A protocol for differential staining of inner cell mass and trophectoderm of embryos for evaluation of health status

Buffalo, an important livestock species for milk and meat production in Asia. Limited work has been done so far regarding *in vitro* fertilization (IVF) in buffaloes. So, there is an urgent need to explore this technology both for research and commercial applications. *In vitro* embryo production (IVEP) technology is now recognized to be the best tool to enhance genetic progress through the maternal lineage, allowing a great increase in the good number of embryos obtainable for transfer. In humans, following the birth of Louise Brown in July 1978 (ref. 1) and the subsequent births, it has become evident that a wider range of infertility problems could be treated by IVF. A proper evaluation of blastocyst quality, however, remains an important challenge for researchers involved in embryology and clinicians who want to select the best embryos for transfer. Today, the primary effort of IVF is to increase blastocyst production with minimum abnormality and proper transfer of embryos into a mother in order to conceive. The current technology available has increased the options and chances of success. We have conducted experiments in buffalo blastocyst to standardize differential staining protocol to count the number of inner cell mass (ICM) and trophectoderm (TE) cells for evaluation of blastocyst health status so that bovine reproductive biologists and infertility clinic researchers can select the best embryos for transfer to a recipient mother.

Chemicals were purchased from Sigma (USA), unless otherwise indicated. (i) PBS, ready to use solution (Sigma D4031). (ii) Dilute Triton X-100 (T8532) in PBS (T solution) to obtain a final concentration of 0.1% (v/v). (iii) Propidium iodide (PI solution) – A 10 mg/ml stock solution was prepared by dissolving PI (Sigma-P4170) in PBS and stored at 4°C. Next, the stock solution was diluted in PBS to give a working concentration of 25 µg/ml. The working solution was stored in a foil-covered tube at 4°C for 1 month. Before use, the working solution was kept in a CO_2 incubator for 1 h. (iv) Hoechst 33258 (H solution) – The stock was prepared by dissolving 25 mg Hoechst 33342 (Sigma B-2261) in 2.5 ml of PBS (10 mg/ml), and stored at –20°C. On the day of use, the working solution was prepared by diluting the stock solution in PBS to give a working concentration of 5 ng/ml. Whatever solution was not used on that day was discarded. (v) Microscope slides and covers slip.

Now, 500 µl of the H solution was placed in the first well of a NUNC four-well plate. The second, third and fourth wells were filled with 500 µl of T solution, PI solution and PBS respectively. The four-well plate was placed on a slide warmer set to 39°C and all solutions were allowed to warm up for 5–10 min prior to beginning the staining procedure. Next 4–5 blastocysts were removed from the culture in as little medium as possible and placed in the well of the H solution for 40 min. After incubation in the H solution, the embryos were washed in the fourth well containing PBS. Then the embryos were placed in 500 µl of the T solution and incubated at room temperature for 1 min. They were washed in PBS. Next the embryos were placed in 500 µl of the PI solution for 35–40 s and washed again in PBS. Finally the embryos were transferred onto microscope slides in a small volume of glycerol solution and the cover slip was mounted. The embryos were viewed as soon as possible under UV light (200–400 nm).

Significant improvement has been achieved in livestock production with the application of reproductive technologies such as IVEP and embryo transfer (ET). The large-scale production of mammals depends upon a large number of high-quality embryos produced *in vitro*[3]. In this regard, the success rate of IVEP systems in terms of blastocyst yield remains modest and ranges from 30% to 40%, which is still lower than that obtained from embryos produced *in vivo*[2–4]. Furthermore, the quality of *in vitro* embryos is inferior to that of embryos produced *in vivo*, as judged by morphology, increased susceptibility to cryoinjury and poor implantation and viability[4]. The low rate of successful pregnancies achieved following the transfer of the IVEP blastocysts indicates that qualitative assessment of the IVEP blastocysts is a crucial factor determining the success of the IVEP and ET programmes[3–5]. Therefore, intensive focus has been paid to provide valuable means for determining the quality of the embryos produced *in vitro* not only for livestock animals, but also for human embryos[6]. Accordingly, a wide range of techniques have been presented for assessment of the total cell number (TCN) as well as viable and dead cells of the blastocysts such as morphological examination[7], differential staining[6,7], tunnel assay[8], metabolic assays[9], etc. Most of these techniques are time-consuming, elaborative and need expensive equipment or reagents and high technical skill. Here, we have described a feasible qualitative approach for determining TCN as well as ICM and TE cells of the blastocysts. This novel technique is based on the natural function and permeability of the cell membrane in response to PI and bisbenzimide (H33342) flourochrome. PI

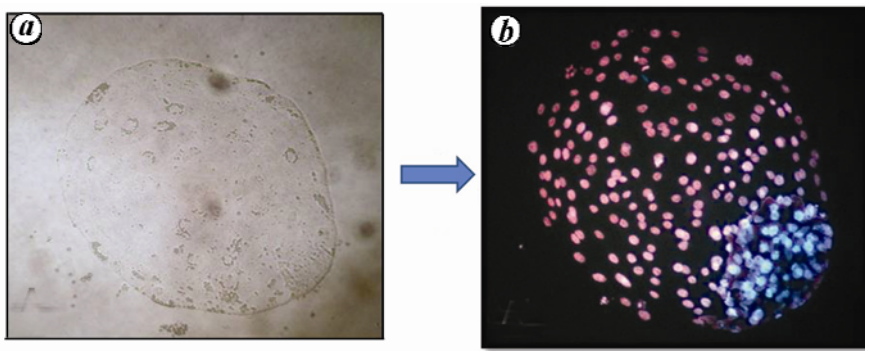

Figure 1. Differential staining of the embryos as a valuable approach for assessment of the blastocyst quality. ***a***, Buffalo blastocysts produced *in vitro*. This embryo is consistent with its expected stage of development (day 7 of embryo development) under bright field. ***b***, Photograph taken using fluorescence microscopy, with excitation wavelength of 330–385 nm and barrier filter 400 nm. ICM cells are shown in blue and TE cells in red.

is not permeable to ICM cells, while H33342 has the complete potency to enter all cells. Keeping this in mind, this approach is proposed as a feasible technique for assessment of blastocyst quality. For examining the quality of the embryos, around 50 stained blastocysts were observed under an epifluorescence microscope with excitation wavelength of 330–385 nm and barrier filter 400 nm. Photographs were taken and total nuclei stained were manually counted. ICM cells are shown in blue colour, while TE cells are shown in red colour (Figure 1). The results of this technique might be useful for examination effect of chemical modifiers, culture system, different physiological conditions on embryos health both for animal and human applications.

1. Edwards, R. G., Bavister, B. D. and Steptoe, P. C., *Nature*, 1969, **221**, 632–635.
2. Steinfeld, H., Wassenaar, T. and Jutzi, S., *Rev. Sci. Technol*., 2006, **25**, 505–516.
3. Garcia-Garcia, R., Ward, F., Fair, S., O'Meara, C., Wade, M. D. P. and Lonergan, P., *Anim. Reprod. Sci*., 2007, **98**, 233–240.
4. Rizos, D., Ward, F., Duffy, P. and Maurice, P., *Mol. Reprod. Dev*., 2002, **61**, 234–248.
5. Farin, P. W., Crosier, A. E. and Farin, C. E., *Theriogenology*, 2001, **55**, 151–170.
6. Royen, E., Mangelschots, K., De Neubourg, D., Valkenburg, M., Van de Meerssche, M., Ryckaert, G. and Eestermans, W. J., *Human Reprod. Embryol*., 1999, **14**, 2345–2349.
7. Van, S. A., Mateusen, B., Leroy, J. and De, K. A., *Reprod. BioMed. Online*, 2003, **7**, 664–670.
8. Fouladi-Nashta, A. A., Alberio, R., Kafi, M., Nicholas, B., Campbell, K. H. S. and Webb, R., *Reprod. BioMed. Online*, 2005, **10**, 497–502.
9. Zuelke, K. A. and Brackett, B. G., *Endocrinology*, 1992, **131**, 2690–2696.

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An RNA isolation protocol for recovery of high quality functional RNA from fungi and plants

Obtaining high quality, intact RNA is the first and often the most crucial step in performing many fundamental molecular biology experiments, including reverse transcriptase PCR (RT–PCR), Northern analysis, nuclease protection assays, RNA mapping, *in vitro* translation and cDNA library construction. However, the presence of high levels of polyphenols, polysaccharides and RNAase in the tissues of both prokaryotes and eukaryotes makes RNA extraction often difficult, challenging and requires extensive alteration in RNA isolation protocols. Thus, RNA recovery differs based on the levels of polyphenols and polysaccharides in different tissues[1].

Trichoderma (class Ascomycota, order Hypocreales, family Hypocreaceae), the free-living, beneficial fungi, commonly found in soil, with capability to produce antibiotics and lytic enzymes (cellulase, hemicellulase, xylanase, chitinase) of industrial interest, are useful for plant protection purposes in agriculture[2]. Recent discoveries show that they are opportunistic, avirulent plant symbionts as well as parasites of other fungi. At least some strains establish robust and long-lasting colonization of root surfaces and penetrate into the epidermis and a few cells below[3]. Unavailability of suitable RNA isolation protocols leads to troubles in obtaining sufficient amounts of good quality RNA from *Trichoderma*, which poses hindrance to several molecular biological studies. Cell walls or fungal structures, viz. conidia, hyphae, etc. which are resistant to lysis slow down high-quality RNA isolation, often leading to modifications of existing protocols[4]. There are several reports on total RNA isolation from filamentous fungi[5]. However, an optimized methodology that could provide large quantity of pure total RNA for gene expression studies is always lacking.

Plants are diverse, and individual species and organs or tissues of plants behave differently during RNA isolation. Hence, a range of extraction protocols has also been devised, depending on the tissue or genotype being extracted. The most common problems encountered while extracting RNA from plants include high levels of RNAases; the presence of large quantities of polysaccharides; different kinds of phenolics, including tannins; low concentrations of nucleic acids (high water content), and tissue, such as lignin (wood), that is difficult to break up. Several CTAB, SDS and GT methods have been developed to isolate total RNA from tissue containing high levels of polyphenols and polysaccharides[6–8]. In addition, there are several commercial kits and triazole (Sigma-Aldrich Co., USA) available for RNA isolation. However, a single protocol for isolation of high-quality RNA both from fungi and plants tissue is not available till date. Therefore, the objective of the present study was to develop a single, rapid and versatile method for the isolation of total RNA from *Trichoderma* spp. and rice (*Oryzae sativa*).

Five millimetre discs of actively growing mycelium of *Trichoderma harzianum* was taken from the margins of fungal growth and transferred to petri dishes (9 cm in diameter) containing about 20 ml PDA (200 g potato, 2% dextrose, 2.5% agar in 1 l of water). The agar percentage was increased (from 2 to 2.5)