Metagenex

Bringing Rare Cells In to Focus

VALIDATION OF AN INNOVATIVE APPROACH FOR NON-INVASIVE PRENATAL DIAGNOSIS OF TRISOMY 21

Scientific Project Presentation

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TABLE OF CONTENTS

1 I- SCIENTIFIC AND TECHNOLOGICAL ASPECTS 3
1.1 I.1. Aims of the project and expected results 3
1.2 I.2. State of the art and Innovative aspects 6
1.3 I.3. Project Flow 8
1.3.1 Stage 1 (months 1 to 18) 8
1.3.2 Stage 2 (months 18 to 24) 12
1.3.3 Stage 3 (months 25 to 36) 13
1.4 I.4. Consortium Description 14
1.5 I.5. Publications linked to the project 15
1.6 I.6. Bioethical aspects 16
2 II- ECONOMICAL ASPECTS .....
2.1 II.1. Qualitative and quantitative assessment of the potential market 16
2.2 II. 2. State of the competition, benchmarking and competitive advantages 17
2.3 II.3. Industrial Innovation Strategy 18
2.4 II. 4. Expected benefits 18
3 III- INTELLECTUAL PROPERTY .....
3.1 III.1. State of the Intellectual property 19
3.2 III.2 Agreements between partners 19
4 IV- REFERENCES .....

1 I- SCIENTIFIC AND TECHNOLOGICAL ASPECTS

1.1 I.1. Aims of the project and expected results

The aim of our project is the validation of an innovative and non-invasive (without any risk of miscarriage) method for early detection of trisomy 21, based on the enrichment and genetic analysis of fetal cells circulating (CFC) in maternal blood. This is a fundamental step in order to develop commercially available kits for medical genetics laboratories.
The proposed work is expected to open the way for the first time to the implementation of a completely reliable genetic screening of trisomy 21 through the analysis of a blood sample. This is a goal which has never been achieved up to now and which will represent a milestone in modern molecular medicine.
The major **social, medical and economical benefits** of the expected results are described below:
The prenatal detection of chromosomal abnormalities, and notably of trisomy 21, is a major public health priority. Indeed, early information to all future mothers concerning pre-natal tests is obligatory, and more than 80% of them accept one type of screening or another. For example, in the Ile de France region (around Paris), the multiplicity of screening tests has led to a major public health problem, with amniocentesis being performed in more than 15% of all pregnant women. This problem is both an economic one for society and a medical and family problem for women, because **one in a hundred normal foetuses will die before birth as a result of amniocentesis.**

The test we propose would provide an alternative to conventional invasive techniques (chorionic villus sampling (CVS) or amniocenthesis). When compared with invasive techniques, **the enormous potential advantages of early-stage, non-invasive screening** are clear:

- abolition of the psychological and physical trauma linked to the iatrogenic loss of a child (miscarriage induced by invasive procedures)
- drastic reduction in therapeutic costs (through the reduction, or abolition, of the number of serum marker tests, amniocenthesis or CVS and karyotypings performed and the number of miscarriages induced)
- reduction in the psychological and physical trauma related to delayed therapeutic abortion, reduction in hospitalisation delays (because of simpler therapeutic abortion techniques).

Unlike other groups which have tried to isolate CFC using immunolabelling and immunomagnetic techniques (which are not entirely specific and damage cell morphology, causing a loss of sensitivity), we isolate fetal epithelial cells on the basis of their size, because they are larger than leukocyte cells. This has enabled us to develop an innovative technique, called **ISET (Isolation by Size of Epithelial Tumor/Trophoblastic cells) (Vona et al., 2000)**. Our company, **Metagenex,** (www.metagenex.fr) has allowed the development of the ISET machine, and was set up to develop innovative tests employing the ISET technology.

Preliminary results already obtained

The ISET method implies that 10 ml of blood are diluted with a special buffer and loaded into 10 wells of a filter block which is sealed on a polycarbonate filter with 8 micron calibrated pores. After filtration, performed by the ISET device, cells are identified on each of the 10 spots of each filter, and can then be counted (N° per ml) and characterised. We have shown that the ISET method allows the isolation of rare CFC (one or more per ml), visualisation of their morphology and their characterisation by immunohistochemistry and FISH (Fluorescence In Situ Hybridisation). CFC can also be microdissected using a laser microscope, and their DNA can be amplified for the detection of deletions or mutations. We have demonstrated that the **ISET technology allows the isolation and genetic characterization of trophoblastic fetal cells**, and have developed and patented a genotyping strategy (using STR markers) for isolated cells to verify their fetal nature (Vona et al., 2002). We have also shown that it is possible to target genetic analyses solely to individual cells whose fetal nature has been demonstrated by genetic genotyping. Twelve women at risk of bearing children with **Spinal Muscular Atrophy (SMA)** were analysed using ISET. This study was performed on only 4 ml of maternal blood. After the genotyping of paternal and maternal blood samples to identify informative primers, the cells isolated by ISET were microdissected and allelotyped, which made it possible to detect a total of 35 fetal cells. Detection of the specific mutation for spinal muscular atrophy (homozygous deletion of the SMN1 gene), performed blindly and in parallel with the invasive method (biopsy of chorionic villi followed by genetic analysis of the fetal cells thus harvested), made it possible to identify the three mothers carrying an affected foetus (Beroud et al., 2003).
Our team has also recently developed the ISET method for the prenatal diagnosis of **Cystic Fibrosis**. The technical details of this method are protected by patent. It was applied to 12 mothers with a risk of bearing an infant with cystic fibrosis. The analysis of just 4 ml of maternal blood and at least 4 to 7 fetal cells per blood sample collected during the 11th or 12th weeks of pregnancy prior to the biopsy of chorionic villi, made it possible to identify (during a study performed entirely under blinded conditions) one mother with an affected foetus, five mothers with foetuses bearing the F508del allele and 6 mothers with completely healthy foetuses (Annex 1, Saker A et al, submitted confidential, see hard copy of this project). Of these 12 mothers, ten came from couples where both parents carried the F508del allele (Annex 1). In one couple, the father carried the F508del allele and the mother an unknown mutation, while in another, both parents carried an unknown mutation. In both cases, however, the Medical Genetics Laboratory held DNA from an affected child of the same family (index cases), which made it possible to locate the mutated alleles and perform a non-invasive prenatal diagnosis using the indirect method (segregation analysis of polymorphic markers situated on chromosome 7).
These results demonstrate the feasibility of applying the ISET method to the non-invasive prenatal diagnosis of cystic fibrosis and, as for spinal muscular atrophy, have led to set up a clinical validation protocol.

Our work has demonstrated that ISET isolates CFC in all the mothers tested up to now (n°=46) using only 3-4 ml of blood.

Preliminary results concerning non-invasive prenatal diagnosis of Trisomy 21

We have shown for the first time that a reliable prenatal diagnosis of SMA and Cystic Fibrosis can be obtained by genetic analysis of CFC. In these cases, the genetic abnormality is a single nucleotide deletion (indicating the loss of the SMN1 gene) for SMA, or a three nucleotide deletion (F508del) for Cystic Fibrosis. In order to develop a non-invasive prenatal diagnosis approach of trisomy 21, the challenge is detection of a quantitative difference (3 from 2 chromosomes 21) at the single cell level, which means using a very low amount of DNA (2 pg).
We have used the following experimental procedures:

- FISH analysis of cells isolated by ISET (non-microdissected),
- Quantitative PCR on laser microdissected cells
- Comparative Genomic Hybridization (CGH) analyses on laser microdissected cells

FISH (fluorescence in situ hybridisation)

FISH analysis of fetal cells concentrated by ISET is undoubtedly a rapid and potentially efficient approach. However, even if the enrichment capability of ISET is very high, a small number of fetal cells are found per spot (from 1 o 6). Since antibodies specifically identifying trophoblastic cells are missing up to now, FISH does not allow to know how many fetal cells are tested (= give a FISH signal), unless a probe specific to chromosome Y is added to the specific probe to chromosome 21. In this case, however, only male fetal cells can be identified. Thus, **if no cells with three chromosomes 21 are identified and the fetus is female, there is no way to know by FISH only if the fetus is really not affected by Trisomy 21.** This implies that if the FISH analysis is negative, **it is necessary to complete the diagnosis by cell microdissection** and genetic characterization. Our preliminary results have shown that the FISH method is applicable to cells isolated using ISET (Vona et al. 2000).

ISET-CGH method.

Our team has developed a method, based on CGH, to be applied to individual cells isolated by ISET. The details of this method are under analysis to be protected by patent.

The method has shown the ability to correctly identify 20 normal cells, isolated by ISET, tested individually (Annex 2, Figure 1). It has then been applied to 10 tumor cells isolated by ISET ISET (HuH7) (Annex 2, Figure 2), with a parallel analysis by CGH of the DNA extracted by the same cell line. These tests have shown the capability of the technique to identify the loss and gain of genetic subchromosomal material. **The ISET CGH method has then been applied to 10 fetal cells isolated by ISET from maternal blood obtained from women pregnant of fetuses with trisomy 21 and has consistently shown the presence of trisomy 21.** The ISET CGH method is very sophisticated, as it allows to perform 50 PCR analyses starting from the DNA of a single cell. It allows to genotype the microdissected cell and to look for the genetic abnormality (trisomy 21) by CGH in genetically identified fetal cells. A critical point of this method is that the reading phase of the test is done on normal metaphases. This part of the test is extremely tedious, slow and delicate. Thus **we have set up a collaboration with the team of Professor Nigel Carter (Cambridge UK) who runs the larger CGHarray platform in Europe.** In fact, reading of GCH analyses by CGH array is much more rapid and susceptible to be automatised.

The Laboratoire de Cytogénétique of the Hôpital Necker (Dr Serge Romana) develops CGH arrays dedicated to the Non Invasive Prenatal Diagnosis of Trisomy 21 on fetal cells microdissected after enrichment by ISET. These CGH arrays will allow a more rapid and specific reading of the ISET-CGH analyses.

ISET-quantitative PCR method

We have developed two different tests of quantitative PCR for NI-PND of Trisomy 21 : a) analysis of STR markers on chromosome 21, b) analysis of genes on chromosome 21.

a) STR markers are amplified by nested PCR. Samples are then analysed by Genotyper (Applied Biosystems) and Genotyper® version 3.7 software. The allele analysis allows to detect the trisomy 21 by a diallelic signal (ratio 2 :1) or a tri-allelic signal (identical signal intensities).

b) We use unique genes on chromosome 21: and Amyloid (Hu Y and al, Prenatal Diagnosis, 2004 ; Zimmermann B et al, Clinical Chemistry, 2002) genes are amplified by nested Real Time quantitative PCR.

The Real Time quantitative PCR is performed by using SYBR Green PCR Master Mix (Applied Biosystems) and the ABI PRISM 7000 (Applied Biosystems). The DNA is quantified

in real time during the PCR by the fluorescence of the probe integrated in the PCR product. Serial dilutions of the DNA from each PCR (1, 10, 100 ng) are used to build standard courbes for the genes DSCR3, Amyloid and GAPDH. Thus, the number of the cycles in the quantitative PCR can be converted to the amount of DNA (ng). The evaluation of the amount of DSCR3 and Amyloid genes is obtained by the calculation of the ratio DSCR3/GAPDH or Amyloide/GAPDH at a single cell level. The application of these two methods to 10 normal lymphocytes and to 10 lymphoïd cells derived from trisomic children has shown a trisomy 21 in the test cells but not in the normal control cells.

In summary:

Our preliminary results show that the development of a reliable test for the **non-invasive prenatal diagnosis of Trisomy 21 is feasible.**

The advantages of the ISET approach are the following:

- **ISET targets epithelial fetal cells, which do not persist in the maternal blood after delivery**, so that diagnosis can be specific to the current pregnancy.
- The detection of a genetic abnormality is performed on **fetal genomes which are not mixed with maternal DNA** (which might mask mutations in the fetal genome).
- T**he genetic diagnosis is repeated on each fetal cell identified,** which thus makes it possible to confirm the results several times.

These research results have opened new perspectives for the early-stage, non-invasive prenatal diagnosis of Trisomy 21, and they **need to be validated on a large scale**.

1.2 I.2. State of the art and Innovative aspects

First fully described in 1958 by Professor Jérôme Lejeune, trisomy 21, or Down's syndrome, **is the most common chromosomal abnormality.** Associated with dysmorphism, psychomotor retardation and a variety of malformations, **it is the leading cause of mental handicap worldwide.** There are 50,000 people with Down's syndrome in France, 400,000 in Europe and 8 million throughout the world (incidence of 1 per 700 newborns). Three types of Down's syndrome can be distinguished:

- Regular trisomy 21: which affects around 95% of all those with trisomy 21. It is characterised by an extra chromosome on the 21st pair. This error in chromosomal distribution occurs during the first cell division (meiosis).
- Mosaic trisomy: which affects approximately 2% of people with Down's syndrome. In this case, the distribution error occurs during the second cell division, which means that only some cells are affected by the presence of a third chromosome.
- Translocation trisomy, which affects 3% of children with trisomy 21. This type of trisomy is more complex, as part of chromosome 21 breaks off and attaches itself to another chromosome, usually chromosome 14.

The prevalence of infantile Down's syndrome increases with the mother's age: 1 in about 2000 at 20 years, 1 in 200 at 38 years and 1 in 16 at 48 years. Until recently, the only strategy for the detection of trisomy 21 was to offer amniocentesis for karyotyping to all women selected on the sole risk criterion of their age (38 years or over) at the time of conception. However, this screening strategy is not wholly reliable, **because the largest proportion (70%) of infants with trisomy 21 are now born to mothers below the age of 35 years.** For this reason, since the early 1990s, other screening strategies have been proposed, and particularly screening using biochemical markers (hCG, AFP and oestriol) in maternal serum during the second trimester of pregnancy (offered since 1996 in France), which is then combined with an ultrasound scan to determine nuchal translucency during the first trimester of pregnancy. These significant advances in the screening of populations at risk from trisomy 21 have had two negative effects: firstly, an excessively high rate of

amniocenteses (and thus of karyotyping), and secondly, increased maternal morbidity linked to the late stage of therapeutic abortions.

In the Ile de France region, screening for trisomy 21 generates karyotyping in 16% of pregnant women (data provided by the health insurance agency, CPAM 78). In addition, throughout France, maternal serum screening alone during the second trimester of pregnancy, which has a sensitivity of approximately 70% for a false-positive rate of 6%, has a positive predictive value of 1/120, i.e. 120 amniocenteses are performed to diagnose one foetus with trisomy 21. If we consider that the risk of miscarriage linked to amniocentesis is 1%, **we need to realise that in order to diagnose one pregnancy involving trisomy 21, a normal pregnancy will have been discontinued.**

A pilot study was set up in 2001 by Drs. Patrick Rozenberg and Laurence Bussières in the Yvelines **(Echo PAPP-A.78),** where so far, more than 20,000 pregnancies have been screened at an early stage by the use of ultrasound markers and new biochemical markers (Pregnancy-Associated Plasma Protein A and free fraction of beta-hCG), which provide a single and global evaluation of the risk. This method exhibits a high degree of sensitivity (approximately 85%), generates karyotyping in approximately 5% of cases and makes it possible to carry out a trophoblast biopsy to determine the fetal karyotype as from the 11th week of amenorrhoea.

At present, the strategy adopted to detect genetic handicap in unborn children involves the study of fetal cells obtained by amniocentesis or choriocentesis, two methods which are associated with a not inconsiderable risk of miscarriage (up to 1-2%). An alternative would consist in identifying and isolating the rare fetal cells which circulate in maternal blood. Indeed, these cells provide a source of fetal DNA which could undergo genetic analysis. However, the number of circulating fetal cells (CFC) is very small, approximately one or two cells per ml, i.e. one or two cells mixed with around 10 million leukocyte cells and 5 billion erythrocytes. Among the fetal cells which cross the placental barrier, four types have been identified and studied: **myeloid and lymphoid progenitors, erythroblasts and trophoblastic cells (**cytotrophoblasts and syncytiotrophoblasts). With respect to myeloid and lymphoid progenitors (CD34 and CD38 positive cells), it has been shown that they may **persist in maternal blood for up to 27 years after a pregnancy** or miscarriage (Bianchi et al., 1996b). For this reason, their isolation is of no value for the prenatal diagnosis of a current pregnancy. However, this problem does not affect erythroblasts and trophoblastic cells.

In view of the rarity of fetal cells in maternal peripheral blood, the first problem encountered when considering development of a non-invasive prenatal diagnostic test (NI-PDT) concerns the isolation of these cells. Most enrichment protocols are based on the use of antibodies directed against the membrane antigens of fetal cells. There is no true consensus as to the most efficient cell enrichment technique, so that several evaluation programmes are currently under way (Bianchi et al., 1996a). Work is also being carried out on the development of new, more specific cellular markers (Huber et al., 1996; Jackson, 2003; Koumantaki et al., 2001; Valerio et al., 1997; Wachtel et al., 2001). Nevertheless, it appears that these protocols, which include several isolation steps, may damage the fetal cells and cause significant cell losses. Micromanipulation/microdissection techniques have been proposed for the genetic analysis of fetal cells. The usefulness of these approaches [the feasibility of which has been demonstrated by the diagnosis of sickle cell anaemia and beta-thalassemia (Cheung et al., 1996)] is in fact limited in terms of their routine application, because of the difficulty in concentrating circulating fetal cells. Isolation of these cells by FACS (Fluorescence Activated Cell Sorter) and/or MACS (Magnetic Activated Cell Sorter) was the subject of a multicentre programme, funded in the past by the US National Institutes of Health. The results of this study, carried out on a total of 2744 blood samples over a period of five years, demonstrated

that these methods were not useful for the isolation of circulating fetal cells (Bianchi et al., 2002) because the specificity achieved (41.4%) was no higher than that of hormone tests performed on maternal serum. Thus, for reasons of cost and complexity, none of these approaches is today applicable on a large scale.
The prenatal detection by FISH of fetal cells with trisomy 21 in maternal plasma was recently described. This approach is interesting, but fetal cells are a rarity in plasma (1 in 500 to 1 in 2000), and are mainly apoptotic (Kolialexi et al., 2004). Use of this technique is therefore limited by its sensitivity and by certain technical aspects (cell permeability). Recently, Krabchi et al. (Krabchi et al., 2006) have performed a FISH/PRINS analysis identifying chromosomes 21 and Y in whole nucleated cells obtained from blood samples of mothers carrying a male fetus with trisomy 21. They found a number of fetal cells varying from 6 to 32 per ml. However, the method is not applicable in a routine manner. In fact they had to screen 66 slides in order to test cells from 3 ml of blood. Another limitation is that this method cannot distinguish fetal cells derived from previous pregnancies from fetal cells corresponding to the ongoing pregnancy. A percentage of fetal DNA (approximately 3.4% of maternal DNA (Lo et al., 2000) is also present in maternal plasma and accessible to genetic screening. Although this fraction is of use to detect the sex of the fœtus, the mixture of fetal and maternal DNA prevents the clinical application of detection of punctual mutations and chromosomal aneuplidies (trisomy 21) in fetal DNA.

Fetal cells can be collected by transcervical sampling (studies performed before termination of pregnancy), but they are quite rare and it is not known at present if this procedure is really non invasive or if it may trigger a miscarriage (Cioni et al., 2005).

1.3 I.3. Project Flow

During the period of this project (3 years), the aim is to successively carry out the technical and clinical validation of the ISET test targeting trisomy 21.

1.3.1 Stage 1 (months 1 to 18)

Technical optimizations

A- Analysis of the possibility to store blood for more than 5 hours before treatment by ISET (Months 1 to 6) (Milestone 1)
Studies already performed by Metagenex have shown that ISET technology allows isolation of CFC (circulating fetal cells) in all the mothers tested to date (N°=53). The method obtains between 1 and 3 CFC (or more) per ml of blood. This very high degree of sensitivity (15-30 CFC per 10 blood sample) is unparalleled up to now and related to the fact that the blood is treated by ISET within 5 hours after collection. A study protocol testing different cell protective agents (the components cannot be revealed here) will be carried out to test the possibility to store blood for more than 5 hours without loosing sensitivity. However, due to the high fragility of CFC (Krabchi et al., 2006), it is difficult to predict the possibility to obtain this goal.

B- Optimisation of the identification and microdissection of circulating fetal cells (Months 1 to 12) (Milestone 2)
We aim to carry out additional developments to render the ISET technique more rapid and applicable reproducibly to routine use. In particular, the step of fetal cells identification on filter and laser microdissection can be speeded up by technical improvements. In this setting, we will test anti-trophoblastic antibodies obtained in collaboration with members of the SAFE European consortium (workpackage 1a) and

anti-CK7 antibodies, described as potentially specific to trophoblastic cells (ref). A collaboration with the Nikon company, which commercializes the laser microscope we use, is planned in order to develop an automated microdissection of single cells based on their morphology, size and immunolabelling.

C- Evaluation of the possibility to perform a very early (6th- 8th WA) non-invasive prenatal diagnosis. (Months 1 to 10) (Milestone 3)

Tests will be performed to confirm our preliminary results which show that non-invasive prenatal diagnosis is feasible at an earlier stage of pregnancy than weeks 10 or 11 of amenorrhoea (WA). Indeed, we are currently collaborating with the team led by Professor Frydman at Hôpital Antoine Béclère, which has allowed us to test blood from mothers after in vitro fertilisation, on a weekly basis as from the 4th week of amenorrhoea. Preliminary results obtained in eight mothers, by genotyping cells identified in the maternal blood, have shown that fetal cells start to circulate at an early stage. We have planned to confirm this result in pregnant women followed by the Obstetricians (Dr A. Benachi) at the Maternity Department of the Necker –EM Hospital. Thirty pregnant women (normal pregnancies) giving informed consent to this study will be tested between the 6th and 8th WA. The number of CFC per ml will be recorded by analyzing 10 ml of blood per mother. Since it has been reported that mothers carrying a trisomic fetus have at least 5 times more CFCs than mothers with normal fetuses (Krabchi et al., 2006), the detection of at least one CFC per ml at the 6th and 8th WA will ensure the possibility to develop a very early non-invasive prenatal diagnosis of Trisomy 21.

D- Evaluation of the possibility to perform a non-invasive prenatal diagnosis on fetal cells isolated from cervical samplings (Months 1 to 8) (Milestone 4)

Our recent unpublished results have shown that it is possible to filter cells obtained by cervical samplings. We have looked for fetal cells (only trophoblastic cells can be found in these samples) and found that a small but significant number of trophoblastic cells can be found by the single cell genetic analysis (STR genotyping) we currently apply to identify CFC. We have optimized the protocol to obtain and identify trophoblastic cells in cervical samples. This approach is interesting as it completely avoids the risk of analyzing CFC derived from previous pregnancies, thus ensuring that genetic analyses are targeted to fetal cells from the ongoing pregnancy. We have planned to apply the development protocols described in step B (identification by antibodies and automated microdissection) and step C (search for fetal cells in cervical samples at the 6th and 8th WA) of this section to further improve this interesting approach.

E- Technical optimization of tests for non-invasive prenatal diagnosis (NI-PND) of trisomy 21 (months 1 to 18) (Milestone 5)

Our preliminary results have shown that three methodological approaches are feasible, each one of them having some **advantages and disadvantages.** We have planned to carry out the **technical validation of these three methodological approaches**. We will use cultured fibroblasts from patients with trisomy 21 and trisomy 18, cultured lymphoid cells from patients with trisomy 21 and cells from cord blood of newborns with trisomy 21, all filtered by ISET.

1) FISH analysis of cells isolated using ISET:

This approach does not require laser microdissection of cells enriched by ISET. It is therefore much more rapid, cheaper and less time consuming. However, Fluorescence In Situ Hybridization (FISH) does not allow to know how many fetal cells have been probed if the fetus is female (not recognizable by Y-specific probes) and requires further developments to obtain that the chromosome 21 and Y specific probes enter the highest number of cells on the filter, potentially 100%, in order to avoid loosing information from the rare cells we are looking for. We have planned to optimize the FISH approach as it will allow a rapid pre-study of the filtered cells. However, both in case of positive signal (cells with 3 chromosomes 21) and negative signals (absence of cells with 3 chromosomes 21 and of cells with Y chromosome), the microdissection will be performed to confirm the diagnosis. Only in case that a relevant number of cells showing the Y signal and 2 chromosomes 21 are clearly detected, we will evaluate the possibility to avoid confirmation by laser microdissection. In a routine screening setting, this strategy should allow to reduce the costs of the non-invasive test.

Our previous studies have shown that the FISH method was applicable to cells isolated using ISET (Vona et al 2000, Vona et al. 2002). However, the different probes have different sensitivities. In collaboration with Dr. Romana and Prof. Vekemans (Cytogenetics Laboratory, Hôpital Necker-Enfants Malades), we are currently producing specific probes for chromosome 21, and developing experimental conditions which should allow us to obtain a FISH signal (>90%) in the majority of cells isolated using ISET. Since the entry of the probe into the vast majority of cells is a critical parameter for FISH, we have also planned to apply the technique Primed in Situ labelling (**PRINS**) to cells isolated by ISET (Koch et al., 1989; Pellestor et al., 1995). PRINS is a highly sensitive approach which implies the in situ hybridization of a specific non labelled primer, followed by in situ elongation with fluorescent nucleotides. Since the size of the primer is smaller than the size of probes used in the FISH approach, a higher number of cells will be labelled by PRINS than by FISH. However, DNA sequences targeted by PRINS have to contain repetitive DNA stretches (centromeric sequences, Alu sequences etc…) in order to obtain a detectable signal. Another way that we will explore to label the majority of cells on the filter and obtain strong signal is the HISOMA technique (in Situ Hybridization of labelled oligonucleotides), which is a variant of PRINS. In order to speed up the optimization of these approaches and their application to cells isolated by ISET, we have planned to discuss a collaborative project with Dr F Pellestor (CNRS, Montpellier) and R Drouin (Canada) who have an extensive experience of PRINS

The molecular techniques performed after single cell microdissection (CGH and quantitative PCR), imply the amplification of single cell genomes. Amplification of the genome of single cells is already in use for preimplantation diagnosis. However, in this setting the fetal origin of the cell is known and the cell is fresh, while **in the case of cells obtained by ISET, the cells are fixed (by the blood dilution buffer).** The sensitivity of PCR is approximately 50 to 100 times less on fixed than on fresh DNA (B. Rocha, personal communication). Nevertheless, our molecular approach allows to consistently amplify single cell DNA from fixed cells (Vona et al, 2000; Vona et al, 2002, Beroud et al, 2003, Vona et al, 2004). Amplification of single cell DNA may imply the risk of **allele drop-out (ADO**, random amplification of the sequence from only one of the two alleles) (Findlay et al., 1998; Gigarel et al., 2004; Hahn et al., 1998; Piyamongkol et al., 2003). **In our laboratory, we have developed a strategy avoiding ADO.** In fact, it has been published that pooling the DNA from 3 fresh single cells practically eliminates the risk of ADO (Piyamongkol et al., 2003). We have therefore developed a method which implies optimized single cell STR genotyping. At

this step, even if ADO occurs (one allele only is detectable), it does not carry the risk of making a wrong diagnosis. The single cell DNA is genotyped with another pair of informative STR primers and, if ADO persists, the cell is discarded. We then **pool the preamplified single cell DNA from 3 STR genotyped single cells proved to be fetal.** In this way, we have shown that occurrence of ADO is practically eliminated, which allows to perform a **reliable NI-PND of genetic diseases** (Saker et al, submitted).
Development of a reliable test for diagnosis of trisomy 21 on CFC implies the possibility to **amplify the single cell DNA in a strict proportional manner.** This will ensure that the relative proportion of DNA copies in the original DNA will remain the same after amplification, thus allowing to identify a trisomic chromosome. We have already performed extensive analyses to compare pre-amplification methods (DOP (degenerate oligonucleotide primed PCR), PEP (primer extension preamplification) techniques and others, looking for the method ensuring a highly proportional amplification. Indeed, **the efficiency of this step is also fundamental to prevent ADO**. These tests have allowed to develop a **specific approach, including cutting and ligation of single cell DNA (CL method),** which allows a highly proportional amplification (we are filing the patent) of the fixed single cell DNA. The CL method has been used to perform the single cell CGH analyses reported below.

2) CGH analyses (microdissection of cells)
This method has the advantage to be focused to single cells proven to be fetal by STR genotyping and to explore the gain and loss of chromosomal material on the whole genome of single fetal cells. It will allow to detect other aneuploidies than trisomy 21 (trisomy 18, etc.…) and to open the way to new studies being able to find a relationship between some pathological findings and genetic abnormalities. However, for the strict purpose of performing a non-invasive prenatal diagnosis of trisomy 21, this approach is presumably too expensive to be able to be applied in a routine and large scale manner. It seems to us important anyway to technically validate this method as it is presumably the most informative analysis that can be developed for non-invasive prenatal diagnosis of aneuploid genetic disorders.

CGH is a highly specific, molecular cytogenetic approach which allows the positional identification of DNA sequence gains and losses at the level of the entire genome. CGH is based on the competitive hybridisation of test DNA and normal DNA, both labelled with different fluorochromes, on normal mitoses. The ratio of fluorescence intensities generated by the two fluorochromes enables the differentiation of chromosomal regions with a normal DNA content, and regions characterised by an increase or decrease in DNA. The spatial resolution of CGH is approximately 10 megabases, so it is therefore an elective approach for the detection of aneuploidy, including trisomy 21. In order to apply CGH to single cells, it is necessary for the DNA from the cell to be preamplified in a proportional manner (see above) and then to label the following PCR products with fluoresceinated (for the reference normal cell) or rhodaminated (for the fetal cell) (dUTP) nucleotides. Fluoresceinated and rhodaminated PCR products then undergo hybridisation, and the mixture serves to hybridise normal metaphases. Reading of the results by image analysis is carried out in collaboration with the Cytogenetics Laboratory at Hôpital Necker, Paris (Dr. Romana, Prof Vekemans).
We are filing the whole technique we have developed for application of CGH to fixed single cells isolated by ISET. Our preliminary results have suggested that the development of a reliable, non-invasive prenatal test to detect trisomy 21, is feasible.

The method has been developed on fibroblast cells with trisomy 18 and 21, isolated using ISET. It has demonstrated its ability to identify correctly the normal cells and aneuploid cells. It has been applied to tumour cells isolated by ISET (HuH7), and in parallel to tumoral DNA from the same line. This test has shown that the method is able to detect losses and gains of subchromosomal genomic material. **It has also been applied successfully to umbilical cord fetal cells from a foetus with trisomy 21.** Thie new method enables 50 PCR analyses on the DNA from a single cell. One critical point of the new approach is that final step of metaphase labelling is extremely slow, laborious, which makes difficult to obtain reproducible results. This problem is not related solely to the single cell CGH, but also to the CGH applied to large amounts of DNA. **CGH array**, a quite expensive chromosomal DNA screening of CGH products performed on chips, allows to bypass this problem. We have then set up a collaborative study with the team led by **Prof. Nigel Carter (Cambridge, UK),** who runs the largest European platform for CGH array**.** This collaboration is aimed to develop the CGH array reading (**pangenomic arrays**, with spotted cloned DNA from all human chromosomes) of CGH products derived from CFC isolated by ISET. A collaborative study is also ongoing with the **Cytogenetics Laboratory at Hôpital Necker in Paris (Dr. Romana, Prof Vekemans),** who develops a **CGH array specifically dedicated to trisomy 21 diagnosis** (chips with spotted cloned DNA from chromosome 21, 18, X, Y and two reference chromosomes) in order to obtain a cheaper and more focused CGH test allowing non-invasive PND of trisomy by the analysis of CFC isolated by ISET.

3) Quantitative PCR (microdissection of cells)
This method has the advantage to be focused to single cells proven to be fetal by STR genotyping and to allow the quantitative analysis of unique sequences on chromosome 21. The technically validated method is predictably **less expensive than the CGH approach, since it avoids the use of CGH arrays**. For this reason its technical and clinical validation is a priority of this project

Quantitative PCR (see Preliminary results, paragraph I.1.)
We use two distinct quantitative PCR procedures: **1) analysis of STR (*Short Tandem repeat)* microsatellite markers on chromosome 21**. STR markers polymorphic loci on chromosome 21 are amplified using nested PCR with external and internal primers. The PCR products are then analysed using the Genotyper system (Applied Biosystems). Allele peaks are visualised using the Genotyper® software, version 3.7. Allele analysis allows the detection of trisomy 21 with a diallelic signal at a dose ratio of 2:1 or a triallelic signal with identical intensities. **2) analysis of a single gene from chromosome 21.** Amplification of unique sequences on chromosome 21 and the GAPDH gene on chromosome 12 is performed using nested, quantitative, real-time PCR with external and internal DSCR3 and GAPDH primers.

Metagenex has planned to develop a multiplex Quantitative PCR using TaqMan probes in order to amplify and GAPDH sequences (as reference) in the same tube. This strategy will ensure highly proportional and relative amplification of the target (on chromosome 21) and reference (on chromosome 12) sequences allowing an extremely sensitive and reliable assessment of the relative proportion of the two sequences from microdissected CFC. This goal will be focused to groups of 10 CFC as a first step and to groups of 3 CFC as a second step.

1.3.2 Stage 2 (months 18 to 24) (Milestone 6)

1.3.2.1 Validation of the reproducibility, sensitivity and specificity of the optimized tests

The optimized tests will be applied to filters containing fibroblasts from patients with trisomy 21 and trisomy 18, lymphoid cells and cord blood cells from subjects with trisomy 21. At least 100 tests will be done for every technical approach (FISH, CGH, quantitative PCR), which will allow to define the reproducibility of the different tests. For the FISH/PRINS method, tests of sensitivity and specificity, performed by mixing 1, 3, 5 and 10 male cells with trisomy 21 to normal blood in triplicate (test repeated 30 times), will be carried out. For the CGH and quantitative PCR approaches, they will be performed **blindly** (the operator will not be aware of the cell chromosomal number) on at least 100 trisomic (trisomy 21) cells and 100 normal cells to test the technical specificity and sensitivity.

These critical aspects of the ISET method for the non-invasive diagnosis of Trisomy 21 have been discussed extensively with Dr. Jais (statistician) and Prof. Thalabar (methodologist) on the Necker campus.

Stage 2 will allow to provide important information about the following items: reproducibility, sensitivity, specificity, costs of the evaluated methods. This assessment will provide the elements to choose the method to be assessed in the clinical validation step. **According to the obtained results, we will decide if FISH/PRINS is an interesting pre-screening approach,** which is worth to be applied or not before the methods on microdissected cells. We will also have the information about **which method targeted to microdissected cells (CGH or quantitative PCR) is the most reliable and cost-effective one to be used in routine non invasive screening of pregnant mothers.**

1.3.3 Stage 3 (months 25 to 36) (Milestone 7)

Clinical validation of the sensitivity and specificity of the method.

For the clinical validation of this method, we will take advantage from a collaborative project with Dr. L. Bussières, Paris Public Hospitals, and Dr P. Rozenberg, Maternity Unit at Poissy-St Germain, who are the organisers of the "Echo-PAPPA 78" protocol. After giving their informed consent, women with a high risk of trisomy 21 are offered a collection of chorionic villi for prenatal diagnosis, and if this test is positive, then a therapeutic abortion. Since 2002, the collection of maternal blood (and paternal) samples for ISET has been offered before the therapeutic abortion, and the blood samples have been sent to the INSERM Unit 370 (now 807)/Biochemistry A Laboratory at Necker Enfants Malades Faculty of Medecine and Hospital. Therefore, **we have available 35 filters obtained from mothers having a trisomic fetus** and the maternal and paternal blood samples for genotyping studies. We will perform the NI-PND diagnosis on these filters to **validate the sensitivity of the method** (N° of samples with at trisomic cells (trisomy 21))
In order to **validate the specificity** of the approach, we will test filters coming from mothers at high risk of having a trisomic child. In this case the maternal and maternal blood is collected before the biopsy of chorionic villi from 200 mothers. Among these 200 mothers, we expect 16 to 20 mothers with a foetus affected by trisomy 21. In order to avoid testing all the 200 mothers, **we will receive from Dr. P. Rozenberg and Dr. Buissières the name of 40 mothers to be tested. These 40 mothers will**

include blindly (we will not be aware of the diagnosis) 15 to 20 mothers with a trisomic fetus and the rest with a normal fetus. This strategy will allow to validate the specificity of the developed method in a clinical setting. The statistical programme of this validation project has been drawn up in collaboration with Dr. Jais and Prof. Thalabar (Hôpital Necker-Enfants Malades) who, together with Dr. Rozenberg, Dr. Buissières, Prof. Y. Dumez, Prof. Munnich and Prof. Vekemans will form the committee evaluating the results.

According to the results of the clinical validation, **the non invasive test will be submitted to the National Ethics committee to be proposed to pregnant women instead of the invasive procedure**, in agreement to the national laws.

1.4 I.4. Consortium Description

The consortium comprises:

1- Metagenex SA
Metagenex participates in the development and validation of test for NI-PND of trisomy 21 (providing instruments, personnel, know how). It also provides for the development, EC compliance and marketing of the ISET apparatus and its consumables which enable the enrichment of CFC from maternal blood, the development of a dedicated ISET device for the specific isolation of CFC (as compared to circulating tumor cells), the implementation of partnerships with major diagnostics companies for the marketing of kits arising from the new diagnostic tests developed by the research team (INSERM U807), partnerships with instrument companies (Nikon for the laser microscope, Beckman for automated devices). Metagenex has the exclusive licence of the patents filed by the Metagenex-Inserm Unit teams, which belong to INSERM, AP-HP, University Paris 5.

2- INSERM Unit 807 at the Faculty of Medicine Necker-Enfants Malades. This team is at the origin of the ISET technology and provides the knowledge required for the technical development of a new non-invasive diagnostic test for trisomy 21, in collaboration with Biochemistry Laboratory A at Hôpital Necker-Enfants Malades (where blood samples are received, treated by ISET and stored). Historically, the two teams (INSERM Unit and Metagenex) have worked together to develop non invasive tests for prenatal diagnosis

3- Cytogenetics Laboratory at Hôpital Necker-Enfants Malades.
The Cytogenetics Laboratory at Hôpital Necker routinely carries out analyses of fetal samples. S. Romana performs karyotyping and FISH tests (preparation of probes, hybridisation on mitoses, image analysis) for trisomy 21. This team will be responsible for collaboration on FISH/PRINS filter analyses and reading of CGH tests on single cells. Dr. Romana is developing dedicated chips (CGH arrays) to the ISET method for the non-invasive diagnosis of trisomy 21.

4- Molecular Genetics Laboratory at Hôpital Necker-Enfants Malades
The Molecular Genetics Laboratory at Hôpital Necker-Enfants Malades is part of the Medical Genetics Department (Prof. A. Munnich), an collaborate with the INSERM Unit 807 to develop non invasive prenatal diagnosis of genetic diseases. It also provides the instrument to perform STR genotyping of microdissected cells

These four partners are working in close collaboration with the two clinical recruitment centres: the **Maternity Unit at Hôpital Necker (**Prof. Yves Dumez and Dr. Alexandra Benachi) and the **Gynaecology and Obstetrics Department at Hôpital Poissy-Saint Germain** (Dr. P. Rozenberg, Prof. Y Ville). These centres send blood samples from mothers at risk of having a child with trisomy 21 to the Biochemistry A laboratory. The partners are also working in collaboration with Dr. Jais (statistician) and Prof. Thalabar (methodologist) concerning the technical and clinical validation of the test.

This is a "prioritary" project for **Metagenex which is in advance at an international level in the area of non-invasive diagnostic tests (no known competitors at present).** Metagenex ensures management of the project, its development and the marketing of machines (ISET) for the enrichment of CFC and kits for non-invasive diagnostic tests.
The feasibility of this study is ensured by the high degree of skill at Metagenex and INSERM Unit 807 in the area of genetic analyses of single fixed cells isolated by ISET, by the high level of experience and renown of the Laboratory of Cytogenetics (Prof. Vekemans), the Laboratory of Molecular Genetics (Prof . Munnich), the department of Maternity (Prof. Dumez), the Department of Satistics (Dr. Jais, Prof. Thalabar) at the Necker Enfants Malades Hospital Paris. The feasibility is also ensured by the availability of the technical facilities necessary to the project (microscopes, Nikon laser microscope, rooms and devices for PCR, sequence analyses device, image analysis systems, etc.). Previous results have shown **that ISET isolates CFC in all the mothers tested up to now (n°=46) using only 3-4 ml of blood.**

1.5 I.5. Publications linked to the project

Metagenex SA and INSERM U807:

Beroud, C., M. Karliova, J.P. Bonnefont, A. Benachi, A. Munnich, Y. Dumez, B. Lacour, and **P. Paterlini-Brechot. 2003.** Prenatal diagnosis of spinal muscular atrophy by genetic analysis of circulating fetal cells. Lancet. 361:1013-4.

Vona, G., C. Beroud, A. Benachi, A. Quenette, J.P. Bonnefont, S. Romana, Y. Dumez, B. Lacour, and P. **Paterlini-Brechot. 2002.** Enrichment, immunomorphological, and genetic characterization of fetal cells circulating in maternal blood. *Am J Pathol*. 160:51-8.

Vona, G., A. Sabile, M. Louha, V. Sitruk, S. Romana, K. Schutze, F. Capron, D. Franco, M. Pazzagli, M. Vekemans, B. Lacour, C. Brechot, and P. **Paterlini-Brechot. 2000.** Isolation by size of epithelial tumor cells : a new method for the immunomorphological and molecular characterization of circulatingtumor cells. Am J Pathol. 156:57-63.

Molecular Genetics Laboratory

Gigarel, N., N. Frydman, P. Burlet, V. Kerbrat, J. Steffann, R. Frydman, A. Munnich, and P.F. Ray. 2004. Single cell co-amplification of polymorphic markers for the indirect preimplantation genetic diagnosis of hemophilia A, X-linked adrenoleukodystrophy, X-linked hydrocephalus and incontinentia pigmenti loci on Xq28. Hum Genet. 114:298-305.

The Cytogenetics Laboratory

Tabet AC, Gosset P, Elghezal H, Fontaine S, Martinovic J, Encha Razavi F, Romana S, Vekemans M, Morichon-Delvallez N. Prenatal diagnosis and characterization of an analphoid marker chromosome 16. Prenat Diagn. 2004 Sep;24(9):733-6.

1.6 I.6. Bioethical aspects

This project is in agreement with the directives of bioethics currently into force. Our activity is framed by the multidisciplinary center of prenatal diagnosis of the Hopital Necker Enfants Malades. Our project received the CPPRB agreement (clinical trial AOR 0160 P011005, positive notice received the April 11, 2002, Hopital Saint Germain en Laye) and the promotion of the AP-HP.

2 II- ECONOMICAL ASPECTS

2.1 II.1. Qualitative and quantitative assessment of the potential market

Genetic abnormalities are relatively common affecting 3-5% of all births. Some, like Down syndrome which affects 1/600 live births and increases in probability with increasing maternal age, are random in nature whilst others such as Cystic Fibrosis (affecting 1/2500 births) are inheritied conditions. 20-30% of all infant deaths are the result of genetic disorders.
In the light of these problems Amniocentesis can be used to take samples of the amniotic fluid or Chorionic villus sampling used to acquire a sample of the placenta for genetic testing. Both techniques however are not without problems. Both significantly increase the likelihood of miscarriage (by up to 3%) and in the case of amniocentesis the test cannot be performed until the 14th to 16th week of pregnancy. As a result, both methods are unsuitable for systematic screening and they are normally only used when other risk factors such as maternal age outweigh the risks of the procedures.

It has long been known that fetal cells can readily be found circulating in the mother's blood and they have therefore been the focus of efforts to achieve non-invasive fetal genetic testing. Unfortunately, these fetal cells can survive for many years in the mother's blood and so there has been no way of uniquely differentiating the cells from different pregnancies over the mother's lifetime. The problem is made worse by the fact that cells also survive from previously naturally aborted pregnancies which are quite common.
The ISET system provides a means to overcome these difficulties. Epithelial cells from the placenta also contain fetal DNA and circulate in the mother's blood in very low concentrations. Critically, these cells have a limited viability lasting only a few weeks in circulation and therefore have a very high probability of being from the current pregnancy. Although low in number, genetic testing can still be carried out on these cells at least as early as the 11th week of pregnancy, providing an early indication of potential problems. Early diagnosis can avoid natural miscarriages which are common when genetic abnormalities exist. In most countries, including France, the UK and the US, the end of the first trimester (~12 weeks) also marks a significant milestone from a legal perspective with strict controls or prevention of pregnancy termination after this point.
With a non-invasive test there are no risks to the ongoing pregnancy and so systematic screening is now possible. Furthermore, the recent development of automated genetic analysis systems has significantly lowered the potential total cost of such a program.
Currently the pricing for amniocentesis based genetic profiling depends on the type of genetic analysis required but typically costs in excess of €500 ($590) in France. ISET based fetal cell analysis can be performed earlier in the pregnancy and without any of the

associated risks providing significantly more value to a highly motivated patient group. Metagenex estimates a premium price is of at least €500 per test.

Country	Total Births /yr
France	760,000
UK	695,000
Italy	543,000
Spain	418,000
Germany	706,000
USA	4,115,000
Total	**7,237,000**

Annual birth statistics for selected countries in the European Union

Considering the current 16% penetration rate of amniocentesis, a non invasive diagnosis method would easily penetrate the market between 30 and 60%. This assumption translates into 2,2 to 4,4 tests ie a market of €1 to €2 bn.

Potential Market value
The potential market of IVD for trisomy 21 presents strong growth opportunities:
- age of pregnancy increases in modern society
- expected child « without abnormality»,
- absence of alternative method
-ISET is not operator dependant (contrary to amniocentesis)
The potential for a future screening test raises this potential market to in excess of €1.8B ($2.1B).
Based on our assumptions the following estimates of market potential for the Metagenex ISET system are considered realistic.
Premium pricing: min 500 Euros / kit ISET
Market dynamics
High speed and rate of test penetration

2.2 II. 2. State of the competition, benchmarking and competitive advantages

Metagenex does not have a known competitor today in the Trisomiy 21 detection market. In addition, the current methods of screening present limits and disadvantages, which generate douts and concern leading to a higher number of amniocenthesis and of miscarriages. The test developed by Metagenex comes in an extremely favourable context, wich should facilitate its introduction into the screening routine and financial reimboursement. Indeed, in France, until recently the only reliable proposed screening of trisomy 21 is based on amniocenthesis or CVS to women older than 38 years. However this screening is not very efficient, since 70% of the babies carrying trisomy 21 occurs now among women younger than 38 years. To the women judged at risk, a chromosomic chart by amniocenthesis is proposed. Significant progress was made, but had perverse effects, in particular an

excessive rate of amniocentesis (11% in France and 16% in Ile de France). Considering that the risk of miscarriage related to amniocentesis is 1%, a **normal pregnancy is stopped for the diagnosis of every trisomic foetus!**
The test proposed by Metagenex will offer a radical change. Indeed, this test has the advantage of offering a reliable diagnosis without risk of miscarriage.

2.3 II.3. Industrial Innovation Strategy

Metagenex has developed and is pursuing enhancements of ISET, a process of isolation of rare cells from the blood and concentrates today in the development on the following field:
Oncology with the isolation and analysis of circulating tumoral cells for the follow up of patients with solid tumors
A non-invasive prenatal screening of genetic disease by isolation and analysis of circulating fetal cells

For these two fields, Metagenex has finalized the development of an ISET machine and its consumables.

The machine's EC labelling process is ongoing and marketing will start by the end of 2006. In the prenatal field, Metagenex already developed two methods of analysis of fetal cells for the diagnosis of Spinal Amyotrophy and Mucoviscidosis. The development of a NI-PND for trisomy 21 is thus in continuity in the strategy of Metagenex.
The development of kits allowing a non invasive mass screening of trisomy 21 is supported by the incidence of trisomy 21 and will allow a generalized diffusion of the test. Metagenex aims to market ISET machine dedicated to CFC isolation to laboratories in Maternity Hospitals, so that maternal blood can be filtered on the spot. The filter can be then sent by post to specifically equipped centers (pluridisciplinary centers for prenatal diagnosis in France). The marketing of ISET machines and consumables will be carried out in partnership.

2.4 II. 4. Expected benefits

Metagenex company was created in 2001 by Professor Patrizia Paterlini-Brechot in order to promote the development of the ISET technology and its marketing. Four people currently work for Metagenex, including the permanent Scientific Advisor, Prof Paterlini-Brechot.

In Metagnex, two scientists are working on this project and expected benefit in term of stable jobs creation is listed in the following table:

	2006	2007	2008	
Scientist	1	2	2	
Technician	2	2	3	
Total	3	4	5	

This project will also develop the company offer in term of IVD enabled by the ISET technology and will allow to position Metagenex as a key player in the field on prenatal screening.

3 III- INTELLECTUAL PROPERTY

3.1 III.1. State of the Intellectual property

Metagenex' intellectual property (IP) portfolio consists of 4 patents and patent applications filed by the Institut National de Recherche et Santé Médecine (INSERM), AP HP University of Paris 5 and Metagenex. They cover the ISET approach, prenatal diagnostic applications.
Patents were originally filed in France but international proceedings have been initiated through subsequent European and North American filings. These various applications are ongoing and are summarized below:

Description	Application Number	Application Date	Patent Status
1. Process, device and reagent for cell separation	9810696	25.08.1998	Awarded 17.05.02
2. Prenatal diagnosis method on isolated fetal cell of maternal blood	0105824	30.04.2001	Pending
3. Non invasive in-vitro detection methodology for cystic fibrosis: healthy carrier or ill	0500512	18.01.2005	Pending
4. Process and device for separation by vertical filtration of biological particles in a liquid	0502945	24.03.2005	Pending

Metagenex has long term, exclusive rights to these patents in an agreement signed with INSERM in 2002. According to the terms and conditions of the licence agreement n°0214A10 signed between the three institutions (INSERM, AP-HAP, Université de Paris 5) and Metagenex on septembre 9, 2003, Metagenex has the exclusive world licence for exploitation of patent n°1 and n°2. Patent n°3 and 4 will be the subject of a new licencing agreement between all parties. As a result Metagenex will obtain the exclusive world licence of exploitation, for all four patents.

It is Metagenex' intention to continue to strengthen this IP portfolio with the filing of further applications and device patents relating to future developments in the domain of rare cell enrichment systems and applications.
An independent patent review to assess any potential weaknesses of the existing portfolio in the light of the current business direction has been initiated and is ongoing. It is anticipated that further filings to strengthen the portfolio may be required but Metagenex is aware of no prior art that currently invalidates our claims or system.

3.2 III.2 Agreements between partners

A formal agreement binding the partners of the NI-PND test for trisomy 21 will be finalised between all parties in order to share the intellectual property generated by the project.

4 IV- REFERENCES

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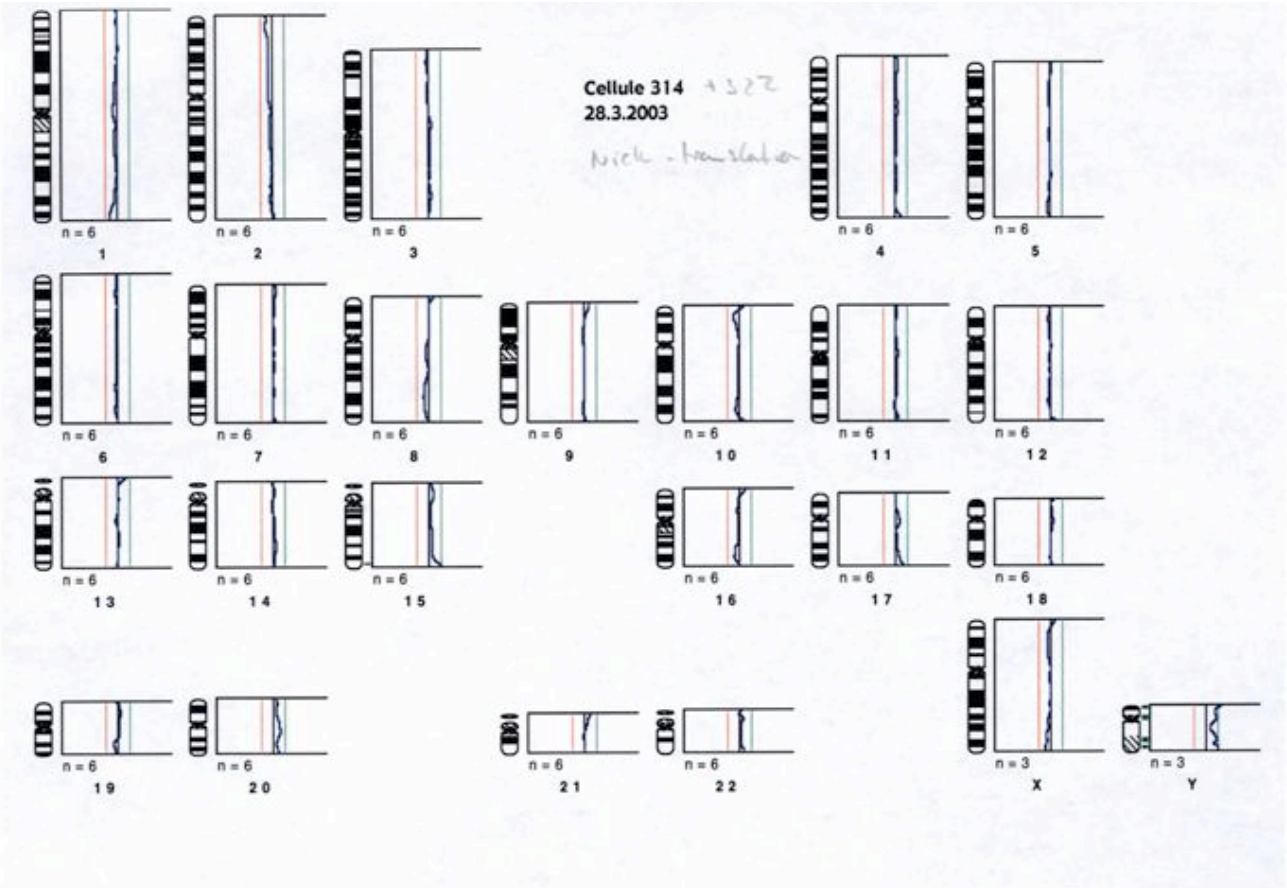
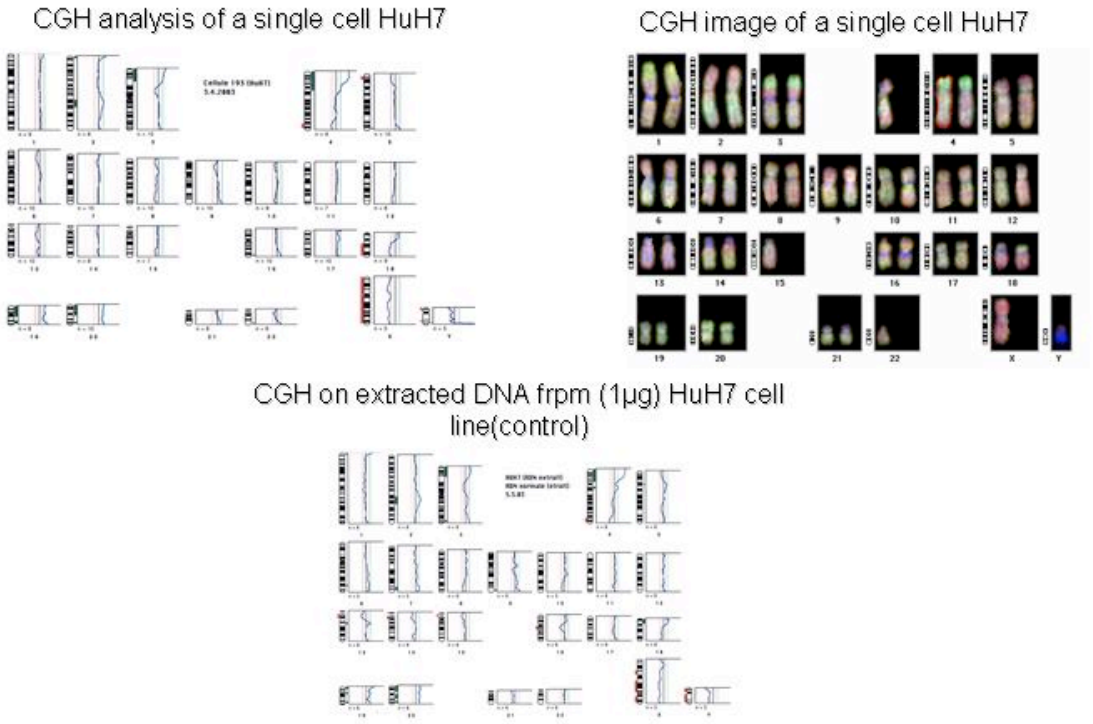
Annexe 2

Figure 1: CGH analysis performed on a single normal cell isolated by ISET and microdissected



Annexe 2

Figure 2: CGH analysis performed on a HuH-7 single tumor cell isolated by ISET and microdissected



Annexe 2

Figure 3: CGH analysis performed on a single fetal cell from a fetus with trisomy 21 isolated by ISET and microdissected