codes) of one of the strands determines the sequence of the complementary and antiparallel strand. The original structure of the ADN enables it to be duplicated in two molecules identical between them and identical to the mother molecule.
The ADN contains the genetic message necessary to synthesis of proteins, elements ensuring the essence of the cellular functions and of the life.

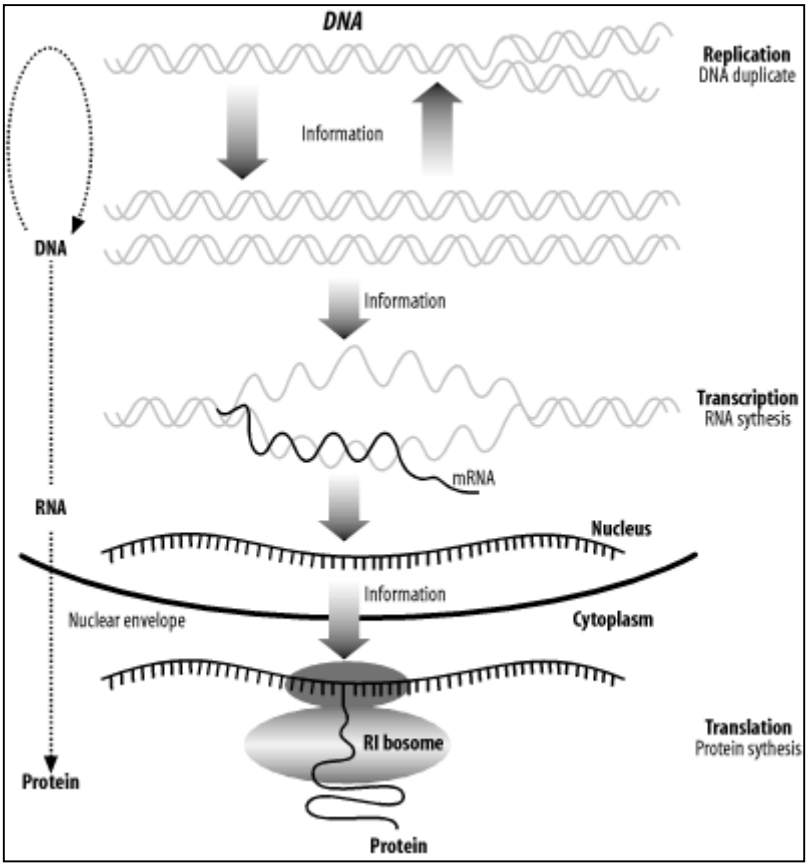

Figure IV. 3: Dogma of molecular biology

RiboNucleic Acids (**RNA**) are the vectors and mediators of the genetic information contained by DNA. They are the intermediaries between the DNA and proteins. One distinguishes several classes of RNA which differ according to their size, localization and function, whose principal ones are:

- **Messengers RNA** (**mRNA**) are a copy (transcription) of the coding sequence of a gene (DNA), which conveys the genetic information, from the place of storage (DNA helix) towards the place of expression (ribosome), where it is used as matrix for the synthesis of proteins (amino acids).
- **Ribosomal RNA** (**rRNA**) and **Transfert RNA** (**tRNA**) provide the structures and the tools necessary to the expression of the messenger RNA into a protein (translation).

Ribonucleic Acids are **polynucleotidic** chains that differ from the DNA 1) by the presence of nucleotidic base of **Uracil (U)** replacing Thymine (T), and 2) by sugars of type **Ribose**, in place of deoxyribose attached to the nucleotidic bases.

The presence of ribose sugar is mainly responsible for the conformational properties that differ from those of DNA. The hydroxyl group on sugar in position 2 ' permits many additional interactions which tend to destabilize the phosphodiester connections 5 ' - 3 ' and prevent the double helix conformation.

RNA is then a molecule made up of only one strand (**monocatenaire**). Except for the messenger RNA which has a linear structure, rRNA and tRNA are made of a strand which is frequently folded up on itself by local pairing of the bases. They thus present a conformation of loops and stems (known as pad or hairpins) and are often associated with specific proteins.

5. Ribosome and ribosomal RNA as molecular marker

Ribosomes, ribonucleoproteic molecules, are present in the eukaryotic and prokaryotic cells. Their function is to synthesize proteins by decoding the information contained in RNA (translation). Ribosome consists of two sub-units: a smaller that “reads” the messenger RNA and a larger which takes care of the synthesis of corresponding protein. These two sub-units consist of ribosomal RNA, which carries the catalytic activity, and of ribosomal proteins.

Ribosomes are free in cytoplasm, or associated whether with the membranes of endoplasmic reticulum, or with the nuclear membrane, or even in certain bacteria with their internal membrane (for example in *Escherichia coli*).

Ribosome is the “machine” ensuring the translation of the molecule of mRNA in the synthesis of proteins. The genetic code ensures the correspondence between the sequence of mRNA and the sequence of synthesized polypeptide. Ribosome uses the tRNA (transfer RNA) like “adapters” between the mRNA and the amino acids.

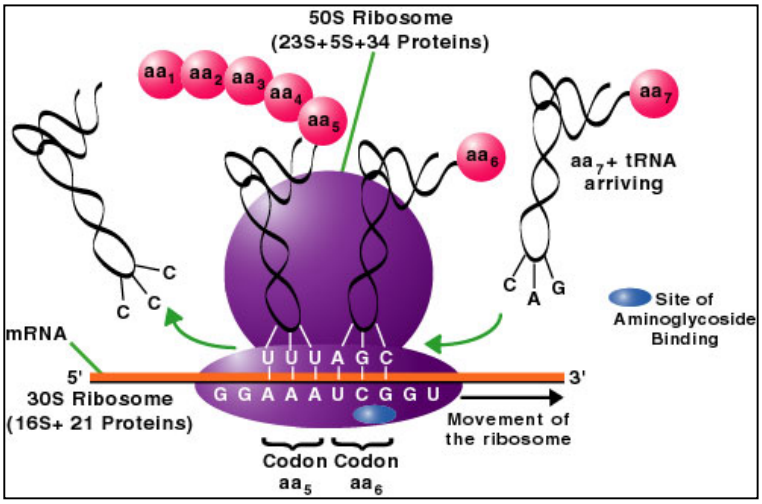

Figure IV. 4: Ribosome and protein translation

The mRNA passes through the small sub-unit which contains the sites of fixing of tRNA. The large sub-unit contains the catalytic part which synthesizes the peptide connection between the consecutive amino acids of protein. The large sub-unit also contains a tunnel by which the proteic chain leaves in the course of synthesis.

At each moment of life of a bacterium, up to 20'000 ribosomes synthesize the proteins necessary to the life. There is thus a very large quantity – up to 20'000 copies – of ribosomal RNA (rRNA) within one living bacterium (or cell). rRNA can represent up to 95% of all RNA present in a bacterium and up to 15% of the total weight of the bacterium.

The IAI PadoTest 4·5® chose to work with the rRNA gene “16S” of the small ribosomal sub-unit as molecular marker. The specific probes for each selected pathogenic bacteria chosen were determined on this gene. The very large quantity and especially the number of copies of rRNA present at every moment in a living bacterium offer several essential advantages for a microbiological test using them:

- Direct and true quantification without modification of the original genetic material neither in quantity, nor in proportion.
- Very specific and sensitive.
- No use of amplification (PCR, for Polymerase Chain Reaction) for the molecular marker. Thus no quantitative bias, no problem of contaminations or others potential artefacts due to amplification.
- Thus, there are no positive forgeries. The test doesn’t count dead bacteria that do not play any role in the infection.
- Measure only living bacteria at the time of the sampling (because RNA is degraded instantaneously when the bacterium dies contrary to the DNA). The buffer contained in the tubes sent for the PadoTest 4·5® fixed immediately and ad æternam the living bacteria and their RNA sampled. RNA is thus not degraded. Thus, there is no false positive (dead bacteria at the time of sampling are not counted).
- Robustness and simplicity of the test.
- Reduced cost.

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