Thermo Scientific
Ampli-Y Bovine Embryo Sexing Kit

Technical Manual

F- 800S sufficient for 25 reactions
F- 800L sufficient for 100 reactions

Product Description

Non-electrophoretic PCR based bovine sex determination
The Thermo Scientific™ Ampli-Y™ Bovine Embryo Sexing Kit is designed for accurate and straightforward bovine embryo sex testing. This PCR based non-electrophoretic method allows to determine sex of preimplantation bovine embryos acquiring biopsy of 1 to 300 cells of *Bos taurus* or *Bos indicus*. The protocol works with compacted morula or blastocyst stage embryos (day 6-8). In contrast to conventional PCR based bovine embryo sexing methods, Ampli-Y workflow does not include electrophoretic analysis making the protocol time-saving and straightforward (Figure 2). After PCR step samples are analyzed under UV light. Pink fluorescence indicates presence of Y chromosome specific sequence in the sample (Figure 1).

The kit consists of lysis solution containing Proteinase K (solution A), PCR solution including polymerase, specific primers, dNTPs, Ampli-Y PCR buffer with $MgCl_2$ and a fluorescent dye (solution B), male and female control DNA tubes and HotStart-tubes.

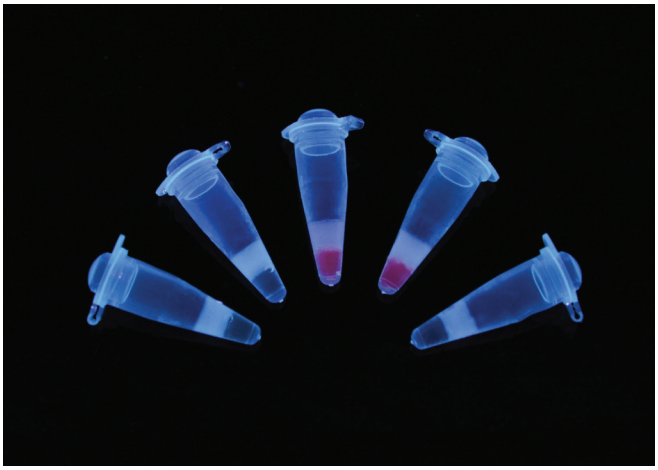

Figure 1. Representative results. Pink fluorescence indicates presence of bovine Y chromosomal DNA in the sample. Female samples do not show fluorescence (inspected in UV 312 nm).

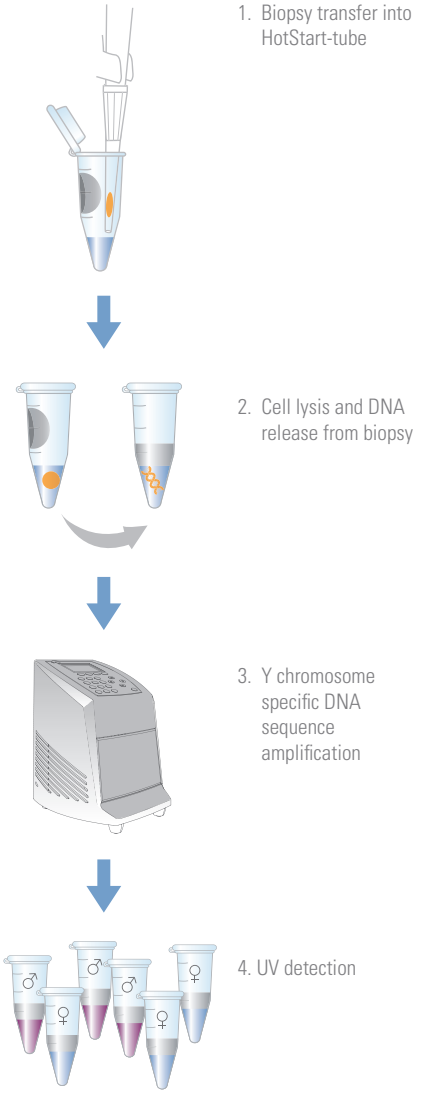

Figure 2. Workflow of the Ampli-Y Bovine Embryo Sexing Kit

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Components

Table 1. Ampli-Y Bovine Embryo Sexing Kit components for F-800S (sufficient for 25 reactions) and F-800L (sufficient for 100 reactions).

Kit Component	F-800S	F-800L
Ampli-Y Solution A (blue cap) Lysis solution including Proteinase K	1 tube × 400 µL	2 tubes × 725 µL
Ampli-Y Solution B (red cap) PCR Master Mix with $MgCl_2$ including polymerase, primers and a fluorescent dye in an optimized buffer	1 tube × 450 µL	2 tubes × 875 µL
Ampli-Y female control tubes (yellow cap) Contains female bovine genomic DNA	5 tubes	10 tubes
Ampli-Y male control tubes (blue cap) Contains male bovine genomic DNA	5 tubes	10 tubes
0.2 mL HotStart-tubes	30 tubes	110 tubes

Shipping and Storage

The Ampli-Y Bovine Embryo Sexing Kit is shipped on dry ice. Upon arrival, store all kit components at -20 °C. Reagents can be refrozen after use. The kit is stable until the expiry date if stored and handled properly.

The A-solution is stable for several weeks and B-solution for about a week when stored at +4 °C.

Materials needed but not supplied

In addition to the Ampli-Y Bovine Embryo Sexing Kit, the equipment and consumables listed below are required for bovine sex testing.

Biopsy microblades:

- Manual biopsy. Use a microsurgery scalpel with a 30 - 45 angle (e.g. Micro-Feather No. 730, feather Safety Razor Co., Ltd, Medical Division, Japan). Self-made microblades can also be used. Up to several hundred biopsies can be made with the same microblade.
- Biopsy with micromanipulator. A microblade can be produced from a standard razorblade and a glass or plastic rod (Williams, T.J. and Moore, L. 1998. Theriogenology 29: 477-484). Commercial microblades are also available.

Sample handling:

- Embryo handling pipette: any pipette used in routine bovine embryo transfer can be used (e.g. Drummond, Model 310).
- Biopsy transfer pipette: a conventional microdispenser (e.g. Gilson Pipetman, 1-10 µL, clear plastic tips)
- Pipette for solutions A and B: any conventional microdispenser covering the range of 10-15 µL can be used.

PCR:

- Disposable gloves.
- Pipettes.
- Aerosol-resistant pipette tips.
- Thermal cycler. The Ampli-Y Bovine Embryo Sexing Kit has been optimized for the Thermo Scientific Arktik™ Thermal Cycler but can be used with most commercially available thermal cyclers.

UV Illuminator

A recommended portable unit is the Spectroline E-series UV Hand Lamp model EBF 280 C/F (Spectronics Corporation, Westbury, New York, USA). This unit has two tubes, one producing UV light of 254 nm, the other 312 nm. Both can be used, but the 312 nm is recommended since 254 nm will more rapidly bleach the fluorescence in the tubes. The 254 nm wavelength tube can be used as an emergency if the 312 nm tube should fail.

Recommended media

Flushing medium
Any standard medium used in bovine embryo flushing, e.g. PBS (phosphate buffered saline, e.g. pH 7.4 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.76 mM KH_2PO_4, H_2O) with 0.1% BSA.

Biopsy medium
When biopsy is performed with proteins or other surfactant, use PBS supplemented with BSA (0.1-0.5%) or PVA (polyvinyl alcohol, 0.1-0.5%) or use SYNGRO Holding medium (Bioniche Animal Health). Any media containing BSA or other bovine derived product may contain traces of DNA. Make sure that your biopsy medium is free of bovine DNA (male).

Release medium
When biopsy is performed in medium free of proteins or other surfactant, medium containing a surfactant should be added to the biopsy medium before picking up the biopsy and embryo. Either add a volume (1:1) of biopsy medium or holding medium, or a small volume of medium with a concentrated amount of surfactant. Make sure that your release medium is free of (male) bovine DNA.

Holding medium
For maintaining biopsied embryos until transfer, place them in a medium designed for this purpose, e.g. SYNGRO Holding medium (Bioniche Animal Health).

Biopsy of day-7 bovine preimplantation embryos for embryo sexing

Many bovine embryo biopsy protocols have been devised. The simplified protocols presented below are compatible with compacted morulae and blastocysts recovered on day 6-8 after insemination. Two options are presented for stabilizing the embryo to the bottom of the dish, one using protein-free medium and the other using media with proteins or other surfactant.

Biopsy using protein-free medium

1. Transfer into biopsy drop.
   Pipet four 300 µL drops of PBS on the lid of a plastic petri dish. Add the embryo in a small amount (1-10 µL) of flushing medium to the first drop of PBS. Wash the embryo in 1 to 3 more drops of PBS until it sticks to the bottom.

   Note: A large drop size will decrease drop movements. If PBS contains Mg^{2+} and Ca^{2+}, the risk of drop movement increases.

2. Alignment of embryo.
   Turn the embryo around with the tip of the blade so that the desired part of it can be cut off. Use trophectoderm for blastocysts. Cells in the perivitelline space can be included in the biopsy.

3. The vertical cut.
   a) Place the microblade over the embryo so that the “heel” of the blade (the proximal part of the cutting edge) is anchored on the bottom of the petri dish (this enhances stability).
   b) Tilt the blade (press down the tip) so that a biopsy of desired size is cut off.

   Note 1: Do not press the tip too hard against the bottom of the petri dish as this may cause the biopsy to stick to the bottom.

   Note 2: If the biopsy sticks to the blade after the cut, it can be removed by a side movement while keeping the heel of the blade at the bottom. This action will produce vibration at the tip of the blade, releasing the biopsied cells.

4. Removal of biopsy and embryo.
   a) Add 10 µL of release medium into the drop, preferably close to the embryo. Pick up the biopsy in 1-2 µL of the medium and pipet it immediately into the reaction tube.
   b) Using another pipette tip, pick up the embryo and place it into holding medium, or if embryo culture environment (incubator) is available, into a proven culture medium.

   Note: If the biopsy sticks to the bottom and cannot be removed by pipetting or by touching it (gently) with the microblade or other instrument, remove the embryo first. Then flush the biopsy with 2-3 µL A-solution. This will help to release the biopsy from the bottom. Pick up the biopsy immediately after it is released.

5. Clean the instruments.
   To minimize sample contamination risk, instruments, if not replaced, should be washed between biopsies serially with distilled water, 70% ethanol and PBS.

Biopsy in medium containing proteins or other surfactant

1. Produce scratches.
   Pipet a drop (100-400 µL) of biopsy medium on the lid of a plastic petri dish. Using your microblade, produce 3 to 5 scratches about 30 to 50 µm apart (embryo diameter is about 150 µm) at the bottom of the dish. The scratches will prevent the embryo from slipping during biopsy.

2. Transfer into biopsy drop.
   Add the embryo in a small amount (1-10 µL) of flushing medium.

3. Alignment of embryo.
   Place the embryo on the scratched surface. Turn the embryo around with the tip of the blade so that the desired part of it can be cut off. Use trophectoderm for blastocysts. Cells in the perivitelline space can be included in the biopsy.

4. The vertical cut.
   a) Place the microblade over the embryo so that the "heel" of the blade (the proximal part of the cutting edge) is anchored on the bottom of the petri dish (this enhances stability).
   b) Tilt the blade (press down the tip) so that a biopsy of desired size is cut off.

   Note 1: Do not press the tip too hard against the bottom of the petri dish as this may cause the biopsy to stick to the bottom.

   Note 2: If the biopsy sticks to the blade after the cut, it can be removed by a side movement while keeping the heel of the blade at the bottom. This action will produce vibration at the tip of the blade, releasing the biopsied cells.

5. Removal of biopsy and embryo.
   a) Pick up the biopsy in 1-2 µL of biopsy medium and pipet it immediately into the reaction tube.
   b) Using another pipette tip, pick up the embryo and place it into the holding medium, or if embryo culture environment (incubator) is available, into a proven culture medium.Clean the instruments.

   Note: If the biopsy sticks to the bottom and cannot be removed by pipetting or by touching it (gently) with the microblade or other instrument, remove the embryo first. Then flush the biopsy with 2-3 µL A solution. This will help to release the biopsy from the bottom. Pick up the biopsy immediately after it is released.

6. Clean the instruments.
   To minimize risk of sample contamination, instruments, if not replaced, should be washed between biopsies serially with distilled water, 70% ethanol and PBS.

Additional notes

Biopsy occasionally sticks to the blade. It can be removed by causing vibration. Using manual technique, vibration can be generated by keeping the "heel" of the blade to the bottom of the plastic dish and producing a scrath with it at the bottom of the dish. If a micromanipulator is used, lift up the blade from the bottom and produce vibration by tapping on the table or micromanipulator. Polyvinylpyrrolidone (PVP) is sometimes used as a surfactant in embryo work. At high concentrations it fluoresces in UV illumination, thus interfering with sex determination based on fluorescence of tube content.

Sexing Protocol

Highly accurate sexing is compatible with embryos of any stage after the first cleavage. Routinely, bovine embryos are flushed around day 7, when they are at the compacted morula or blastocyst stage. These stages are ideal for microblade-assisted biopsy. Ampli-Y provides highly accurate results when 1 to 300 biopsy cells are used for sex determination. After biopsy, DNA release and Y chromosome specific sequence amplification takes place:

1. Add 10 µL of A-solution into each 0.2 mL HotStart-tube (except the control tubes).

2. Add the biopsy in 1-2 µL of PBS with surfactant (e.g. PVA).

3. Place tubes into PCR cycler and run the DNA release from biopsy program (see page 5).

4. Add control tubes (female and male) into PCR cycler and start the DNA amplification program (see page 5).

5. Add 15 µL B-solution into each tube (including controls) during the first denaturation step (2 minutes at 94 °C). Do not go through the melted wax layer with the pipette as the solution will go through automatically as the wax melts.

6. After the PCR amplification allow the tubes to cool to below 35 °C, then inspect the tubes under UV illumination (preferably 302-312 nm). Pink fluorescence indicates the presence of a male sample.

Table 2. Cycling parameters

Step	Temperature	Time	Number of cycles
DNA release from biopsy	55 °C 95 °C	5 min 5 min	1
Amplification[1]	94 °C	2 min	1
	94 °C 50 °C 75 °C	20 s 40 s 20 s	10
	96 °C 60 °C 75 °C	10 s 30 s 25 s	35
	75 °C	5 min	1

[1] This program is optimized with a Arktik Thermal Cycler for 0.2 mL reaction tubes. Other thermal cyclers can also be used, but may require optimization of cycling protocol.

A cooling step (4 °C) may be added at the end to facilitate faster cooling of the tubes.

Interpretation of the results

After PCR samples are analyzed using UV illumination (preferably 302-312 nm). Pink fluorescence indicates the presence of a male sample. Successful PCR is confirmed inspecting control tube, containing bovine male DNA. Male control must show pink fluorescence, whereas female control must show none.

Important Notes

- The use of disposable gloves is recommended.
- The A-solution contains Proteinase K, which is toxic to embryos. Use different pipettes for handling the biopsy and embryo to avoid contamination of the embryo holding medium.
- For transfer of biopsies into reaction tubes we recommend pipettes with plastic disposable tips (e.g. Finnpipette, Gilson).
- Use isotonic medium for transfer of biopsy (e.g. PBS). To decrease stickiness of the biopsy the use of a surfactant is highly recommended. However, avoid using serum or serum albumin as surfactants unless they are proven free of bovine DNA. Other usable surfactants include PVA (1 mg/mL) and SYNGRO Holding medium (Bioniche Animal Health). The B-solution should be added during the first step of the amplification program. In case the time (2 minutes) is too short this step can be prolonged either by programming this step to last 3-5 minutes, or use a “hold” function (pause) during this step. In the latter case make sure the program continues normally after this.
- The B-solution contains ethidium bromide, which is a suspected mutagen. Handle carefully.

Frequently asked questions about bovine embryo sexing

What developmental embryonic stage is most suitable for biopsy?

Any stage after the first cleavage is compatible with highly accurate sexing. Routinely, bovine embryos are flushed around day 7, when embryos are at the compacted morula or blastocyst stage. These stages are ideal for microblade-assisted biopsy. The consensus seems to be that biopsy of blastocysts is preferred over biopsy of compacted morula.

What part of the embryo should be selected as the site of biopsy?

For Grade 1 compacted morula it does not matter. If dead or extruded cells are seen, they can be included in the biopsy. In that case it is advisable to also include live cells from the compacted mass (there is a reason for the cells to die or get extruded, perhaps they have lost (sex) chromosomes).

How large biopsy can one take?

For successful sexing one cell is enough. However, a single cell biopsy can create problems with later stage embryos (compacted morulae and blastocysts), as a small cell can get lost on its way to the assay tube. With these embryos one should take about 10-25% of the total cell mass, but less than that (5-20%) if the embryos are to be frozen after sexing.

What is the decrease in viability after biopsy?

Not very much data has been published on this. Under ideal conditions (good quality embryos and recipients, etc.) a pregnancy rate of 75-80% with intact embryos may drop to 70-75% when embryos are biopsied. Less ideal conditions may cause a more significant drop and require that biopsy size is smaller.

Is it possible to biopsy in vitro produced embryos?

Yes. Pregnancy rates of about 45-50% are possible with a good culture system. Retarded embryos may be extremely sensitive to biopsy.

What pregnancy rate can one expect after freezing biopsied embryos?

Under ideal conditions up to 50% pregnancy rates may be achieved. Most workers seem to rely on ethylene glycol as the cryoprotectant for biopsied embryos.

Can one sex embryos after thawing with acceptable embryo viability?

Sexing is no problem and preliminary data indicate that a 40-50% pregnancy rate is achievable. However, this approach may include some very critical measures. The biopsied embryo should be allowed to recover from biopsy in a growth supporting medium (e.g. ViGro or EmCare at room temperature, or incubated at 38-39 °C in a proven culture medium and gas atmosphere.

What is the accuracy of embryo sexing?

It is possible to have an accuracy of 95-99.9%.

Why is the accuracy of embryo sexing not 100%?

There can be several reasons and it also depends on the techniques used. For instance, if the DNA is released from cells by heating or snap-freezing, nucleases are not deactivated and may partially degrade the DNA. As fragmented DNA cannot be amplified by PCR, Y-chromosomal DNA may not be amplified resulting in a male diagnosed as a female. Keeping the tubes on ice will help. A better way to solve this problem is to release the DNA with a lysis medium containing proteinase K which also deactivates nucleases.

A more likely problem is that previously amplified Y-chromosomal DNA may enter equipment and media used in biopsy or PCR (carry-over contamination). Such contamination will result in females diagnosed as males.

How can one avoid contamination problems?

If using electrophoresis to analyze PCR products, keep equipment used for electrophoresis in a separate place, away from where the biopsy and PCR takes place. Also, extra care should be taken if the same person who did the electrophoresis is working with biopsy equipment, PCR machine or tubes.
One way to completely avoid contamination by amplified products is to use non-electrophoretic sexing.

What is non-electrophoretic sexing?

In non-electrophoretic sexing the result can be seen directly after PCR by exposing the sample tubes to UV light. Fluorescent samples are male.

How can one make sure that the biopsy goes into the tube?

The tip of the pipette should enter the lysis medium without touching the walls of the tube. It is a good idea to siliconize the pipet tips, especially if glass capillaries are used as tips. The tube can also be inspected while the biopsy is pipetted into the tube. However, sometimes the biopsy will enter the tube even if one cannot see it.

References

1. Bredbacka P, Kankaanpää A, Peippo J. (1995) PCR-sexing of bovine embryos: A simplified protocol. *Theriogenology* 44: 167-176.

2. Bredbacka P. (1998) Recent developments in embryo sexing and its field application. *Reproduction Nutrition Development*. 38: 605-613.

3. Hasler JF, Cardey E, Stokes JE, Bredbacka P. (2002) Nonelectrophoretic PCR-sexing of bovine embryos in a commercial environment. *Theriogenology*. 58:1457-69.

Product use limitations

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