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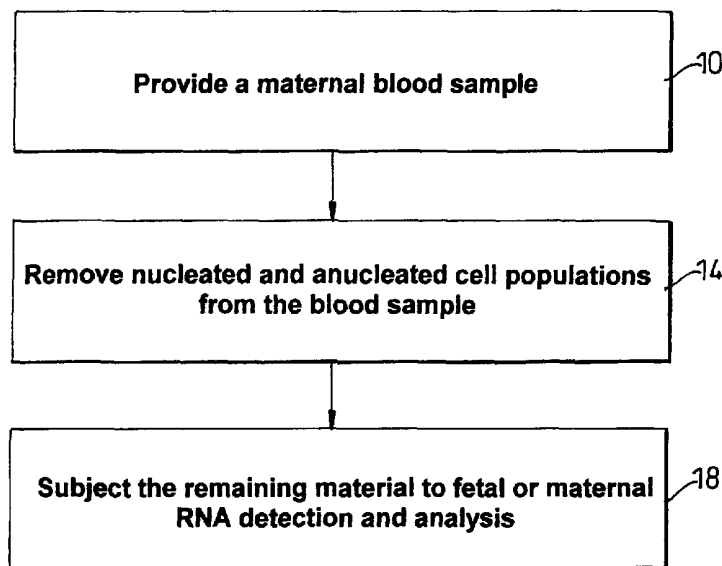
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(54) Title: NON-INVASIVE PRENATAL MONITORING



(57) Abstract: Embodiments of the present invention are directed to the detection of fetal or maternal RNA in a blood sample from a pregnant subject, and may involve subjecting the sample to a test for fetal or maternal analysis indicative of a fetal or maternal condition or characteristics. For instance, the RNA analysis may involve the assessment of the gene expression pattern of an unborn fetus by analysing a blood sample from the mother. The prenatal monitoring technology allows, for the first time, the detection of genes which are expressed by the fetus, just by analysis of a sample of maternal blood. In specific embodiments, the prenatal monitoring technology is based on the discovery of circulating RNA of fetal origin in the plasma of pregnant women. In general, the detection method performed on a maternal serum or plasma sample from a pregnant female comprises detecting the presence of RNA of fetal or maternal original in the sample.



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## NON-INVASIVE PRENATAL MONITORING

### BACKGROUND OF THE INVENTION

This invention relates to prenatal detection methods using noninvasive  
5 techniques and, more particularly, to the detection of fetal or maternal RNA in a blood  
sample from a pregnant subject, including the assessment of the gene expression pattern of an  
unborn fetus by analyzing the blood sample.

We have previously described the detection of circulating fetal DNA in  
maternal plasma and serum. Y.M. Dennis Lo et al., "Presence of Fetal DNA in Maternal  
10 Plasma and Serum," Lancet 1997, 350:485-7. We have demonstrated the use of this  
technology for non-invasive prenatal diagnosis. Y.M. Dennis Lo et al., "Prenatal Diagnosis  
of Fetal RhD Status by Molecular Analysis of Maternal Plasma," New England Journal of  
Medicine 1998, 339:1734-8. The discovery of fetal DNA in maternal plasma has opened up  
a new horizon on prenatal molecular diagnosis. A number of groups have since shown that  
15 fetal genetic traits, such as RhD status and inherited genetic diseases, can be determined from  
fetal DNA in maternal plasma.

The main limitation of this existing technology is that fetal DNA detection  
will only allow one to tell the presence and quantity of genetic material of fetal origin in the  
maternal circulation. It does not give information regarding the gene expression profile of  
20 the baby. Gene expression patterns can be expected to be affected by physiological or  
pathological processes affecting the baby and mother. Such direct monitoring of fetal gene  
expression is beyond the reach of conventional non-invasive technologies.

### SUMMARY OF THE INVENTION

25 Embodiments of the present invention are directed to the detection of fetal or  
maternal RNA in a blood sample from a pregnant subject, and may involve subjecting the  
sample to a test for fetal or maternal analysis indicative of a fetal or maternal condition or  
characteristics. For instance, the RNA analysis may include the assessment of the gene  
expression pattern of an unborn fetus by analyzing a blood sample from the mother. The  
30 utility of the invention has been demonstrated from the second trimester and the third  
trimester of pregnancy, and may be used for the diagnosis and monitoring of a wide variety  
of diseases, including pre-eclampsia and preterm labor.

The new prenatal monitoring technology according to embodiments of the present invention allows, for the first time, the detection of genes which are expressed by the fetus, just by analysis of a sample of maternal blood. This will become a platform technology upon which a new generation of non-invasive prenatal tests can be built.

5           The prenatal monitoring technology is based on the discovery of circulating RNA of fetal origin in the plasma of pregnant women. Heretofore, it was not known whether fetal RNA was also present in maternal plasma. Using a two-stepped reverse transcriptase polymerase chain reaction (RT-PCR) assay, we demonstrate the presence of fetal-derived, male-specific mRNA in plasma of pregnant women carrying male fetuses. As described  
10       herein, fetal RNA is detected by RT-PCR; but in principle any RNA detection method can be used.

          This technology is expected to have application in all cases of pregnancy for monitoring the physiological or pathological status of a fetus. It is further anticipated that in certain scenarios, more than one such monitoring may be desirable or necessary during  
15       pregnancy. Thus, the number of potential clients and possibilities of multi-usage is potentially enormous.

          In accordance with an aspect of the present invention, a method of performing prenatal monitoring or testing of a blood sample obtained from a pregnant subject comprises removing all or substantially all nucleated and anucleated cell populations from the blood  
20       sample to obtain a remaining material. The remaining material is subjected to a test for fetal or maternal RNA analysis indicative of a fetal or maternal condition or characteristic. The remaining material may comprise plasma or serum.

          In accordance with another aspect of the invention, a detection method performed on a maternal serum or plasma sample from a pregnant female comprises  
25       detecting the presence of RNA of fetal origin in the sample. The fetal RNA may be amplified to facilitate detecting the presence of RNA of fetal origin in the sample. For instance, the fetal RNA may be converted into complementary DNA by a reverse transcriptase and then detected by a polymerase chain reaction.

          In some embodiments, the fetal RNA is detected using a sequence specific  
30       probe. The RNA detection may involve detecting the presence of a fetal RNA transcribed from the Y chromosome, or a fetal RNA from a gene or other DNA sequences inherited from either the father or the mother. The RNA may be detected by any one of a physical method, an immunological method, and a biochemical method.

In accordance with another aspect of the present invention, detection method performed on a maternal serum or plasma sample from a pregnant female comprises detecting the presence of RNA of fetal or maternal origin in the sample. The RNA detection may involve detecting the presence of RNA transcribed from genes on chromosome 6. The RNA may be transcribed from the HLA-G gene.

Another aspect of the invention is directed to a method of performing prenatal monitoring or testing. The method comprises providing a maternal blood sample, and separating the sample into a predominantly cellular fraction (fraction 1) and a predominantly non-cellular fraction (fraction 2). The presence of RNA of fetal origin is detected in fraction 2. The method further includes providing a diagnosis based on at least one of the following: presence, quantity, concentration, sequence, and biochemical composition of the detected RNA.

In accordance with another aspect of the present invention, a method of performing a prenatal monitoring or testing on a maternal blood sample comprises obtaining a non-cellular fraction of the maternal blood sample, and performing an RNA analysis for expressed human genes on the fraction.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a flow diagram illustrating a noninvasive prenatal monitoring method according to an embodiment of the present invention; and

Fig. 2 illustrates the detection of fetal-derived, Y chromosome-specific zinc finger protein (ZFY) mRNA in maternal plasma and for RT-PCR assay for HLA-G mRNA according to an embodiment of the present invention.

#### DESCRIPTION OF THE SPECIFIC EMBODIMENTS

As shown in Fig. 1, the method of performing prenatal monitoring or testing of a blood sample obtained from a pregnant subject according to an embodiment of the present invention begins with providing a maternal blood sample (step 10). In step 14, all or substantially all nucleated and anucleated cell populations are removed from the blood sample. The remaining material is subjected to a test for fetal or maternal RNA analysis indicative of a fetal or maternal condition or characteristic in step 18. The remaining material to be subjected to the RNA analysis may be a cellular matter or a non-cellular matter. In specific embodiments, the remaining material may include plasma or serum. The serum may be obtained, for instance, by allowing clotting in the maternal blood sample.

The maternal serum or plasma sample may be tested to detect the presence of RNA of fetal or maternal origin in the sample. To facilitate the detection of RNA in the sample, the RNA may be amplified. In one example, the fetal or maternal RNA is converted into complementary DNA by reverse transcriptase and then detected by a polymerase chain reaction. Amplifying the fetal or maternal RNA may involve the use of at least one fetal or maternal sequence specific oligonucleotide.

The fetal or maternal RNA may be detected using a sequence specific probe. In one example, detecting the presence of RNA of fetal origin includes detecting the presence of a fetal RNA transcribed from the Y chromosome. More specifically, the fetal RNA may be transcribed from the ZFY gene. The sex of the fetus can be determined from the RNA of fetal origin in the sample. In another example, detecting the presence of RNA of fetal origin in the sample involves detecting the presence of a fetal RNA from a gene or other DNA sequences inherited from the father. In another example, detecting the presence of RNA of fetal or maternal origin in the sample includes detecting the presence of RNA transcribed from genes on chromosome 6. The RNA may be transcribed from the HLA-G gene. The detection of RNA may be performed by any one or more of a physical method, an immunological method, and a biochemical method.

In yet another example, the maternal or fetal RNA detection and analysis is performed on a non-cellular fraction of the maternal blood sample. The non-cellular fraction may be completely or predominantly non-cellular, and is obtained by separation from the completely or predominantly cellular fraction in the blood sample. The RNA analysis may involve a diagnosis based on one or more of the presence, quantity, concentration, sequence, and biochemical composition of the detected RNA. In a specific embodiment, the RNA analysis is performed for expressed human genes on the non-cellular fraction of the sample.

It should be noted that fetal or maternal RNA detection and analysis are still possible even after filtration of the blood sample removing all cellular material to obtain a plasma or serum (i.e., the predominantly cell-free fraction of blood).

The following example is provided to illustrate various features of the present invention, and does not in any way limit the scope of the invention.

In the example, pregnant women attending the Prenatal Diagnosis Unit at the Department of Obstetrics and Gynecology, Prince of Wales Hospital, Hong Kong were recruited with informed consent. The study was approved by the Clinical Research Ethics Committee. Twenty-one and thirty-seven women in early and late pregnancies, respectively, were recruited in this study. The mean gestational ages of the subjects in early and late

pregnancies were 16 weeks (range, 11-19 weeks) and 33 weeks (range, 26-40 weeks), respectively. All early pregnancy samples were obtained before any invasive procedure. On the other hand, late pregnancy samples were collected either from women who had invasive procedures in early pregnancy (n=21) or from women who did not have any prenatal invasive procedure (n=16). All plasma samples were harvested within 30 minutes from EDTA blood samples as described previously. See Y.M. Dennis Lo et al., "Presence of Fetal DNA in Maternal Plasma and Serum," *Lancet* 1997, 350:485-7. Total RNA from plasma samples was isolated with the Trizol LS Reagent (Gibco BRL) as instructed by the manufacturer. In general, RNA isolated from 1 mL of plasma was dissolved in 50  $\mu$ L of RNase-free water.

In this study, we chose to detect fetal-derived, Y chromosome-specific zinc finger protein (*ZFY*) mRNA in maternal plasma. See D.C. Page et al., "The Sex-Determining Region of the Human Y Chromosome Encodes a Finger protein," *Cell* 1987, 51:1091-104; M.S. Palmer et al., "Comparison of Human *ZFY* and *ZFX* Transcripts," *Proc. Nat'l Academy Science USA* 1990, 87:1681-5. As shown in Fig. 2, RT-PCR products corresponding to *ZFY* mRNA were observed only when male placental total RNA was used in the RT-PCR assay (Fig. 2, lane 1). By contrast, no positive signal was detected when either RT was omitted (Fig. 2, lane 2) or female placental total RNA was used (Fig. 2, lane 3) in the RT-PCR assays. Among 20 women carrying male fetuses in late pregnancy, *ZFY* positive signals were detected (Fig. 2, middle panel, lanes 6-10) in 13 plasma samples. Positive signals were observed in 2 out of 9 women carrying male fetuses in early pregnancy. The identity of *ZFY* mRNA-specific RT-PCR products in the positive cases were confirmed by DNA sequencing (data not shown). By contrast, of 20 women carrying female fetuses either in early (n=12) or in late (n=8) pregnancy, all but one case were negative in the assay (Fig. 2, middle panel, lanes 11-14). The only false-positive case was presumably due to contamination during RNA processing. As a control for the quality of the extracted RNA, we also subjected all samples to an RT-PCR assay for *HLA-G* mRNA. See T.V.F. Hviid et al., "Co-Dominant Expression of the HLA-G Gene and Various Forms of Alternatively Spliced HLA-G mRNA in Human First Trimester Trophoblast," *Hum. Immunol.* 1998, 59:87-98. The *HLA-G* gene is expressed by both fetal (e.g. trophoblast (see *id.*)) and maternal (e.g. lymphocytes (see M. Kirszenbaum et al., "An Alternatively Spliced Form of HLA-G mRNA in Human Trophoblasts and Evidence for the Presence of HLA-G Transcript in Adult Lymphocytes," *Proc. Nat'l Academy Science USA* 1994, 91:4209-13)) tissues. As shown in the lower panel of Fig. 2,

RT-PCR products specific for *HLA-G* mRNA were detected in all tested plasma samples, demonstrating the presence of amplifiable RNA in these samples.

Recently, it has been demonstrated that a proportion of maternal plasma fetal DNA circulates in the form of intact fetal cells. See I.J. van Wijk et al., "Detection of Apoptotic Fetal Cells in Plasma of Pregnant Women [Technical Brief], Clin. Chem. 2000, 46:729-31. Thus, theoretically it is possible that the fetal RNA that we detected in the current study could have originated from these 'plasma-derived' cells. To conclusively test if fetal RNA can be detected in *cell-free* form in maternal circulation, maternal plasma samples were filtered by a 0.2  $\mu\text{m}$  membrane (Nalgene) and the RNA extracted from these filtered plasma samples was tested by the *ZFY* RT-PCR assay. Of 9 filtered plasma samples collected from women carrying male fetuses in late pregnancies, positive *ZFY* mRNA signals were detected in 6 samples (data not shown). These results indicate that at least a portion of fetal RNA in maternal plasma exists in *cell-free* form.

Our data demonstrate that fetal RNA can be detected in maternal plasma. The detection rate of plasma fetal RNA in early and late pregnancies were 22% and 63%, respectively. The detection rate of fetal RNA in early pregnancy cases was lower than that in late pregnancy cases, suggesting that the concentration of plasma fetal RNA is lower in early pregnancy. This observation is similar to our previous finding that the concentration of fetal DNA in maternal plasma increases with gestation. See Y.M. Dennis Lo et al., "Quantitative Analysis of Fetal DNA in Maternal Plasma and Serum: Implications for Noninvasive Prenatal Diagnosis," Am. J. Hum. Genet 1998, 62:768-75. We also realized that the detection rate of plasma fetal RNA in this study is lower than that of plasma fetal DNA. It is possible that fetal RNA is more susceptible to degradation in maternal blood. As a result, the amount of fetal RNA in plasma is much lower than plasma fetal DNA. This is supported by the fact that Y-specific DNA was detected in all plasma samples from women carrying male fetuses in this study (data not shown). A highly sensitive real-time quantitative RT-PCR assay may be developed to improve the sensitivity of maternal plasma fetal RNA detection.

In conclusion, we have shown, for the first time, that fetal RNA can be detected in maternal plasma, and our data provide a novel way for noninvasive prenatal diagnosis. Plasma fetal DNA analysis can provide data on the presence and concentration of fetal genetic material in the maternal circulation. Plasma fetal RNA analysis, in addition, can provide valuable information regarding the gene expression patterns of fetal tissues. For example, abnormal pregnancies, such as preeclampsia, are often associated with abnormal



gene expression patterns in fetal tissues. B.K. Rinehart et al., "Expression of the Placental Cytokines Tumor Necrosis Factor Alpha, Interleukin 1 Beta, and Interleukin 10 is Increased in Preeclampsia," Am. J. Obstet. Gynecol. 1999, 181:915-20. Thus, with the development of further RNA markers, maternal plasma RNA analysis may allow the noninvasive monitoring of fetal gene expression in a multitude of physiological and pathological conditions. It is understood that the term "plasma fetal RNA analysis," as used herein, encompasses analysis of fetal RNA in maternal plasma or maternal serum.

The above-described arrangements of apparatus and methods are merely illustrative of applications of the principles of this invention and many other embodiments and modifications may be made without departing from the spirit and scope of the invention as defined in the claims. For example, while we have demonstrated the feasibility of the concept, further research may elucidate the spectrum of conditions in which this technology can be applied. In addition, further refinement of the technology may be made, for instance, to improve the reliability of the method without departing from the scope of the invention. The scope of the invention should, therefore, be determined not with reference to the above description, but instead should be determined with reference to the appended claims along with their full scope of equivalents.

WHAT IS CLAIMED IS:

- 1                   1.       A method of performing prenatal monitoring or testing of a blood  
2 sample obtained from a pregnant subject, the method comprising:  
3                    removing all or substantially all nucleated and anucleated cell populations  
4 from the blood sample to obtain a remaining material; and  
5                    subjecting the remaining material to a test for fetal or maternal RNA analysis  
6 indicative of a fetal or maternal condition or characteristic.
- 1                   2.       The method according to claim 1 wherein all nucleated and anucleated  
2 cell populations are removed from the blood sample to obtain the remaining material.
- 1                   3.       The method according to claim 1 wherein the remaining material  
2 comprises non-cellular matter, and wherein the RNA analysis is performed on the non-  
3 cellular matter of the remaining material.
- 1                   4.       The method according to claim 1 wherein the remaining material  
2 comprises cellular matter, and wherein the RNA analysis is performed on the cellular matter  
3 of the remaining material.
- 1                   5.       The method according to claim 1 wherein the remaining material  
2 comprises plasma.
- 1                   6.       The method according to claim 1 wherein the remaining material  
2 comprises serum.
- 1                   7.       A detection method performed on a maternal serum or plasma sample  
2 from a pregnant female, the method comprising:  
3                    detecting the presence of RNA of fetal origin in the sample.
- 1                   8.       The method according to claim 7 further comprising amplifying the  
2 fetal RNA to facilitate detecting the presence of RNA of fetal origin in the sample.
- 1                   9.       The method according to claim 8 wherein the fetal RNA is converted  
2 into complementary DNA by a reverse transcriptase and then detected by a polymerase chain  
3 reaction.

1                   10.     The method according to claim 8 (claim 8 or claim 9) wherein at least  
2 one fetal sequence specific oligonucleotide is used in amplifying the fetal RNA.

1                   11.     The method according to claim 7 (any one of claims 7 to 10) wherein  
2 the fetal RNA is detected using a sequence specific probe.

1                   12.     The method according to claim 7 (any one of claims 7 to 11) wherein  
2 detecting the presence of RNA of fetal origin in the sample comprises detecting the presence  
3 of a fetal RNA transcribed from the Y chromosome.

1                   13.     The method according to claim 12 wherein the fetal RNA is  
2 transcribed from the ZFY gene.

1                   14.     The method according to claim 12 (claim 12 or claim 13) further  
2 comprising determining the sex of the fetus from the RNA of fetal origin in the sample.

1                   15.     The method according to claim 7 (any one of claims 7 to 11) wherein  
2 detecting the presence of RNA of fetal origin in the sample comprises detecting the presence  
3 of a fetal RNA from a gene or other DNA sequences inherited from the father or the mother.

1                   16.     The method according to claim 7 (any one of claims 7 to 11) wherein  
2 the RNA is detected by any one of a physical method, an immunological method, and a  
3 biochemical method.

1                   17.     A detection method performed on a maternal serum or plasma sample  
2 from a pregnant female, the method comprising:  
3                   detecting the presence of RNA of fetal or maternal origin in the sample.

1                   18.     The method according to claim 17 further comprising amplifying the  
2 fetal or maternal RNA to facilitate detecting the presence of RNA of fetal or maternal origin  
3 - in the sample.

1                   19.     The method according to claim 18 wherein the fetal or maternal RNA  
2 is converted into complementary DNA by reverse transcriptase and then detected by a  
3 polymerase chain reaction.

1                   20.     The method according to claim 18 (claim 18 or claim 19) wherein at  
2     least one fetal or maternal sequence specific oligonucleotide is used in amplifying the fetal or  
3     maternal RNA.

1                   21.     The method according to claim 17 (any one of claims 17 to 20)  
2     wherein the fetal or maternal RNA is detected using a sequence specific probe.

1                   22.     The method according to claim 17 (any one of claims 17 to 21)  
2     wherein detecting the presence of RNA of fetal or maternal origin in the sample comprises  
3     detecting the presence of RNA transcribed from genes on chromosome 6.

1                   23.     The method according to claim 22 wherein the RNA is transcribed  
2     from the HLA-G gene.

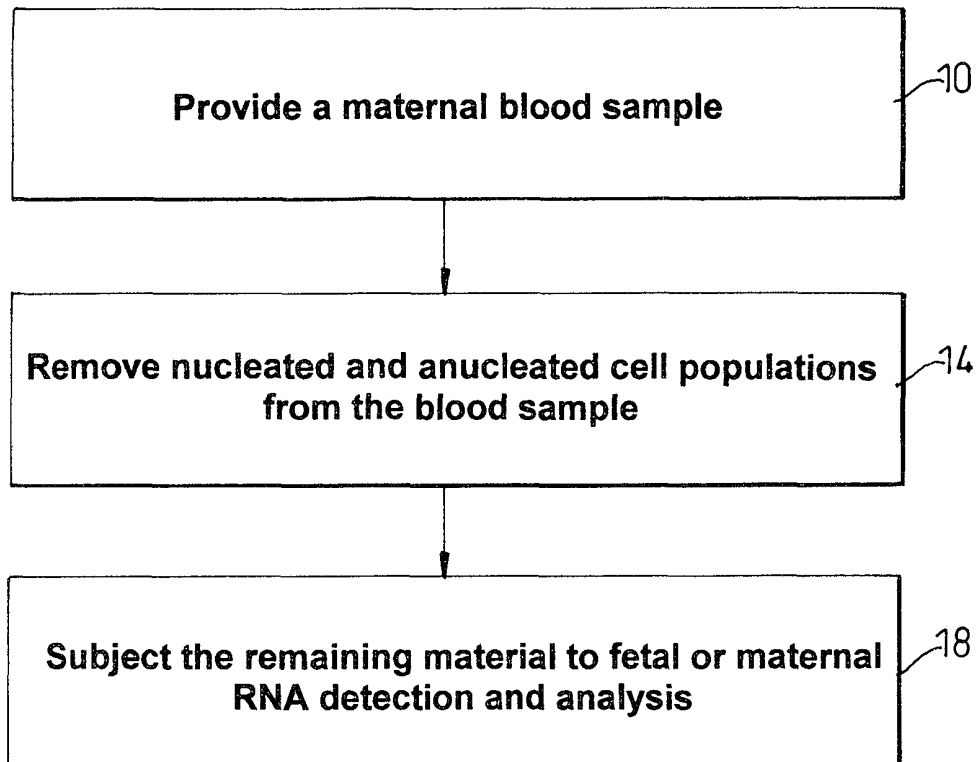
1                   24.     A method of performing prenatal monitoring or testing, the method  
2     comprising the steps of:

- 3                   (i) providing a maternal blood sample;  
4                   (ii) separating the sample into a predominantly cellular fraction (fraction 1)  
5     and a predominantly non-cellular fraction (fraction 2);  
6                   (iii) detecting the presence of RNA of fetal origin in fraction 2;  
7                   (iv) providing a diagnosis based on at least one of the presence, quantity,  
8     concentration, sequence, and biochemical composition of the detected RNA.

1                   25.     The method according to claim 24 further comprising the step of  
2     allowing clotting in the maternal blood sample to obtain a serum and using the serum for  
3     steps (iii) and (iv).

1                   26.     The method according to claim 24 wherein step (iii) further comprises  
2     detecting the presence of RNA of fetal or maternal origin in fraction 2.

1                   27.     A method of performing a prenatal monitoring or testing on a maternal  
2     blood sample, the method comprising:  
3                   obtaining a non-cellular fraction of the maternal blood sample; and  
4                   performing an RNA analysis for expressed human genes on the fraction.

**1/2*****Fig. 1***

