EMMA FAQs

1. What is the test?
EMMA (Endometrial Microbiome Metagenomic Analysis) determines if the uterine microbial environment is optimal for embryo implantation. EMMA provides a complete view of the endometrial bacteria, including pathogens causing chronic endometritis that can be specifically investigated in ALICE.

2. What are the indications for EMMA?
The impact of the endometrial microbiome in patients with repeated implantation failure (RIF) has been demonstrated (Moreno et al. 2016). However, EMMA can be beneficial for any patient wishing to conceive, by assessing the microbiological environment that the embryo will encounter at implantation.

3. Which is the novelty provided by the test?
EMMA test analyses the bacterial composition in the uterine cavity providing a new microbiological view of the endometrial factor. Also, a single endometrial sample contains endometrial and bacterial cells, which that can be analyzed using deep sequencing to simultaneously predict endometrial receptivity (ERA test) and the endometrial microbiome (EMMA test). This evaluation (EndomeTRIO test) offers an extended view of the endometrium to improve clinical management of the patient.

4. What are the benefits of NGS microbiome vs. microbial culture?
Microbial culture is the current method for identification of bacteria and infection. However, it has been demonstrated that, depending on location, between 20% and 60% of bacteria cannot be cultured. Among them, *Mycoplasma* spp. and *Ureaplasma* spp., some of the most common pathogens of the reproductive tract, cannot be cultured by standard microbiological methods. Molecular assessment of the microbiome using NGS allows detection of culturable and non-culturable bacteria (Moreno et al., 2018).

5. How should the endometrial biopsy be collected?
Endometrial biopsies should be collected from the uterine cavity using Pipelle catheters from Cornier Devices (CCD Laboratories) or similar, under sterile conditions, either in a natural cycle or in an HRT cycle.

6. How should the patient be prepared for endometrial biopsy collection?
If the ERA test is to be performed together with EMMA, a single endometrial biopsy should be used, and the ERA protocol followed for sample collection (in case of first biopsy, P+5 for HRT or LH+7, hCG+7, Ovo+6 for natural cycle). If EMMA is requested alone, the endometrial biopsy should be obtained either following the ERA protocol, or between days 15 and 25 in a natural cycle.

7. Do any drugs affect or interfere with the EMMA analysis?
Yes – specifically, antibiotics – so they should not have been administered to the patient during the 3 months prior to sample collection. If the patient has taken any antibiotic during this timeframe, the name of the active substance, its dosage, mode of administration and duration must be recorded in the Test Requisition Form. This includes prophylactic antibiotics for egg retrieval. Other drug, such as those altering the patient's microbiota or immunological condition, could also affect results, so should also be recorded in the Test Requisition Form.

8. Endometrial biopsy collection

- **What type of pipelle should be used to take the endometrial biopsy?**
  - We recommend the Pipelle de Cornier.
- **Is it necessary to cut the tip and then expel the sample into the vial/solution provided?**
  - No, this is not necessary. Just leave the tissue inside the cryotube.
- **How should the endometrial biopsy be taken?**
  - Once the pipelle has been introduced into the endometrial cavity, repeatedly scratch downwards from the fundus in each endometrial wall. Aspiration should be maintained during scratching, to ensure that the tissue enters the pipelle.
- **Is it possible to do a biopsy for EMMA during a hysteroscopy?**
  - Yes, but we would recommend collecting the biopsy at the beginning of the procedure, before distending the uterine cavity.
- **What happens if the doctor does not have an EMMA kit?**
  - **First option:**
    - Igenomix Customer Support could contact a local clinic to borrow a kit.
  - **Second option:**
    - Perform the biopsy and place the sample in a sterile, DNAse- and RNAse- free, freezer-resistant, dry tube (no solution). Place this in the freezer immediately at –20°C. Once the clinic receives a kit, transfer the sample (without thawing it) into the Igenomix cryotube and refrigerate at 4°C for a minimum of 24 hours. This method is not highly reliable, and there is a strong chance that an 'Invalid sample' result will be obtained.
  - **Third option:**
    - Arrange for overnight delivery of a kit to the clinic and perform the biopsy the following day. Medication should be continued as usual, and it is important to note the exact progesterone administration time (only applicable if ERA is also being analysed in the same sample).
- **How to avoid excess blood or mucus in the biopsy sample?**
  - It is important not to introduce an excessive amount of blood or mucus into the cryotube, as this can prevent the stabilizing buffer from preserving the endometrial tissue sample. To avoid this situation, do not penetrate too deeply into the tissue when collecting the biopsy.
- **Can endocervical samples be analyzed?**
  - The EMMA test has been validated for endometrial tissue from the uterine cavity, so we cannot process endocervical samples.
- **Can a single sample be split between two different tubes?**
  - We recommend not taking too much tissue; the amount should not exceed the white line on the tube.
  - If the sample is too big, it can be split into two different cryotubes. We would analyze the first sample and, if 'Invalid', would analyze the second sample. It is important that there is a sufficient sample size in each cryotube and that the clinic indicates that this is how they want to proceed.
- **Can lidocaine be used when taking the biopsy?**
  - No.
- **Can Cytotec be used when taking the biopsy?**
  - No.
- **Can betadine be used when taking the biopsy?**
  - No.
- **How to proceed if the cervical canal is very narrow and difficult to access?**

- Use of a smaller catheter (i.e. an embryo transfer catheter with or without the guide inside) may be helpful. If the cervical canal is not accessible, a cervicohysteroscopy should be performed.
- For patients undergoing ART cycles, the cervical canal will need be accessible for final embryo transfer. Therefore, regular protocols should be used to prepare it in advance. A mock transfer should be done during the first appointment to ensure the accessibility of the endometrial cavity.
- Alternatively, it may be possible to relax the cervical os with a laminaria stick (although this procedure should never be performed in the transfer cycle).

- **Can EMMA be performed in an atrophic endometrium (an endometrium < 6 mm)?**
  - Yes.

9. EMMA Sample storage and shipment

- **At what temperature and for how long should the sample be stored?**
  - After retrieval, it is important that the sample is immediately placed in the refrigerator at 4–8°C for a minimum of 4 hours.
- **For how long can the sample be stored in a fridge?**
  - Once the sample has been stored for 4 hours at 4–8°C, it can then be stored at 4–8°C for up to 3 weeks.
- **For how long can the sample be stored in a freezer?**
  - Once the sample has been stored for 4 hours at 4–8°C, it can then be stored at –20°C for up to 1 year, however the microbiota can change along time so the storage of sample for a long period of time is not recommended.
- **For how long can the sample be stored at room temperature?**
  - If samples are delayed during shipping, keep in mind that they are typically stable at room temperature for 5-7 days. We always try to extract DNA and perform the analysis, but there is no guarantee that results will be valid.
- **Is it possible to analyze samples that have been preserved in a paraffin block?**
  - No.

10. What is the turnaround time for the test?

TAT is 15 days.

11. Will Igenomix release new EMMA reports?

A report will be released to provide information about EMMA (which will include pathogens reported in ALICE). Igenomix will release an additional ERA report if both tests are ordered.

12. What information is provided by the EMMA report?

The EMMA report will provide information about the overall microbial health of the uterine cavity, including:

- The percentage of *Lactobacillus*.
- The percentages of the bacteria detected in the endometrial sample in amount higher than 1%.
- Whether the endometrial microbiota is normal (high percentage of *Lactobacillus*), abnormal (significant presence of bacterial pathogens or dysbiotics), mild dysbiotic (low percentage of *Lactobacilli*) or ultralow biomass (the amount of the endometrial flora is extremely low).
- Suggested probiotic/antibiotic therapy. Recommendations for antibiotic therapy will be always guided by an expert clinical microbiologist, who will counsel the patient or the doctor on an individual basis.

- ALICE test results: because EMMA includes ALICE, the results of CE diagnosis and abundance of CE causing bacteria are also shown in the EMMA report.

An example EMMA report report it is shown in annex I.

13. If the microbiome is abnormal, do we recommend cancelling the embryo transfer? Do we recommend repeating the test?
The EMMA report will recommend personalized treatment for each particular case. Please refer to the decision tree (annex II).

14. What is the suggested clinical solution if the microbiota is abnormal?
Based on EMMA results, a list of recommended antibiotics and/or probiotics will be provided at the end of the report:

- Probiotics: vaginal suppositories containing *Lactobacillus* strains (*L. rhamnosus*, *L. crispatus*, *L. reuterii*, *L. plantarum*, etc.).
- Antibiotics: please see the 'Antibiotic therapy' document. In complex cases, the recommended therapy will be guided by microbiology counselling.

15. If the patient has previously had an ERA test, do we need to collect a new endometrial biopsy?
Yes, we recommend performing the EMMA test with a new sample, because the microbiome varies over time. There is, however, a risk of contamination if a previous sample is used, due to previous manipulation.

16. If a second sample is required, will it be collected under the same cycle/day (i.e. HRT, P+5)?
If only the EMMA test needs to be repeated, the sample can be collected between 15-25 days of the natural cycle, or in P+5 in an HRT cycle (or the recommended day for ERA). However, we recommend that samples are collected in similar conditions. If the ERA test also needs to be repeated, then the sample should be obtained following the recommendations of the ERA report. We anticipate that up to 40% of patients will require a second sample.

17. Is there any scientific evidence supporting this test?
There is a paper published by Igenomix in the American Journal of Obstetrics and Gynecology (Moreno et al, 2016), showing the impact of endometrial microbiota on ART success in a pilot study with 35 RIF patients. This paper was published as a Report of Major Impact.

18. Endometrial pathologies and EMMA

- **The patient has a uterine surgical intervention planned prior to the transfer. When should the EMMA test be performed?**
    - If any surgical interventions are necessary, they should be performed before sample collection, so that the endometrium is in a suitable condition for embryo transfer.
- **Does endometriosis affect the EMMA result?**
- Yes, some reports demonstrate a higher prevalence of microbiota alterations in patients with endometriosis, although further research is needed. This relationship is currently under investigation by our group. We recommend performing the EMMA test in these patients, and request that clinicians record endometriosis in the TRF.
- **If the patient has fluid in the endometrial lining, can the biopsy be taken?**
    - Yes. This fluid is most likely caused by an infection of the endometrium.
- **Could the biopsy be performed if there is no trilaminar appearance?**
    - Yes.

19. Are previous EMMA results still valid after D&C?

If a D&C takes place after an EMMA sample, the results could change. Therefore, we recommend repeating the EMMA test after D&C procedures, ideally in the cycle immediately before the embryo transfer.

20. Do chemotherapy/radiation affect the EMMA results?

We currently have no data about the effect of chemotherapy or radiation on the endometrial microbiota, but there may be an effect. Therefore, if a patient has undergone chemotherapy or radiation after the EMMA test, we would recommend that the endometrial biopsy is repeated once treatment is complete and patient is close to embryo transfer.

21. Non-endometrial pathologies and EMMA

- **Does chronic thyroiditis increase the risk of abnormal microbiota?**
    - We do not have any cases of this to our knowledge, so we are unaware of the effects of this disease over the endometrial microbiota.
- **Does polycystic ovary syndrome increase the risk of an abnormal microbiota?**
    - We do not currently have data on this.

References

Moreno I, Cicinelli E, Garcia-Grau I, Gonzalez-Monfort M, Bau D, Vilella F, De Ziegler D, Resta L, Valbuena D, Simon C. The diagnosis of chronic endometritis in infertile asymptomatic women: a comparative study of histology, microbial cultures, hysteroscopy, and molecular microbiology. Am J Obstet Gynecol 2018; doi: 10.1016/j.ajog.2018.02.012.

Moreno I, Codoñer FM, Vilella F, Valbuena D, Martinez-Blanch JF, Jimenez-Almazán J, Alonso R, Alamá P, Remohi J, Pellicer A, Ramon D, Simon C. Evidence that the endometrial microbiota has an effect on implantation success or failure. Am J Obstet Gynecol 2016; 215:684-703.

ANNEX I

ENDOMETRIAL MICROBIOME METAGENOMIC ANALYSIS (EMMA)

Patient information	Sample information	Clinic information
Unique pat id:	**Date received:**	**Clinic:**
Patient name:	**Report date/time:**	**Clinician:**
Patient DOB:	**Sample type:** Endometrial Biopsy	
Allergic to antibiotics	**Cycle type:**	
	No. Biopsy:	
	Date of biopsy:	

EMMA TEST RESULT MILD DYSBIOTIC MICROBIOME PROFILE

ALICE TEST RESULT NEGATIVE FOR BACTERIAL PATHOGENS CAUSING CHRONIC ENDOMETRITIS

EMMA

Most abundant bacteria	%
Lactobacillus	4,04% *
Flavobacterium	43,60%
Bacillus	8,72%
Propionibacterium	5,97%
Streptococcus	3,36%
Corynebacterium	3,16%
Gemmata	2,97%
Others	28,17%

** For reference intervals, please refer to Moreno et al., Am J Obstet Gynecol. 2016.*

ALICE

Chronic Endometritis pathogens	%
Enterobacteriaceae	Not Detected
Escherichia	Not Detected
Klebsiella	Not Detected
Chlamydia	Not Detected
Neisseria	Not Detected
Ureaplasma	Not Detected
Mycoplasma	Not Detected
Enterococcus	Not Detected
Staphylococcus	Not Detected
Streptococcus	3,36%

INTERPRETATION OF YOUR RESULT AND RECOMMENDATION

The percentage of Lactobacilli is below the standard described for endometrial health. However, the amount of pathogenic bacteria is not significant, so antibiotic therapy is not recommended.

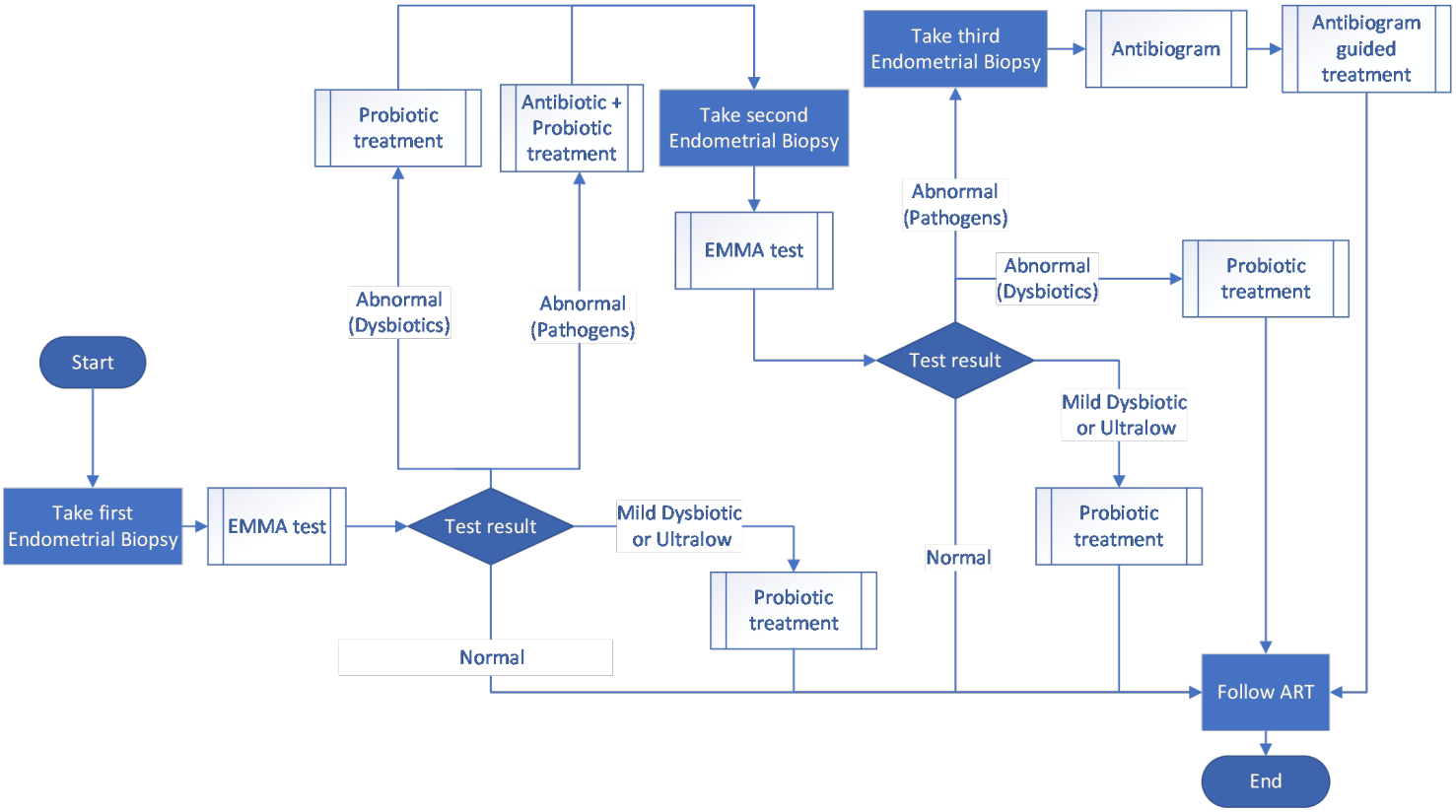
Probiotic treatment with vaginal Lactobacillus is recommended to increase the proportion of Lactobacilli in the endometrium. A second biopsy is not required.

For more information and individualized advice, we strongly recommend you consult our clinical microbiological counselor at microbiologycounselling@igenomix.com.

SUGGESTED THERAPY

Probiotic treatment with vaginal Lactobacillus is recommended. A list with recommended probiotics of vaginal administration is provided at the end of this report.

ANNEX II


Start
Take first Endometrial Biopsy
EMMA test
Test result
Abnormal (Dysbiotics)
Probiotic treatment
Abnormal (Pathogens)
Antibiotic + Probiotic treatment
Mild Dysbiotic or Ultralow
Probiotic treatment
Normal
Take second Endometrial Biopsy
EMMA test
Test result
Abnormal (Pathogens)
Take third Endometrial Biopsy
Antibiogram
Antibiogram guided treatment
Abnormal (Dysbiotics)
Probiotic treatment
Mild Dysbiotic or Ultralow
Probiotic treatment
Normal
Follow ART
End