**ALICE FAQs**

1. What is the test?
ALICE (Analysis of Infectious Chronic Endometritis) detects the most frequent bacteria that cause chronic endometritis (CE). This expands the service offered by Igenomix, by evaluating the endometrium at the microbiological level, with the aim of improving the clinical management of patients with this silent disease.

2. What is chronic endometritis?
Chronic endometritis is the persistent inflammation of the endometrium, mainly caused by bacterial infection. It affects approximately 30% of infertile women, but prevalence in patients with Repeated Implantation Failure (RIF) and Recurrent Pregnancy Loss (RPL) can reach 60%. This disease is asymptomatic and is not detectable by ultrasound during standard gynaecological checks. For this reason, it is often overlooked and, therefore, not treated. Diagnosis of CE is traditionally based on histology, hysteroscopy and/or microbial culture. However, these three methods provide inconclusive or misleading results in 80% of cases.

3. Which bacteria cause CE?
Bacteria that cause CE include: *Enterococcus* spp., *Enterobacteriaceae*, *Streptococcus* spp., *Staphylococcus* spp., *Mycoplasma* spp., and *Ureaplasma* spp. Other bacteria also include genital pathogens associated with sexually transmitted infections (STI), such as *Chlamydia* and *Neisseria*.

4. What are the indications for ALICE?
ALICE can be beneficial for any patient wishing to conceive, by assessing the microbiological environment that the embryo will encounter at implantation. ALICE could also be beneficial for patients with a history of RPL and/or RIF, because CE has been linked to these events.

5. What does the change imply for the methodology?
A single endometrial sample contains both endometrial and bacterial cells, which can be analyzed using deep sequencing to simultaneously predict endometrial receptivity and bacterial infection. This assessment provides an extended view of the endometrium, with the aim of improving clinical management of the patient.

6. What are the benefits of NGS pathogen detection vs. classical methods?
While histology usually underdiagnoses CE, hysteroscopy usually overdiagnoses the disease. Molecular microbiology presents equivalent results to the concordant results obtained by using combined histology, hysteroscopy and microbial culture (Moreno et al., 2016 and 2018).

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

9. How should the patient be prepared for EB?
If the ERA test to be performed together with ALICE, 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 ALICE is requested alone (without ERA), the EB should be obtained either following the ERA protocol, or between days 15 and 25 in a natural cycle.

10. Do any drugs affect or interfere with ALICE?
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, route of administration and duration must be recorded in the Test Requisition Form (TRF). This includes any prophylactic antibiotics for egg retrieval. Other drugs, such as those altering the patient’s microbiota or immunological condition, may also affect results, and should also be recorded on the TRF.

11. EB collection
- What type of pipelle should be used to take the endometrial biopsy?
  o We recommend the Pipelle de Cornier.
- Is it necessary to cut the tip and then expel the sample into the cryotube/solution provided?
  o No, this is not necessary. Simply expel the tissue inside the cryotube.
- How should the EB be taken?
  o 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 ALICE during a hysteroscopy?
  o Yes, but we would recommend collecting the biopsy at the beginning of the procedure, before distending the uterine cavity and without antibiotic treatment during or after the procedure.
- What happens if the doctor does not have an ALICE kit for sample collection?
  o First option:
    ▪ Igenomix Customer Support could contact a local clinic to borrow a kit.
  o 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.
  o 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 analyzed in the same sample).
- How can excess blood or mucus in the biopsy sample be avoided?
  o 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?**
  - ALICE has been validated for endometrial tissue from the endometrial 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 ALICE be performed in an atrophic endometrium (an endometrium < 6 mm)?**
  - Yes.

12. **ALICE sample storage and shipment**

- **At what temperature and for how long should the sample be stored?**
  - After biopsy, 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 over 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 will 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.
13. What is the turnaround time for the test?  
TAT is 15 days.

14. Will Igenomix release new reports for ALICE?  
A specific report will be released to provide information about ALICE.

15. What information is provided in the ALICE report:
   • Presence and abundance of specific bacteria that cause CE: Enterococcus spp., Enterobacteriaceae, Streptococcus spp., Staphylococcus spp., Mycoplasma spp., and Ureaplasma spp. Other genital pathogens associated with sexually transmitted infections, such as Chlamydia and Neisseria will also be reported.
   • For any pathogens detected, recommendations for antibiotic/antimicrobial therapy will be provided by an expert clinical microbiologist, who is available to counsel the doctor on an individual basis, as needed.

An example of ALICE report it is shown in annex I.

16. If CE is diagnosed, do we recommend canceling the embryo transfer? Do we recommend repeating the test?  
The ALICE report will recommend personalized treatment for each case. Please refer to the decision tree (annex II). Yes, we recommend postponing embryo transfer until CE is successfully treated. A second test is needed to confirm that treatment has been successful.

17. What clinical solution is recommended following a positive CE diagnosis?  
   • Antibiotics: the most appropriate antibiotic therapy is recommended in the ALICE report, according to the profile of pathogens found in the sample, including the type of antibiotic, dose and duration.
   • Probiotics: vaginal suppositories/capsules containing Lactobacillus strains (L. rhamnosus, L. crispatus, L. reuterii, L. plantarum, etc.) are recommended after antibiotic treatment. At the end of the ALICE report, a probiotics list is provided.

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

19. If a second sample is required, will it be collected under the same cycle/day (i.e. HRT, P+5)?  
If only the ALICE test needs to be repeated, the sample can be collected between 15 and 25 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, the sample should be obtained following the recommendations of the ERA report. We anticipate up to 40% cases will require a second sample.

20. Is there any scientific evidence supporting this test?  
Yes, a recent publication by Igenomix in the American Journal of Obstetrics and Gynecology (Moreno et al, 2018), demonstrates the relevance of an accurate diagnosis of CE. In this paper, molecular microbiology shows equivalent results to performing the three classical tests together in 65 patients. This paper has been selected as the Editor’s Choice and will appear on the cover page of the June 2018 issue of this journal.
21. Endometrial pathologies and ALICE
• The patient has a uterine surgical intervention planned prior to the transfer. When should the ALICE test be performed?
  o The ALICE test should be performed following any surgical interventions, so that the endometrium is in a suitable condition for embryo transfer.
• Does endometriosis affect ALICE results?
  o Yes. Some reports describe a higher prevalence of CE in patients with endometriosis. For this reason, we recommend performing ALICE in these patients, and request that clinicians record endometriosis in the TRF.
• If the patient has fluid in the endometrial lining, can the biopsy still be taken?
  o Yes. This fluid is most likely caused by an infection of the endometrium.
• Could the biopsy be performed if there is no trilaminar appearance?
  o Yes.

22. Are previous ALICE results still valid after D&C?
If a D&C takes place after an ALICE sample, the results could change. Therefore, we recommend repeating ALICE after D&C procedures, ideally in the cycle immediately before the embryo transfer.

23. Do chemotherapy/radiation affect ALICE 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 ALICE test, we recommend that endometrial biopsy is repeated once treatment is complete and the patient is close to embryo transfer.

24. Non-endometrial pathologies and ALICE
• Does chronic thyroiditis increase the risk of CE?
  o We do not know, but it is possible because of the immunological depletion that chronic thyroiditis can cause.
• Does polycystic ovary syndrome increase the risk of CE?
  o We currently have no information about the relationship between these two pathologies.

References
ANNEX I

ANALYSIS OF INFECTIOUS CHRONIC ENDOMETRITIS (ALICE)

<table>
<thead>
<tr>
<th>Patient information</th>
<th>Sample information</th>
<th>Clinic information</th>
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ALICE TEST RESULT

POSITIVE FOR BACTERIAL PATHOGENS CAUSING CHRONIC ENDOMETRITIS

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<th>Chronic Endometritis pathogens</th>
<th>%</th>
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<tbody>
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<tr>
<td>Esherichia</td>
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<tr>
<td>Klebsiella</td>
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<tr>
<td>Chlamydia</td>
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</tr>
<tr>
<td>Neisseria</td>
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</tr>
<tr>
<td>Ureaplasma</td>
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</tr>
<tr>
<td>Enterococcus</td>
<td>Not Detected</td>
</tr>
<tr>
<td>Staphylococcus</td>
<td>Not Detected</td>
</tr>
<tr>
<td>Streptococcus</td>
<td>13.66%</td>
</tr>
</tbody>
</table>

INTERPRETATION OF YOUR RESULT AND RECOMMENDATION

DNA from chronic endometritis causing bacteria has been detected in a significant amount in the endometrial sample.

Antibiotic therapy followed by probiotic treatment is recommended before continuing with ART. Please find below the suggested therapy based on the bacteria detected. We also recommend the analysis of a second sample after treatment, to confirm the restoration of a favourable environment for implantation.

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

SUGGESTED THERAPY

Amoxycillin / Clavulanic acid 500mg/125mg/8h for 7 days followed by probiotic treatment 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 → ALICE test → Test result

- Positive: Antibiotic + Probiotic treatment → Take second Endometrial Biopsy → ALICE test → Test result
  - Positive: Take third Endometrial Biopsy
  - Negative: Antibiogram → Antibiogram guided treatment

- Negative: Follow ART → End