SAGWADI
GENETICS & EMBRYOLOGY

Advanced Embryo Transfer & IVF Manual

Optimized for Zebu (Bos indicus) Genetics & Advanced Diagnostics

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1 The Zebu Difference & Facility Handling

At Sagwadi, we recognize that Zebu cattle require a fundamentally different and highly specialized approach. Because our cattle possess higher sensitivity to FSH, smaller follicles at the time of deviation (~ 6 mm), and an acute physiological reactivity to stress, our protocols are specifically tailored to maximize oocyte viability for our laboratory IVP (In Vitro Production) pipeline.

Pre-Procedure & Chute-Side Etiquette

- **Low-Stress Procurement:** We utilize the "Bud Williams" method. Our handlers must work the edges of the flight zone. Do not use dogs or electric prods. Absolute silence in the Sagwadi facilities lowers cortisol, a known embryotoxin.
- **Environmental Control:** Round up our cattle in the early morning (before 08:00) to mitigate heat stress. Hold them in shaded pens with ad-libitum water and roughage for 2 hours pre-procedure.
- **Fasting Preparation:** Withhold feed for 12 to 24 hours and water for 12 hours prior to procedures to reduce rumen fill and intra-abdominal pressure.
- **Thermal Thresholds:** If ambient temperature exceeds 28°C (82°F) or the Temperature-Humidity Index (THI) > 72, all Sagwadi handling must be completed before peak heat hours.
- **Oocyte Transport:** If transporting aspirated tubes to our lab, portable incubators must be pre-heated for 45 minutes and hold precisely 37.5°C to 38.5°C. Maximum transport time is 4 hours.
- **Sanitation:** Evacuate the rectum completely. Scrub the vulva and perineum with a 2% chlorhexidine scrub.
- **Epidural Block:** Administer 5 to 7 cc of 2% Lidocaine (without epinephrine) between the 1st and 2nd coccygeal vertebrae using an 18G x 1.5-inch needle. Allow 5 to 10 minutes for full anesthetic effect.

Strict Laboratory Warning

NO ALCOHOL OR IODINE prior to OPU (Oocyte Pick-Up). Vapors entering the vagina are highly toxic to oocytes.

2 Materials & Equipment Inventory

At Sagwadi, maintaining our standard of excellence requires uncompromising quality control. From the moment our veterinary team steps into the field for oocyte collection to the highly controlled environment of our IVF lab, every instrument must perform flawlessly. We rely on the following comprehensive inventory of specialized apparatuses, micro-tools, and media to bridge the gap between our outside cattle work and our inside laboratory precision.

Phase	Equipment / Media	Sagwadi Intended Use & Parameters
Field Procurement & Transfer (Chute-Side)		
Chute-Side	**Bovine Ultrasound**	Portable unit with 5.0 to 7.5 MHz convex transvaginal probe. Allows our technicians to visualize the Zebu ovary and perfectly target follicles 2mm and larger.
Chute-Side	**Vacuum Aspirator Pump**	OPU pump strictly calibrated to 55 to 65 mmHg. Provides gentle, constant suction to extract oocytes without stripping the delicate surrounding cumulus cells.
Chute-Side	**OPU Needles**	18G, short-bevel disposable needles. Changed every 2 to 3 donors to remain razor sharp and minimize ovarian trauma during follicle puncture.
Chute-Side	**Aspiration Filters**	70 μm EmCon filters used to safely catch the oocytes as the aspirated follicular fluid flows through the warmed silicone lines.
Chute-Side	**Portable Incubator**	Pre-heated to precisely 38.0°C to safely transport aspirated fluid and oocytes from the chute to our lab without risking cold shock.
Chute-Side	**Cassou ET Guns**	Stainless steel transfer syringes used with sterile chemises to safely bypass the cervix and deposit the embryo in the uterine horn.
Laboratory Equipment (In-Vitro Culture & Diagnostics)		
Laboratory	**Tri-Gas CO_2 Incubator**	Keeps embryos alive at exactly 38.5°C in a controlled Tri-Gas atmosphere (5% CO_2, 5% O_2, 90% N_2). Low oxygen is critical for blastocyst success.
Laboratory	**Laminar Flow Hood**	Class II biological safety cabinet providing sterile airflow. Must run for 15 minutes prior to use to clear particulates and prevent contamination.
Laboratory	**Stereo Microscope**	Equipped with a heated stage calibrated to exactly 38.5°C. Used to search, locate, and grade oocytes from the aspirated follicular fluid.
Laboratory	**Inverted Microscope**	High-magnification optics ($400\times$) used in tandem with our micromanipulators and advanced laser biopsy system.
Laboratory	**Micromanipulator System**	Joystick-operated micro-tools used by our technicians to hold the embryo steady, insert sperm into eggs, and extract cells for sexing.
Laboratory	**Centrifuge**	Separates healthy, motile sperm from dead sperm and freeze extenders. Rotor is pre-warmed to 37°C and spun at $300 \times g$ for 10 minutes.
Laboratory	**LN_2 Dewars**	Liquid nitrogen storage tanks for the permanent cryogenic vitrification and storage of Sagwadi Grade 1 and 2 embryos at -196°C.
Micro-Tools, Biologicals & Media		
Consumable	**Micropipettes**	135μm to 150μm polycarbonate stripper tips used to physically move embryos and carefully denude cumulus cells.
Consumable	**Nunc & Petri Dishes**	4-well dishes for IVC micro-drops and 100mm gridded dishes for systematic visual searching under the microscope.
Biological	**EMXCELL Media**	Specialized OPU aspiration media. Store at 2°C to 8°C. Must be pre-warmed to 38.5°C for 2 hours prior to OPU.
Biological	**SOF Culture Media**	Synthetic Oviductal Fluid. Chemically replicates the cow's oviduct to sustain the embryo for the 7-day maturation culture.
Biological	**Lysis Buffer**	Used post-biopsy to break open extracted trophectoderm cells and expose the DNA for PCR sexing. Store at room temperature.

3 The Sagwadi OPU Pre-Stimulation Protocol (IVF Prep)

At Sagwadi, we exclusively produce our elite embryos *in vitro* (in the lab) using Oocyte Pick-Up (OPU) rather than relying on traditional *in vivo* uterine flushing. While Zebu can undergo un-stimulated OPU, our standard utilizes a precise FSH super-stimulation protocol prior to aspiration. This maximizes the yield, uniformity, and developmental competence of the oocytes retrieved, directly increasing our lab's blastocyst conversion rates.

Folltropin Sourcing, Storage & Preparation

Procurement: Folltropin (highly purified porcine pituitary extract) must *only* be acquired through authorized veterinary biopharma distributors (e.g., Vetoquinol). Do not accept generic substitutes, as the Sagwadi standard demands extremely low LH (Luteinizing Hormone) contamination.
Cold Chain Management: Folltropin must be shipped and stored continuously between 2°C and 8°C. If the cold chain is broken, the protein degrades, and the batch must be discarded.
Reconstitution: Mix the diluent with the lyophilized powder only within the sterile confines of the laminar flow hood. Once reconstituted, the vial is viable for exactly 5 days under refrigeration.

To prevent ovary shut-down in our sensitive Zebu donors, we administer a strictly controlled **descending dose** of FSH over a shortened timeline, followed by a "coasting" period before aspiration.

The Sagwadi OPU Stimulation Timeline

- **Day 0 (08:00):** Insert CIDR (1.38g Progesterone) + Administer 2mg Estradiol Benzoate to reset the follicular wave.
- **Day 4 (08:00 & 20:00):** FSH Day 1: 2.5mL AM / 2.5mL PM
- **Day 5 (08:00 & 20:00):** FSH Day 2: 2.0mL AM / 2.0mL PM
- **Day 6 (08:00 & 20:00):** FSH Day 3: 1.5mL AM / 1.5mL PM **(+ 5mL PGF2α in AM)**
- **Day 7 (08:00):** Pull CIDR. *Coasting Phase Begins:* No FSH is given. This 48-hour window allows follicles to reach optimal size without premature ovulation.
- **Day 8 (08:00 to 12:00): OPU (Oocyte Pick-Up) Performed.** Using transvaginal ultrasound, all follicles $\geq$ 2mm are aspirated. *Note: No Artificial Insemination (AI) is performed on the donor.*

4 The Sagwadi In Vitro Production (IVP) Pipeline

Once the aspirated follicular fluid arrives from the chute, it immediately enters the Sagwadi laboratory pipeline. Creating elite embryos entirely *in vitro* demands exact chemical, chronological, and thermal replication of the maternal tract. The following detailed phases must be executed flawlessly by our embryologists.

Phase 1: COC Search, Grading & Washing

The aspirated fluid is carefully poured into 100mm gridded Petri dishes and examined under our stereo microscopes on stages heated precisely to 38.5°C.

- **Identification:** Technicians systematically scan the grid to locate Cumulus-Oocyte Complexes (COCs). We select only Grade 1 and 2 COCs, characterized by a dark, homogeneous, ungranulated cytoplasm tightly enveloped by at least three uniform layers of compact cumulus cells.
- **Washing:** Selected COCs are immediately transferred through three distinct wash drops of HEPES-buffered media. This critical step removes toxic erythrocytes (red blood cells) and cellular debris, preventing oxidative stress.

Phase 2: In Vitro Maturation (IVM) & Metaphase II

Washed COCs are placed into micro-drops of TCM-199 maturation media (supplemented with gonadotropins and FCS), heavily overlaid with sterile mineral oil to prevent evaporation and stabilize pH.

- **Incubation:** The dishes are placed in the CO_2 incubator for **22 to 24 hours**.
- **Achieving Metaphase II (MII):** During this window, the oocyte undergoes crucial nuclear and cytoplasmic maturation. The cumulus cells undergo "expansion" (becoming puffy and producing a sticky hyaluronic acid matrix), and the oocyte extrudes its first polar body into the perivitelline space. Reaching the MII stage means the oocyte is officially mature and ready to accept a sperm cell.

Phase 3: Sperm Capacitation & In Vitro Fertilization (IVF)

Simulating the journey through the female reproductive tract is required to make the sperm capable of fertilizing the egg.

- **The Percoll Gradient:** Frozen semen is thawed at $37°$C for 45s and layered onto a Percoll density gradient (typically 45% over 90%). Centrifuging this gradient strips away the toxic cryoprotectants, seminal plasma, and dead sperm, isolating only the highly motile, viable sperm at the bottom of the tube.
- **Capacitation & Co-Incubation:** The washed sperm is added to the expanded COCs in drops of IVF-TALP media. Heparin in the media triggers sperm capacitation (destabilizing the sperm head to allow for the acrosome reaction). They co-incubate for **18 to 20 hours**.

Phase 4: In Vitro Culture (IVC) & Embryogenesis

After fertilization, the resulting presumptive zygotes must be cultivated for 7 days.

- **Denuding:** Zygotes are mechanically denuded using 135μm stripper micropipettes to physically shear away the dead cumulus cells and excess attached sperm.
- **Tri-Gas Culture:** The cleaned zygotes are transferred into SOF (Synthetic Oviductal Fluid) drops and placed into our strictly monitored Tri-Gas incubator. Low 5% O_2 is critical here to prevent free-radical damage to the rapidly dividing cells.
- **Development Checks:** At 48 hours, we check for *cleavage* (successful division into 2-to-4 cell stages). At Day 7 (168 hours), we evaluate final blastocyst conversion for grading and biopsy.

5 Advanced Diagnostics: Embryo Sexing Protocol

At Sagwadi, once our embryos reach the blastocyst stage, we perform sex determination utilizing our micromanipulator and laser systems. **Time limit:** Embryos must not remain outside the incubator for more than **10 minutes** to prevent harmful pH and temperature shifts.

Post-Fertilization Biopsy Workflow

1. **Stabilization:** Hold the embryo firmly under the inverted microscope (stage heated to 38.5°C) using the holding pipette.
2. **Zona Thinning:** Thin or breach the *Zona Pellucida* (outer layer) using targeted laser pulses (duration: **0.5 to 1.5 milliseconds** per pulse) to avoid thermal damage to the cells.
3. **Extraction:** Pass the micropipette through the breached zona to extract 3 to 5 cells exclusively from the *trophectoderm* layer (avoiding the inner cell mass).
4. **Healing:** Retract the pipette. After cell removal, the embryo will close and seal naturally within **2 to 3 hours** back in our incubator.
5. **Cell Prep:** Submerge extracted cells in **lysis buffer**. Incubate at 37°C for **10 minutes**, then heat-inactivate at 95°C for **5 minutes**.
6. **Determination:** Utilize PCR (Polymerase Chain Reaction) on the lysed DNA. Thermal cycling (Denaturation: 95°C, Annealing: 60°C, Extension: 72°C) takes approximately **90 minutes**.

6 Embryo Grading & Transfer Procedures

Every *in vitro* produced (IVP) embryo exiting the Sagwadi lab must be rigorously evaluated by our technicians prior to biopsy, freezing, or fresh transfer.

Grade	Classification	Morphological Appearance
1	**Excellent**	Symmetrical, spherical mass. Uniform blastomeres in size/color. Ideal for biopsy or freezing.
2	**Good**	Minor imperfections. Few extruded blastomeres ($< 20\%$ of mass).
3	**Fair**	Noticeable irregularities. 20 to 50% extruded/dead cells. Fresh transfer only; do not vitrify.
4	**Degenerate**	Collapsed mass, non-viable. Do not transfer, freeze, or biopsy.

Transfer Preparation & Execution

Thawing (If Frozen): Hold in ambient air for 10 seconds, then submerge in a 37°C water bath for exactly 30 seconds.
Straw Loading Sequence: Holding Media (30mm) $\rightarrow$ Air (5mm) $\rightarrow$ Media w/ Embryo (30mm) $\rightarrow$ Air (5mm) $\rightarrow$ Holding Media (30mm).
Implantation Time Limit: Transfer the loaded embryo into the recipient within **15 minutes** to ensure maximum viability.
Implantation: To meet Sagwadi criteria, the recipient cow *must* have a confirmed, palpable Corpus Luteum (CL). Deposit the embryo gently into the **ipsilateral uterine horn** (the horn on the same side as the CL).

Technical Support & Consultations

For any questions, troubleshooting, or advanced technical assistance regarding these protocols, please reach out to our team at:

contact@sagwadi.com